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Tryptophan Metabolism: Implications for Biological Processes, Health and Disease

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Preface

Tryptophan Metabolism: Implications for Biological Processes, Health and Disease

In many organs and tissues, the major route for the metabolism of tryptophan is the kynurenine pathway. One of the initial enzymes for this pathway is indoleamine-2,3-dioxygenase, present in most organs and tissues except the liver. The second enzyme, tryptophan-2,3-dioxygenase, is almost exclusively found in the mammalian liver and is responsible for tryptophan catabolism. A small portion of tryptophan is used for the synthesis of serotonin. Serotonin is a key neurotransmitter that modulates a wide variety of functions in both peripheral organs and the central nervous system. In response to signals from the circadian clock, N-acetylserotonin is converted to melatonin, which is synthesized not only in the pineal gland but also in many other parts of the body. Melatonin shows a strong antitumor activity by decreasing tumor cell viability and reactive oxygen species generation.

Most of the endogenous metabolites of tryptophan particularly derived from kynurenine pathway are implicated in cell damage in a wide range of psychiatric, neurological, and systemic disorders such as osteoporosis, neurodegenerative diseases, allergic and infectious diseases, brain injury, ischemic stroke injury, depression, immune response modulation, and immune tolerance. Additionally disrupted circadian rhythm, sleep restriction, and sleep deprivation-associated metabolic disorders are the subject of current research; however, extremely limited data has been obtained concerning the immune modulation, immune escape mechanisms, spontaneous immune tolerance, and the biosynthesis of quorum-sensing molecules.

Extensive screening of the tryptophan degradation pathway components aimed to clarify and update the selected topics within the scope of recent opinions. However, reappraisal of conceptualized definitions of tryptophan-related disorders within the current perspectives surprisingly revealed that several details of tryptophan metabolism still remain unknown. Last of all, complementary investigations

are required to comprehend the complex interaction between tryptophan-derived metabolites among themselves and within the central nervous system and in the periphery. Overall this publication focuses on the critical and controversial points of tryptophan metabolism. We believe that the reassessment of tryptophan metabolism may lead to new perceptions.

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Ayse Basak Engin, Ph.D., is Associate Professor of Toxicology and currently working in Gazi University, Faculty of Pharmacy, Department of Toxicology, in Ankara, Turkey. Dr. Engin has several publications related to pteridine and tryptophan metabolism, immunological alterations in biological systems, application of these biomarkers in cancer diagnosis and prognosis, oxidative stress, nanotoxicology-nanomedicine, and mycotoxins. She has participated as principal investigator or researcher, in many research projects funded by national and international organizations and her findings have been cited in numerous international journals. While some of these were registered and included into scientific databases, some of them won national and international awards. She edited and wrote two chapters in a book related to endothelium. Dr. Engin is currently the Secretary General of Turkish Society of Toxicology.

Chapter 1
Tryptophan-Related Signaling Molecules: Targets and Functions

Atilla Engin

Abstract Most of the daily dietary tryptophan (Trp) is oxidatively degraded through the kynurenine (Kyn) pathway, and the remaining may be consumed either in serotonin synthesis or in conversion into melatonin through the methoxyindole pathway. Trp degradation products along the Kyn pathway include three neuroactive metabolites: the neuroinhibitory agent kynurenic acid (KA), the free radical generator 3-hydroxykynurenine (3HK), and the excitotoxin quinolinic acid (QA). Kyn is the major metabolite of Trp and is readily transported across the blood–brain barrier into the brain where it can be further metabolized in perivascular macrophages, microglia, and astrocytes, also to generate neuroactive intermediates. In contrast to Kyn, QA, KA, and 3-hydroxyanthranilic acid (3HAA) penetrate through the blood–brain barrier only poorly due to its polar nature. Although the cytokines do not pass through the blood–brain barrier, their signals reach the brain through humoral, neural, and cellular pathways and stimulate Trp degradation by interacting with a cytokine network in the brain. The induction of Kyn pathway by indoleamine 2,3-dioxygenase (IDO) activity exhausts L-Trp in the medium and produces toxic metabolites. While Kyn to Trp ratio reflects IDO activity, Kyn to KA ratio indicates the neurotoxic challenge. Alpha7 nicotinic acetylcholine receptor (alpha7nAChR) constitutes a crucial link between excessive KA formation and reduction in glutamate. KA-induced reduction in prefrontal glutamate levels emerges as a result of alpha7nAChR inhibition. Changes in the endogenous concentrations of KA, as a potent alpha7nAChR and N-methyl-D-aspartate (NMDA) receptor antagonist, affect extracellular dopamine levels in the brain. The entire monoaminergic neurotransmission involves functional interactions between serotonin, norepinephrine, and dopamine systems (Fig. 1.1). Serotonin transporter (SERT) reuptakes biogenic amine neurotransmitters following release in the nervous systems and terminates the action of serotonin. SERT can be regulated by a membrane-bound G-protein-coupled receptor, and this occurs via nitric oxide (NO) and cyclic guanosine monophosphate (cGMP). Desensitization and re-sensitization of G-protein-coupled

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receptors (GPCRs) can modulate receptor responsiveness in regulation of many cellular functions. Diet restriction-induced exaggerated feedback control over serotonin synthesis decreases serotonin neurotransmission at postsynaptic sites by reducing availability of Trp. Enterochromaffin (EC) cells of the intestinal mucosa respond to chemical and mechanical stimuli by releasing serotonin. The enteric serotonin transporter plays a critical role in serotonergic neurotransmission and in the initiation of peristaltic and secretory reflexes.

Keywords Tryptophan • Kynurenine • Kynurenic acid • Quinolinic acid • Indoleamine 2,3-dioxygenase • N-Methyl-D-aspartate receptor • Serotonin • Serotonin transporter • Serotonin receptors

1.1 Introduction

Amino acids are not only regulators of gene expression and the protein phosphorylation cascade but are also cell signaling molecules. Carbon skeletons of essential amino acids cannot be synthesized by animal cells and, therefore, must be provided from the diet (Wu 2010). The average daily nutritional requirement of L-tryptophan (Trp) as an essential amino acid is 5 mg/kg. In order to improve mood or sleep, many adults may consume Trp much more, up to 4–5 g/day (60–70 mg/kg) (Fernstrom 2012). Ninety-five percent of dietary Trp is oxidatively degraded in the liver through the kynurenine (Kyn) pathway. Actually there are two rate-limiting enzymes of Kyn formation: first, tryptophan 2,3-dioxygenase (TDO) and, the second, indoleamine 2,3-dioxygenase (IDO) (Marazziti et al. 2013). TDO reaction generates nicotinamide adenine dinucleotide [NAD^+] following Trp oxidation. A small amount of Trp degradation can also occur extrahepatically by the enzyme IDO. IDO is expressed by a large variety of cells and can be directly activated by proinflammatory cytokines such as interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha, whereas TDO is only located in the liver cells and is activated by stress hormones (Wirleitner et al. 2003). Degradation of Trp mainly occurs along the Kyn pathway. Eventually Kyn is metabolized along one of two catabolic branches, leading to the formation of either hydroxykynurenine (3HK) and quinolinic acid (QA) or kynurenic acid (KA). The cerebral Kyn pathway is driven mainly by blood-borne L-Kyn, which enters from the circulation to the brain using the large neutral amino acid transporter, whereas QA, KA, and 3-hydroxyanthranilic acid (3HAA) cannot pass the blood–brain barrier easily (Fig. 1.1) (Fukui et al. 1991). In the brain, L-Kyn is then rapidly taken up by astrocytes and, presumably, by microglial cells. Almost all enzymes of the Kyn pathway are primarily contained in astrocytes and microglial cells (Schwarcz 2004). However, astrocytes do not contain kynurenine 3-hydroxylase and therefore favor KA synthesis, whereas microglial cells have very little kynurenine aminotransferase (KAT) activity which catalyzes the irreversible transamination of L-Kyn to KA and preferentially forms intermediates of the QA (Guillemin et al. 2001). KA can antagonize the neuronal degeneration mediated by excessive stimulation of N-methyl-D-aspartate (NMDA) receptors in vivo (Lekieffre et al. 1990). During the stress response

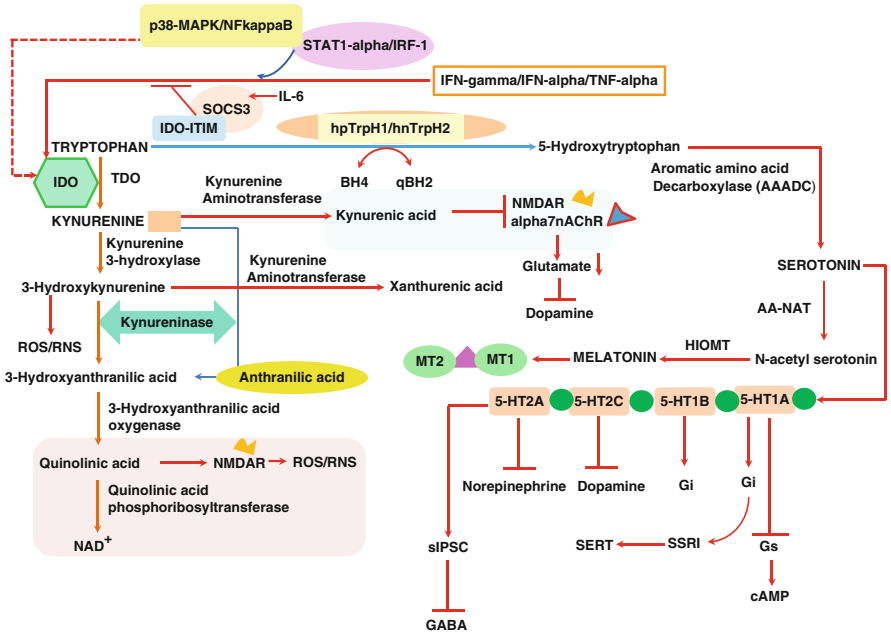

Fig. 1.1 Catabolic cascade of tryptophan metabolism. A simplified version of the kynurenine, serotonin, and methoxyindole pathways demonstrating the major enzymes, intermediates, and receptors. *TDO* tryptophan 2,3-dioxygenase, *IDO* indoleamine 2,3-dioxygenase, *SOCS* suppressor of cytokine signaling, *STAT1-alpha* signal transducer and activator of transcription 1-alpha, *IRF-1* interferon regulatory factor-1, *NF-kappaB* nuclear factor kappa B, *p38-MAPK* p38 mitogen-activated protein kinase, *IDO-ITIM* immunoreceptor tyrosine-based inhibitory motif for IDO, *IFN-gamma* interferon gamma, *IFN-alpha*, interferon alpha, *TNF-alpha* tumor necrosis factor alpha, *IL-6* interleukin-6, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *NMDAR* N-methyl-D-aspartate receptor, *NAD*$^+$ nicotinamide adenine dinucleotide, *hpTrpH1* human peripheral tryptophan hydroxylase1, *hnTrpH2* human neural tryptophan hydroxylase2, *BH4* tetrahydrobiopterin, *qBH2* quinonoid dihydrobiopterin, *alpha7nAChR* alpha7 nicotinic acetylcholine receptor, *AA-NAT* arylalkylamine-N-acetyltransferase, *HIOMT* hydroxyindole-O-methyltransferase, *5-HT2A, 5-HT2C, 5-HT1B, 5-HT1A* serotonin receptors, *Gi* inhibitory G protein, *Gs* stimulatory G protein, *SSRI* selective serotonin reuptake inhibitor, *SERT* serotonin transporter, *sIPSC* spontaneous inhibitory postsynaptic currents, *GABA* gamma-aminobutyric acid, *cAMP* cyclic adenosine monophosphate, *MT1, MT2* membrane-bound melatonin receptors

100- to 1,000-fold elevations in 3HK and QA occur upon microglial cell activation or macrophage infiltration to the brain (Schwarcz 2004). 3HK generates free radical species that can cause oxidative stress and lipid peroxidation. QA-induced excitation and neurotoxicity are mediated by N-methyl-D-aspartate receptor (NMDA) receptors. Because of the absence of effective removal mechanisms for extracellular QA (Foster et al. 1984), its ability to induce concentration-dependent increases in reactive oxidative species (ROS) formation (Santamaría et al. 2001), and its specific interaction with the NMDA receptor (De Carvalho et al. 1996), QA is particularly excitotoxin, whereas KA acts as a competitive blocker of the glycine co-agonist site of the NMDA receptor (Kessler et al. 1989) and as a noncompetitive inhibitor of the

alpha7 nicotinic acetylcholine receptor (alpha7nAChR) (Hilmas et al. 2001). Therefore, KA is considered to be neuroprotective.

In the second metabolic pathway of L-Trp degradation, a small amount of Trp is converted to 5-hydroxytryptophan by the tetrahydrobiopterin (BH4)-dependent tryptophan hydroxylase (TrpH). Subsequently aromatic amino acid decarboxylase (AAADC) catalyzes the second step of serotonin synthesis (Chen and Miller 2012).

The third metabolic pathway of L-Trp degradation involves its conversion into melatonin through the methoxyindole pathway. Biosynthetic steps of melatonin comprise two major rate-limiting enzymes: arylalkylamine-N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT). Although transforming of Trp into melatonin originally occurred in pinealocytes, it has been also detected in many other parts of the body, including the eyes, bone marrow, skin, lymphocytes, and enteroendocrine cells of the gastrointestinal tract (Konturek et al. 2007; Srinivasan et al. 2011). However, cytokine-driven Trp degradation pathways and how they influence each other under different physiologic and pathologic conditions are open to debate.

1.2 Cytokine-Mediated Signaling

Contrary to Kyn, cytokines are relatively large molecules that do not freely pass through the blood–brain barrier. Nevertheless, cytokine signals are able to reach the brain through humoral, neural, and cellular pathways and interact with a cytokine network in the brain consisting of neurons, microglia, and astrocytes (Capuron and Miller 2011). Considering the abovementioned issues, cytokine signals reach to the brain with five different mechanisms: (1) passage of cytokines through the leaky regions of the blood–brain barrier, (2) active transport with cytokine-specific transport molecules on brain endothelium, (3) activation of endothelial cells, (4) transmission of cytokine signals via afferent nerve fibers, and (5) entry into the brain parenchyma and involvement of microglia and astrocytes (Rivest et al. 2000; Konsman et al. 2002; Plotkin et al. 1996).

Cytokine overexpression in the brain due to inflammation is an important factor in the pathogenesis of neurotoxic disorders. However, peripheral and central cytokine compartments appear to be integrated, and their effects might synergize or inhibit each other (Szelényi 2001). Although numerous cytokines and their receptors have been identified in the brain, interleukin-1 (IL-1), IL-6, and TNF-alpha have been implicated in the central control of responses to neuroendocrine, immune, and behavioral alterations (Rothwell et al. 1996). Actually the innate and adaptive immune responses are triggered by microglia in the central nervous system including the release of proinflammatory mediators. In this case toll-like receptor (TLR)-induced activation of microglia and the release of proinflammatory molecules are responsible for neurotoxic processes (Lehnardt 2010). Following activation of the immune system pathways, a number of cytokines alone or in combination including IFN-alpha, IFN-gamma, and TNF-alpha through activation of a number of inflam-

matory signaling pathways such as signal transducer and activator of transcription 1-alpha (STAT1-alpha), interferon regulatory factor (IRF)-1, nuclear factor (NF) kappa B, and p38 mitogen-activated protein kinase (MAPK) stimulate IDO (Fig. 1.1) (Fujigaki et al. 2006). IDO breaks down Trp into Kyn. Kyn is preferentially converted to KA and QA in astrocytes and in microglia, respectively (Schwarcz and Pellicciari 2002). As mentioned above, activated microglia is a chronic source of multiple neurotoxic molecules, including TNF-alpha, nitric oxide (NO), IL-1beta, and ROS, which cause progressive neuron damage (Lull and Block 2010). Initially released cytokines, IL-1beta and TNF-alpha, signal neuroendocrine, autonomic, limbic, and cortical areas of the central nervous system to control neural activity, behaviors, hormone release, and autonomic functions (Lorton et al. 2006).

Acute activation of pattern-recognition receptors, TLR-4 and TLR-2, by exposing to bacterial lipopolysaccharide and peptidoglycan, respectively, also increases circulating levels of IFN-gamma and potently activates IDO in both the periphery and the brain (Lestage et al. 2002). Indeed glial cells and TLRs are vital components of immune response in the central nervous system. Intrauterine infection/inflammation promotes inflammatory processes in glial cells by upregulating cytokines and by activating signaling pathways and transcriptional factors (Yuan et al. 2010).

Response to cytokines seems to be related to the hypothalamic–pituitary–adrenal (HPA) axis activation. Thus IL-1 administration increases noradrenaline secretion and stimulates indoleamine metabolism and most prominently increases the metabolism of serotonin (5-hydroxytryptamine, 5-HT). IL-6 also induces a short-lived activation of the HPA axis. Its effects on Trp and serotonin metabolism are similar to those of IL-1 (Dunn et al. 1999). Furthermore suppressors of cytokine signaling (SOCS) proteins are critical modulators of cytokine-mediated processes, and janus kinase 2 (JAK)–STAT–SOCS signaling modules can have diverse effects on inflammatory diseases (O'Shea and Murray 2008). In the long term, IL-6-dependent upregulation of SOCS3 is responsible for inhibiting the IFN-gamma-driven transcriptional expression of IDO (Fig. 1.1) (Orabona et al. 2004). Hence, an inverse correlation between SOCS3 and IDO expression is evident. Immunomodulatory mechanisms extensively use negative regulators in the form of signaling proteins bearing one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs). IL-6 upregulates SOCS3 and promotes SOCS3 binding to ITIMs of IDO. This process causes shortening of the half-life and proteasome-mediated degradation of IDO (Orabona et al. 2008).

1.3 IDO-Mediated Signaling

The extrahepatic Trp degradation enzyme IDO is induced by IFN-gamma-mediated effects of the STAT1-alpha and IRF-1. The induction of IDO can also be mediated through an IFN-gamma-independent mechanism which may be related to the activity of the p38-MAPKinase pathway and NF-kappaB (Fujigaki et al. 2006). Actually the enzymatic activity of IDO is enhanced in conditions of acute or chronic

activation of the immune system, including immunotherapy, acquired immunodeficiency syndrome, atherosclerosis and coronary heart disease, rheumatoid arthritis, and obesity (Wirleitner et al. 2003). In particular secretion of IFN-gamma is significantly higher in the obese than that of the control subjects. Initially this might be partly dependent on the action of leptin that shifts T-helper (Th) cells toward a Th1 phenotype. A shift to Th1-cytokine profile is dominated by the production of IFN-gamma and is related to insulin resistance in obesity (Pacifico et al. 2006). Hereby T cells and IFN-gamma participate in the regulation of the chronic inflammatory response in obese individuals (Rocha et al. 2008). Chronic inflammation might trigger and maintain the transcriptional induction of IDO-mediated Trp catabolism. Consequently chronic immune activation is the cause for reduced Trp plasma levels in morbidly obese patients (Brandacher et al. 2007). In case of obesity, activation of IDO simultaneously causes excessive synthesis of kynurenines (Brandacher et al. 2006). Furthermore, decrease in Trp levels and subsequent reduction in serotonin due to shift to Kyn pathway provoke satiety dysregulation and ultimately lead to increase in caloric intake and favor obesity (Brandacher et al. 2007). Even after weight reduction in morbidly obese patients, Trp depletion persists (Brandacher et al. 2006). The induction of the Kyn pathway by IDO activity and subsequent decrease in the Trp availability in the brain results in the IFN-alpha-induced depressive symptoms. While Kyn to Trp ratio reflects IDO activity, the Kyn/KA indicates the neurotoxic challenge (Wichers et al. 2005). Higher IDO activity has also been implicated in immune tolerance because it can inhibit the immune response, either by exhausting L-Trp in the medium or producing toxic metabolites. Trp metabolites in the Kyn pathway, such as 3HAA and QA, induce the selective apoptosis in vitro of murine thymocytes and of Th1 but not Th2 cells (Fallarino et al. 2002). As stated above IDO activity is characterized best by the Kyn to Trp ratio, but considering the immune tolerance, it should be correlated with the concentration of immune activation marker such as neopterin (Schröcksnadel et al. 2006).

Until recently, the conversion of Trp to N-formylkynurenine was thought to be performed by either of two enzymes, TDO and IDO. However a third enzyme, indoleamine 2,3-dioxygenase-2 (IDO2) [indoleamine 2,3-dioxygenase-like protein (INDOL1) or proto-indoleamine 2,3-dioxygenase (proto-IDO)], with the Trp degradation activity has been described (Ball et al. 2009). Although IDO2 is not as widely expressed as IDO (IDO1), it is also expressed in antigen-presenting dendritic cells where Trp catabolism drives immune tolerance. Like IDO, IDO2 catabolizes Trp and triggers phosphorylation of the translation initiation factor eIF2alpha. Trp restoration switches off this signaling pathway when activated by IDO, but not IDO2, arguing that IDO2 has a distinct signaling role (Metz et al. 2007). IDO2 has 43 % similarity to classical IDO protein and shares the same critical catalytic residues. Although IDO2 enzyme activity is weaker than IDO, it is less sensitive to dextro-methyl tryptophan inhibition than IDO. Thus a more recent study indicated that human CD4+ and CD8+ T-cell proliferation was inhibited by IDO2, but both levo-1-methyl tryptophan and dextro-methyl tryptophan which are the gold stan-

dard inhibitors of IDO enzyme activity could not reverse IDO2-mediated arrest of cell proliferation, even at high concentrations (Qian et al. 2012). In fact, IDO-dependent tolerogenic effects induced by transforming growth factor beta (TGF-beta) are abolished by IDO gene silencing, but not by the use of 1-methyltryptophan. TGF-beta/IDO/phosphotyrosine phosphatase SHP-1 axis activates the anti-inflammatory NF-kappaB pathway by inhibiting the IL-1 receptor-associated kinase-1 (Orabona et al. 2012).

1.4 Aryl Hydrocarbon Receptor Activation

Gene transcription in response to xenobiotics can be stimulated by aryl hydrocarbon receptor (AhR) which is one of the several ligand-dependent intracellular responsive elements (Denison and Nagy 2003). In this respect Trp photoproducts modulate light-dependent regulation of circadian rhythm through triggering of AhR signaling. Thus these by-products, including 6-formylindolo(3,2-b)carbazole, have high affinity for AhR (Mukai and Tischkau 2007). Ligand activation provokes the AhR to migrate from cytosol to the nucleus and form a complex with the aryl hydrocarbon nuclear translocator (ARNT) that can bind dioxin-responsive elements in the promoter regions of xenobiotic-metabolizing cytochrome P450 (CYP1A) enzymes and 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible poly (ADP-ribose) polymerase (PARP7) (TiPARP) (Diani-Moore et al. 2010). TiPARP is an AhR target gene that can mediate a 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity. TCDD suppresses glucose metabolism-related pathways such as hepatic glucose production, expression of key gluconeogenic genes, phosphoenolpyruvate carboxykinase, and glucose-6-phosphatase activities, and NAD^+ levels. Nicotinamide, a known precursor of NAD^+, is an AhR antagonist. There is a link between signaling pathways for AhR toxicity and nutrient homeostasis NAD^+/peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1alpha), regulator of mitochondrial biogenesis, and function/silent mating type information regulation 2 homolog 1 [(SIRT1), NAD-dependent deacetylase sirtuin-1] via the AhR target gene TiPARP (Diani-Moore et al. 2010). Consequently the effects of TCDD are mediated through its binding to the AhR, as a ligand-activated transcription factor. Subsequent to binding AhR, TCDD inhibits CD4+ T-cell differentiation into T helper (Th)1, Th2, and Th17 effector cells while inducing forkhead transcription factor (Foxp3)-negative and/or preserving Foxp3+ regulatory T cells (Tregs) (Marshall and Kerkvliet 2010). The AhR is a key transcriptional regulator of Th17-cell differentiation. Th17 cells express kynurenine 3-monooxygenase, which is an enzyme involved in catabolism of the Trp metabolite Kyn (Stephens et al. 2013). On the other hand, activation of AhR induces IDO and IDO2 expression of dendritic cells. Hence AhR activation is an important signaling pathway for IDO expression and displays a critical role in the mechanism leading to the generation of Tregs. Eventually induction of Tregs mediates the immune suppression through the activation of AhRs (Vogel et al. 2008).

1.5 Glutamate Neurotransmission

Inflammatory cytokines and their signaling pathways have significant effects on the synthesis, release, and reuptake of serotonin, dopamine, and glutamate (Miller et al. 2013). In this context, higher glutamate receptor, mGluR1alpha, and lower guanine nucleotide-binding protein (G-protein)-coupled receptor, regulator of G-protein signaling 4 (RGS4) mRNA levels, play an important role in regulating gamma-aminobutyric acid (GABA) and glutamate neurotransmission in the brain cortex by initiating intracellular signaling cascade (Fig. 1.1). Suppression of GABA release in GABA neurons of the prefrontal cortex and diminished glutamate neurotransmission due to NMDA receptor hypofunction are evident in certain cognitive deficits (Volk et al. 2010). Activation of serotonin 5-HT1A receptors or dopamine D (4) receptors downregulates the function of NMDA receptor channel in pyramidal neurons of the prefrontal cortex. Blocking RGS4 function significantly potentiates the 5-HT1A regulation of NMDA receptor. Conversely, overexpression of RGS4 couples RGS4 to serotonin signaling in cortical neurons and attenuates the 5-HT1A effect (Gu et al. 2007). Furthermore elevated levels of KA in the prefrontal cortex may contribute to the abnormal glutamatergic and nicotinic functions in cognitive deficits (Schwarcz et al. 2001; Erhardt et al. 2009). This concept is partly based on the finding that endogenous KA is an astrocyte-derived metabolite of Trp degradation via Kyn pathway (Kiss et al. 2003). As already mentioned above, Trp degradation products along the Kyn pathway include three neuroactive metabolites: the neuroinhibitory agent KA, the free radical generator 3HK, and the excitotoxin QA. Inhibition of kynurenine 3-hydroxylase shifts Kyn pathway metabolism from 3HK formation toward enhanced KA formation in the mature brain. Therefore acute kynurenine 3-hydroxylase inhibition effectively increases KA formation (Ceresoli-Borroni et al. 2007). Following the systemic administration of Kyn, a significant reduction in prefrontal glutamate occurs. Alpha7nAChRs constitutes a crucial link between excessive KA formation and reduction in glutamate. Subsequent to peripheral administration, Kyn penetrates the blood–brain barrier and dose dependently raises extracellular KA levels in the prefrontal cortex. Actually systemic Kyn administration duplicates the reduction in extracellular glutamate seen after a local perfusion of Kyn in the prefrontal cortex. Resultant KA-induced reduction in prefrontal glutamate levels emerges as a result of alpha7nAChRs inhibition (Konradsson-Geuken et al. 2010). The cognitive deficits are likely related to abnormal glutamatergic and cholinergic neurotransmission in the prefrontal cortex. These defects may be secondary to increased levels of the astrocyte-derived KA, which inhibits alpha7A-ChR and may thereby reduce glutamate release. Fluctuations in endogenous KA formation bidirectionally influence cortical glutamate concentrations. Consequently selective attenuation of cerebral KA production by increasing glutamatergic tone might improve cognitive functions (Wu et al. 2010). Endogenous glutamate acts locally within the striatum via ionotropic receptors to control impulse-independent and transporter-mediated mode of dopamine release. When the KA inhibits the release of glutamate, low glutamate level may inhibit the secretion of dopamine

(Borland and Michael 2004). Modulation of glutamate release by the alpha7nAChRs on striatal glutamatergic terminals, in turn, activates presynaptic ionotropic glutamate receptors on striatal dopaminergic nerve terminals (Kaiser and Wonnacott 2000). Decrease in extracellular levels of striatal dopamine due to KA-induced blockade of alpha7nAChRs can be enhanced by stimulating the endogenous formation of KA via kynurenine 3-hydroxylase inhibition (Rassoulpour et al. 2005). Blood-derived Kyn rapidly accesses to KAT II-containing astrocytes, and KA synthesis takes place in astrocytes (Guidetti et al. 2007). Fluctuations in KA indirectly regulate extracellular dopamine levels in the striatum. Acute inhibition of KAT II reduced the de novo synthesis of KA; thus, KAT II is a critical determinant of functionally relevant KA fluctuations (Amori et al. 2009). On the other hand, cytokine-activated Kyn pathway not only depletes Trp but also generates neuroactive metabolites that can significantly influence the regulation of dopamine and glutamate (Miller et al. 2013). Excitotoxic damage is a common pathologic event in a number of neurologic diseases occurring after accumulation of excess extracellular glutamate in the central nervous system and subsequent overstimulation of glutamate receptors. High extracellular glutamate increases risk of glutamate excitotoxicity. However, astrocytes can take up synaptically released glutamate and maintain glutamate homeostasis (Pitt et al. 2003). Nevertheless astrocytes can release glutamate together with the various chemical transmitters which may mediate communication between neurons and astrocytes (Ida et al. 2008). There are complex cross talks between microglia and astrocytes during neuroinflammatory insults which would influence glutamate-dependent responses in astrocytes (Tilleux et al. 2007). Astrocytosis due to the destruction of neurons is accompanied by microglial activation. Actually in proinflammatory processes, activated microglia stimulates the increase in number of astrocytes and enhances mRNA expression of IL-6 (Röhl et al. 2007). Cytokine release from microglia also causes downregulation of mGluR5, mGluR5 protein, and mRNA expression in astrocytes (Tilleux et al. 2007). On the other hand, the inhibition of inducible nitric oxide synthase (iNOS) eliminates the cytokine-induced enhancement of glutamate release, whereas treatment with a NO donor, even in the absence of cytokines, increases glutamate release (Ida et al. 2008). Nonspecific NOS inhibitors decrease the homocysteine-induced lipid peroxidation more than does the selective neuronal nitric oxide synthase (nNOS) inhibitor. In this case homocysteine can induce oxidative injury to nerve terminals, and this effect involves the NMDA receptor stimulation, NOS activation, and associated free radical formation (Jara-Prado et al. 2003). Higher concentration of QA induces concentration-dependent increases in ROS formation in all synaptosomes, but the increase in the production of peroxidized lipids only emerges in the striatum and the hippocampus. These findings suggest that the excitotoxic action of QA involves regional selectivity in the oxidative status of brain synaptosomes (Santamaría et al. 2001). However, NMDA receptor antagonists completely inhibit the increase of QA-induced lipid peroxidation (Santamaría and Ríos 1993).

1.6 Serotonin Neurotransmission

Trp is only available in the diet. It is therefore likely that excessive diet restriction and malnutrition decrease brain serotonin stores. Evidence shows that diet restriction-induced exaggerated feedback control over serotonin synthesis and the smaller availability of Trp decrease serotonin neurotransmission at postsynaptic sites, leading to hyperactivity, depression, and behavioral disorders (Haleem 2012). Conversely excessive L-Trp ingestion raises brain Trp levels and stimulates its conversion to serotonin in neurons. Adverse effects may be seen at higher doses (70–200 mg/kg) and include tremor, nausea, and dizziness. When Trp is taken alone or with a drug that enhances serotonergic effects, it may provoke side effects (Fernstrom 2012). In fact serotonin neurotransmission comprises multiple consecutive processes including synthesis, storage/release, signaling, reuptake, and metabolism, of which the first step, synthesis, is a critical modulator of serotonin neurotransmission (Chen and Miller 2012). Serotonin is synthesized by a two-step enzymatic reaction. Firstly, the essential amino acid L-Trp is hydroxylated into 5-hydroxy-L-tryptophan by the limiting enzyme TrpH. Two isoforms of TrpH enzyme, TrpH1 and TrpH2, have been characterized so far: TrpH1 is mainly expressed in the gastrointestinal tract and the pineal gland, whereas TrpH2 is primarily expressed in the central nervous system (Watts 2009). TrpH2 polymorphisms directly influence serotonergic function and thus impact on mood disorders. TrpH2-deficient mice display alterations in anxiety-like behavior which is accompanied by adaptational changes of 5-HT1A receptors and its associated signaling pathway (Waider et al. 2011). Genetic inactivation of TrpH2 function in mice led to the identification of phenotypic changes, ranging from growth retardation and late-onset obesity to enhanced conditioned fear response, increased aggression, and depression-like behavior (Lesch et al. 2012). In fact TrpH, a BH4-dependent amino acid hydroxylase, is the key regulator of serotonin biosynthesis (Carkaci-Salli et al. 2006). 5-Hydroxy-L-tryptophan is converted to serotonin by AAADC. Actually AAADC deficiency is a severe genetic neuro-metabolic disorder that is characterized with combined deficiency of serotonin, dopamine, and catecholamines (Manegold et al. 2009). Furthermore endothelial AAADC plays an important role in cardiac synthesis of serotonin and possibly in serotonin-dependent regulation of NO generation. 5-Hydroxy-l-tryptophan administration in mice increased phosphorylation of aortic endothelial NOS (eNOS) at Ser-1177 as well as accumulation of nitrates in cardiac tissue (Rouzaud-Laborde et al. 2012). eNOS is known to be stimulated by serotonin via 5-HT1B receptor/eNOS pathway (McDuffie et al. 1999). Phosphorylation of eNOS produces NO without requiring any changes in $[Ca^{2+}]i$ (Boo et al. 2003). Actually 5-HT2B receptor stimulation plays a critical role in the phosphorylation of both extracellular signal-regulated kinase 1/2 (ERK1/2) and eNOS (Asada et al. 2009). In human endothelial cells, serotonin markedly stimulates eNOS expression and the phosphorylation of eNOS, Akt, and ERK1/2. Consequently serotonin induces angiogenesis through activation

of Akt in endothelial cells. Selective inhibition of 5-HT2A causes induction of the eNOS/Akt pathway via the endothelial 5-HT1B receptors and enhances vasodilation in diabetes mellitus (Iwabayashi et al. 2012).

Increased activity of the liver enzyme TDO is stimulated by an excess of circulating corticosteroids. In hypercortisolemic conditions, metabolism of Trp turns to the Kyn pathway from serotonin synthesis. Upregulation of the Trp-Kyn pathway and diminished availability of Trp are the primary causes of serotonin deficiency (Oxenkrug 2010). Hypercortisolism affects the gene encoding TrpH2 and the expression of TrpH2. Also chronic corticosterone intake disrupts the diurnal variation of TrpH2 mRNA expression in the brain stem dorsal raphe nucleus and of plasma adrenocorticotropin and corticosterone levels in a dose-dependent manner (Donner et al. 2012). The hippocampus plays a central role in regulation of the HPA axis and release of endogenous glucocorticoids. Exposure to serotonin increases the glucocorticoid receptor mRNA levels in hippocampal neurons. Eventually synthetic and endogenous glucocorticoids, as well as serotonin, influence glucocorticoid receptor expression during hippocampal development (Erdeljan et al. 2005). Stress significantly increases extracellular serotonin release in the basolateral amygdaloid nucleus and the prefrontal cortex (Kawahara et al. 1993). Indeed serotonin dramatically enhances frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) in the basolateral amygdala through 5-HT2A receptors. Because of the basolateral amygdaloid GABAergic inhibition is blocked by selective 5-HT2A receptor antagonists, the stress-induced effect appeared to be specific to 5-HT2A receptor downregulation (Jiang et al. 2009).

Monoaminergic neurotransmission involves functional interactions between serotonin, norepinephrine, and dopamine systems. First of all serotonin system exerts negative effect on norepinephrine system through 5-HT2A and on dopamine system through 5-HT2C receptor-mediated mechanisms. Positive and negative effect of norepinephrine system on serotonin neurotransmission is mediated through alpha1- and alpha2-adrenergic receptors, respectively (Hamon and Blier 2013). Actually BH4 is an essential cofactor in the synthesis of serotonin, dopamine, epinephrine, norepinephrine, and NO. BH4 availability influences many cells, including neurons. Following peripheral nerve damage, BH4 dramatically increases in sensory neurons and causes pain hypersensitivity (Latremoliere and Costigan 2011). Fatigue and impaired executive functions are commonly linked to disturbed cerebral dopaminergic and noradrenergic neurotransmission. Moreover selective serotonin reuptake inhibitors (SSRIs) contribute to fatigue, which is a common residual symptom associated with depression (Stenman and Lilja 2013). During the prolonged exercise, fatigue is attributed to the muscle glycogen depletion. “Central fatigue hypothesis” previously was based on the increase in the concentration of brain serotonin during exercise. However according to the revised central fatigue hypothesis, an increase in central ratio of serotonin to dopamine is associated with feelings of tiredness and lethargy (Meeusen and Piacentini 2003). Actually a complex interplay between the different neurotransmitter systems induces fatigue: dopamine and noradrenaline rather than serotonin alone (Roelands and Meeusen 2010).

Diet restriction-induced exaggerated feedback control over serotonin synthesis and the reduced availability of Trp decrease serotonin neurotransmission at postsynaptic sites. A compensatory upregulation of postsynaptic 5-HT1A receptors and hypophagic serotonin receptors may be involved in suppression of appetite (Haleem 2012). In this case although the levels of Trp in the plasma and of serotonin in the hypothalamus decrease, no effect is found on the levels of Trp in the hypothalamus. Diet restriction-induced decrease of serotonin is due to an increase in the responsiveness of negative feedback control over serotonin, not due to smaller availability of Trp (Haleem 2009). Likewise 20–25 % reduction in body weight due to food restriction decreases serotonin concentration in the brain of male but not female rats (Haider and Haleem 2000). Conversely in sugar-diet-treated rats, when the cumulative food intakes increase, body weights decrease. Hyperphagic effects of selective 5-HT1A agonist are greater in normal diet than sugar-diet-treated rats. However serotonin and 5-hydroxyindole acetic acid levels are not changed. Desensitization of pre- as well as postsynaptic 5-HT1A receptors in rats treated with sugar diet causes the precipitation of obesity (Jabeen and Haleem 2008). Actually long-term consumption of sugar diet results in a decrease in the effectiveness of pre- as well as postsynaptic 5-HT1A receptor-dependent responses (Inam et al. 2006). Malnourished offspring have a significant elevation of L-Trp, TrpH activity, and serotonin in the brain stem. Both isoforms of TrpH (TrpH1 and TrpH2) are expressed at birth in both groups; however, TrpH1 expression is significantly higher in offspring with intrauterine malnutrition when compared to the controls. Malnourished offspring show reduced expression of TrpH2 compared to controls. Thus it has been confirmed that intrauterine malnutrition produces an increase in serotonin in the brain stem and also shows increased expression of TrpH1 at birth, with decreased expression of TrpH2 (Manjarrez-Gutiérrez et al. 2012).

The dorsal raphe nucleus (DRN) is the largest serotonin-containing nucleus in the brain and has extensive ascending projections that innervate most forebrain structures. Targets of DRN innervation receive input from both serotonergic and nonserotonergic cells. Selective serotonergic neurotoxins, including 5,7-dihydroxytryptamine (5,7-DHT), have been shown to disrupt axonal transport in serotonergic neurons (Callahan et al. 2001; Araneda et al. 1980). Human LIM homeobox transcription factor 1-beta (Lmx1b)-encoded gene is essential for the development of central serotonergic neurons. This gene is required for the normal biosynthesis of serotonin in the adult brain and for regulating normal functions of central serotonergic neurons. Lmx1b deletion in the adult brain leads to reduction in central serotonin levels. However the overall number of serotonergic neurons is not affected by deleting Lmx1b, and Pet1 promoter expression in the adult brain is independent of Lmx1b. Reduction in central serotonin levels seems to be the consequence of TrpH2 downregulation (Song et al. 2011). In fact Pet1 in the brain is necessary for terminal differentiation of serotonergic neuron phenotype during embryonic development (Hendricks et al. 2003). Considering the serotonergic signaling mechanisms, ETS domain transcription factor Pet1 is also required for maintaining the serotonergic neurotransmitter system during adult stages as well as for expression of the presynaptic 5-HT1B autoreceptor. Therefore adult central nervous system expression of

TrpH2 and serotonin transporter (SERT) is restricted to Pet1-expressing serotonin neurons and is rate limiting for the essential serotonergic functions of serotonin synthesis and reuptake (Liu et al. 2010). Pet1 RNA co-localizes with TrpH-positive neurons in raphe nuclei. Loss of Pet1 in the serotonergic neurons leads to a decrease of TrpH2 expression but no change in Lmx1b expression (Song et al. 2011). Virtually serotonergic and nonserotonergic axons innervate distinct but partially overlapping fields within vestibular nuclei (Halberstadt and Balaban 2007). Both local GABAergic and glutamatergic cells project onto DRN serotonergic neurons (Jolas and Aghajanian 1997). 5-HT1A receptors are present on nonserotonergic as well as serotonergic DRN neurons. While the majority of serotonin-immuno-positive cells are double-labeled for 5-HT1A receptor, small but significant population of serotonin-immuno-negative cells express the 5-HT1A receptor (Kirby et al. 2003). Both 5-HT1A and alpha1b adrenergic mRNA are highly expressed throughout the DRN, and the vast majority of serotonergic neurons express both receptors. A smaller percentage of GABAergic neurons also express 5-HT1A or alpha1b adrenergic mRNA. A small amount of catecholaminergic cells express either 5-HT1A or alpha1b adrenergic mRNA (Day et al. 2004).

Hence, serotonin not only affects neuronal excitability through activating postsynaptic receptors (Guo and Rainnie 2010) but also affects presynaptic excitatory or inhibitory neurotransmission in the central nervous system, because of the serotonin activating 5-HT1A and/or 5-HT1B receptors located on the presynaptic terminals. Serotonin exerts significant control over the synaptic inputs and the autonomous activity of subcortical pallidal neurons (Bouryi and Lewis 2003; Hashimoto and Kita 2008). The serotonin receptors have been divided into 7 subfamilies, 6 of which include 13 different genes for G-protein-coupled receptors (GPCR). Postgenomic modifications create 20 more G-protein-coupled serotonin receptors. Consequently there are at least 30 distinct serotonin receptors that signal through G proteins (Raymond et al. 2001). 5-HT1A and 5-HT1B receptors interface primarily with inhibitory G proteins (Gi) to decrease adenylyl cyclase activity. Subsequently the action of the SSRI is mediated through the 5-HT1A receptor (Blier and Ward 2003; Monaca et al. 2003). While SSRI inhibiting the SERT density and function, it maintains the normal firing rates and release of serotonin and immediately increases activation of postsynaptic serotonin receptors (Nemeroff and Owens 2003). All of the seven specific serotonin receptors mediate SSRI effects; however, the second-class receptors, 5-HT6 and 5-HT7, primarily interact with stimulatory G proteins (Gs) to increase adenylyl cyclase activity. In particular the 5-HT6 receptor is involved in neuronal serotonergic transmission and may have effects on anxiety and mood (Yoshioka et al. 1998). The 5-HT7 receptor is involved in hippocampal function (Gill et al. 2002), and has been implicated in the regulation of the glucocorticoid receptors (Laplante et al. 2002). SSRIs also affect the function of the 5-HT2C receptor (Bristow et al. 2000) with some adverse effects potentially mediated by 5-HT2C. Other than for the 5-HT3 receptor, most of the downstream effects of serotonin are mediated by G proteins (Raymond et al. 2001). Actually G proteins are a family of guanine nucleotide-binding regulatory components that couple neurotransmitter receptors to various types of intracellular effector systems. Gs/Gi

mediates stimulation/inhibition of adenylate cyclase system, which forms cyclic adenosine monophosphate (cyclic AMP) as a second messenger (Lesch and Lerer 1991). There are 16 genes for G-protein alpha subunits, 5 for beta, and 12 for gamma (Downes and Gautam 1999). SSRIs have been associated with increased transcription of adenylyl cyclase 1. 5-HT1A receptor mediates inhibition of basal and Gs-induced cAMP formation in the absence of adenylyl cyclase 2. 5-HT1A activation decreases activity of neuronal adenylyl cyclase 2 (Albert et al. 1999). Among serotonin receptors, the 5-HT3 receptor is a member of the Cys-loop family of ligand-gated ion channels and located in both the peripheral and central nervous systems. Chronic activation of 5-HT3 receptor produces significant desensitization of 5-HT3 and postsynaptic 5-HT1A receptors without major changes in the expression of SERT and TrpH-2 genes (Kondaurova et al. 2012).

Human SERT reuptakes biogenic amine neurotransmitters following release in the nervous systems and terminates the action of serotonin (Murphy et al. 2004).

SERTs are tightly controlled by multiple signaling pathways, including G-protein-coupled receptor-linked pathways (Blakely et al. 2005). Two protein kinase G (PKG)-dependent pathways have been proposed to support rapid SERT regulation by A3 adenosine receptors (ARs). The first enhances SERT surface trafficking to clear serotonin following vesicular release, and the second is a separate, p38 MAPK-dependent process which augments SERT intrinsic activity (Zhu et al. 2004). p38 MAPK activation downstream of PKG via SERT catalytic regulatory pathway in a trafficking-independent mode is distinct from events controlling SERT surface density. Protein phosphatase 2A is a critical component of the pathway responsible for p38 MAPK upregulation of SERT catalytic activity (Zhu et al. 2005). Thus A3 ARs activation stimulates serotonin uptake via PKG- and p38 MAPK-linked pathway (Zhu et al. 2004). MAP kinase kinase (MAPKK) superfamily molecules, MKK3, MKK3b, and MAPKK6, can act as a specific activator for p38. Furthermore as a major activator for p38, the MAPKK6/p38 kinase cascade is activated strongly by TNF-alpha and H_2O_2 (Moriguchi et al. 1996). Eventually PKG-linked and p38 MAPK-linked pathways provide a rapid increase in SERT surface expression and function. In contrast, the activity of protein phosphatase 2A inhibitors attenuates MAPK or other signal transduction pathways and facilitates the stimulation of serotonin transport (Zhu et al. 2005), whereas activated protein kinase C (PKC) interacts with SERT and alters the subcellular localization of the transporter resulting in a reduction of serotonin transport. SERT proteins are rapidly phosphorylated in parallel with transporter redistribution and loss of functional uptake capacity. Indeed loss of surface SERT protein after PKC activation reflects transporter redistribution rather than irreversible loss of transporter protein via degradation (Haase et al. 2001; Blakely et al. 1998). In brief, one of the well-known mechanisms in the termination of the stimulation of monoamine neurotransmitters is the removal from the synapse by transporter molecules. Transporters are located within the plasma membrane of presynaptic cells and may be readily regulated by a variety of receptor-mediated intracellular signals.

SERT can be rapidly regulated by a membrane-bound G-protein-coupled receptor and this occurs via NO and cyclic guanosine monophosphate (cGMP). A3 AR is

coupled to NO and cGMP (Miller and Hoffman 1994). ARs, A1, A2A, A2B, and A3, are widely distributed throughout the brain and periphery (Fredholm et al. 2001) and have been implicated in a variety of physiological and pathological conditions, including modulation of neural signaling (Okada et al. 1999). It was shown that IL-1 receptors couple via the p38 MAPK pathway to activate SERT. Regulation of SERT is achieved by the multiple AR subtypes in the brain (Fredholm et al. 2005). In particular, A3 AR activation stimulates SERT function in the brain. Inhibition A3 ARs may be able to selectively diminish elevations in SERT activity in a region-dependent manner without affecting basal serotonin clearance or steady-state serotonin levels (Zhu et al. 2007). Consequently A3 AR activation leads to the induction of the serotonin transport by a p38 MAPK-dependent pathway. Stimulation of SERT by A3 AR activation in the brain suggests a functional relationship between A3 AR activation, SERT activity, and serotonin signaling (Zhu et al. 2007). SERT does not have a significant contribution to serotonin uptake in vascular smooth muscle cells of human brain and peripheral vessels. The lack of SERT activity in these vascular smooth muscle cells suggests that different mechanisms may be responsible for serotonin uptake in different vascular beds. In this regard more likely candidates responsible for non-SERT-dependent serotonin uptake are organic cation transporters (OCTs). The polyspecific organic cation transporters OCT1, OCT2, and OCT3 mediate bidirectional diffusion of small organic cations such as acetylcholine and monoamine neurotransmitters (Lee et al. 2009). The mRNA of OCT3 is also called "extraneuronal monoamine transporter" and is expressed in vascular smooth muscle cells of the human brain but not OCT1 and OCT2. In addition to OCT3, most probably the mRNA of plasma membrane monoamine transporter is expressed and contributes to serotonin uptake in these cells (Li et al. 2013).

1.7 Desensitization and Re-sensitization of Serotonin Receptors

Desensitization and re-sensitization of GPCRs can modulate receptor responsiveness in regulation of many cellular functions. These processes depend on the availability of functional receptors at the cell surface and on their mode of activation. Chronic stimulation of receptor agonists causes GPCR desensitization. Actually receptor desensitization can occur by a series of events such as downregulation of the receptor, internalization of the receptor, or uncoupling of the receptor from its signaling proteins (Sibley et al. 1987; Damjanoska et al. 2004). Desensitization process is well described for serotonin receptors. The 5-HT1A is expressed both as a pre- and postsynaptic receptor in neurons. The presynaptic receptor is preferentially desensitized compared to postsynaptic receptors. Desensitization is dependent on internal Ca^{2+} ions and PKC-dependent agonist-induced uncoupling of the 5-HT1A receptors (Wu et al. 2013). In a similar manner chronic treatment with 5-HT2A/2C receptor agonists disrupts the receptor-to-G-protein interaction. Possible

mechanism underlying this desensitization process may be phosphorylation of the 5-HT2A receptor and/or G alpha q/11 proteins. The desensitization of 5-HT2A receptors is most likely due to posttranslational modifications of the 5-HT2A receptor and G alpha q/11 proteins altering the 5-HT2A receptor-to-G alpha q/11 protein interface (Damjanoska et al. 2004). Activation of 5-HT2A receptors stimulates activation of G alpha q/11, which in turn activates effector enzyme phospholipase C (PLC). Desensitization of 5-HT2A receptor-stimulated PLC activity is dependent on activation of the JAK–STAT pathway and is associated with increases in RGS7 protein levels. This increase in RGS7 protein plays a role in the desensitization of 5-HT2A receptor signaling by terminating the activated G alpha q/11 proteins (Singh et al. 2009). Recycled internalized receptors return to the cell surface and recover their ability to couple with G proteins that involve in the re-sensitization process (Bhattacharyya et al. 2002). On the other hand in the absence of serotonin, PKC-activated receptors also recycle to the cell surface. Even in the presence of 5-HT, blocking the activation of PKC prevents the receptor internalization. Therefore PKC activation is necessary for the internalization of serotonin receptors. In order to internalize the receptor, PKC-mediated phosphorylation occurs in the absence of serotonin or G-protein activation (Bhattacharyya et al. 2002). Eventually 5-HT2A receptors become available again at the cell surface after both serotonin- and PKC-mediated processes.

1.8 Enterochromaffin Cell and Serotonergic Signaling

Actually one of the predominant sites of serotonin synthesis, storage, and release is the enterochromaffin (EC) cells of the intestinal mucosa. Serotonin released from EC cells activates neural reflexes associated with intestinal secretion, motility, and sensation. In this respect 5-HT3 and 5-HT4 are the two important receptors for serotonin signaling in pathologic conditions (Costedio et al. 2007). Hence serotonin is not taken up by mucosal nerve fibers (Gershon and Sherman 1982). EC cells activate both intrinsic and extrinsic primary afferent neurons through their release of serotonin. Upon stimulation of 5-HT1P receptors by serotonin, submucosal intrinsic primary afferent neurons trigger peristaltic and secretory reflexes. Serotonin also enhances the release of transmitters through 5-HT4 receptors in prokinetic reflex pathways. However in inflammatory conditions, serotonergic signaling is specifically diminished within the mucosa due to decrease of transcripts encoding tryptophan hydroxylase-1 and 5-HT reuptake transporter. Stimulation of serotonin secretion and desensitization of its receptor can account for the symptoms seen in diarrhea-predominant and constipation-predominant irritable bowel syndrome, respectively (Gershon 2004).

Th17 cells, a novel subtype of proinflammatory T-helper cell, seem to have an important role in the development of inflammatory bowel diseases (Brand 2009). Increase in the plasma IL-17 and mRNA levels of the Th17-specific transcription factor, retinoic acid-related orphan receptor gammat (RORgammat), is an evident

finding in patients with active ulcerative colitis. The levels of p-STAT3 and p-STAT5 in peripheral blood mononuclear cells, as well as the ratio of p-STAT3/p-STAT5, are also elevated in these patients. Rising circulating Th17 and the aberrant activation of the STAT pathway may be effective in the progression of inflammatory bowel diseases (Dong et al. 2013). Despite the importance of STAT3 signaling, it should be emphasized that this stimulus alone is not sufficient to drive Th17 differentiation. STAT3 is necessary but not sufficient for IL-17 expression (Chen et al. 2007). Thus IL-27 inhibits the development of proinflammatory Th17 cells by suppressing in a STAT1-dependent manner the expression of the Th17-specific transcription factor of RORgammat (Diveu et al. 2009). Stimulation of intestinal epithelial cells with IL-27 results in the activation of the MAPK signaling pathways p38 and ERK as well as of the phosphoinositol-3-kinase (PI3K)-Akt pathway. IL-27 also activates the transcription factors STAT1, STAT3, and STAT6. IL-27-mediated IDO1 enzymatic activity is also strongly dependent on STAT1 as determined by the IL-27-induced Kyn levels. While silencing of STAT3 has a weak positive effect on IDO1 mRNA and protein expression, silencing of STAT6 does not influence IL-27-activated IDO expression and enzymatic activity (Diegelmann et al. 2012). STAT1 DNA-binding site in the IDO promoter is identical to a described STAT1-binding site following IFN-gamma stimulation (Chon et al. 1995). The response of the IDO gene promoter region to IFN-gamma is dependent on two regulator elements IFN-gamma-activated site and the IFN-stimulated response element. The location of the IFN-gamma-activated site-related sequence is important in relation to the IFN-stimulated response element sequence for a response to IFN-gamma. A cooperative role of IFN-gamma-IRF1 and STAT1 is described in the induction of the IDO1 gene by IFN-gamma (Chon et al. 1996).

EC cells are the sensory transduction elements in the gastrointestinal mucosa and respond to chemical and mechanical stimuli by releasing serotonin. The uptake of serotonin by SERT-dependent mechanisms is a key factor in controlling serotonin availability in the gastrointestinal tract. EC cell numbers increase in the ileum of these rats (Bertrand et al. 2011). In obesity a significant decrease in the total number of EC cells per crypt and a reduction in the levels of serotonin occur in western type of diet-fed rats compared with in chow-fed rats. SERT protein levels and SERT-dependent uptake of serotonin are constant. Although there is no change in tryptophan hydroxylase 1 mRNA, SERT mRNA increases. Reduction of serotonin availability is associated with decreased intestinal motility in vivo (Bertrand et al. 2012). The enteric SERT is the only transporter expressed in the bowel with a high affinity for serotonin. In SERT deficient bowel expresses dopamine transporter (DAT) and OCT3 that transport serotonin, although they lack the selectivity and affinity of SERT for 5-HT. DAT and the OCTs might thus compensate, at least partially, for the absence of SERT. Although there is an excessive increase in colonic motility and watery diarrhea in the majority of SERT-deficient subjects, a striking decrease in colonic motility and constipation may be evident in a minority of these animals (Chen et al. 2001). No difference in OCT1 expression is detected between SERT deficient and control animals. Upregulation of OCT3 expression and enhanced low-affinity serotonin uptake may limit the adverse effects of elevated extracellular

serotonin in the absence of SERT (Schmitt et al. 2003). Consequently OCT3 contributes to serotonin clearance if the expression of the SERT is low or absent.

As mentioned already, OCTs and the plasma membrane monoamine transporters are capable of clearing biogenic amines from extracellular fluid and may serve to buffer the effects of selective serotonin reuptake inhibitors (Daws 2009). Proliferation of intestinal mucosal cells is significantly greater in mice with lack of the serotonin reuptake transporter and in mice given selective serotonin reuptake inhibitors. On the other hand serotonin promotes growth and turnover of the intestinal mucosal epithelium. These processes are mediated by neuronal rather than mucosal serotonin (Gross et al. 2012). Likewise, constitutive gastrointestinal motility depends on neuronal rather than on mucosal serotonin, and the development of dopaminergic, GABAergic, and calcitonin gene-related peptide (CGRP)-expressing enteric neurons requires neuronal serotonin (Li et al. 2011).

Since EC cells are sensitive to oxygen, alterations in oxygen levels differentially activate hypoxia-inducible factor 1alpha (HIF-1alpha) and TpH1, as well as NF-kappaB signaling. Changes in the amount of serotonin production and secretion determine the oxygen sensing role of EC cells. Decrease in oxygen concentration elevates serotonin secretion by 2–3.2-fold, as well as protein levels of HIF-1alpha by 1.7–3-fold. Whereas rising of the oxygen concentration to 100 % reduces serotonin secretion, inhibits hypoxia transcriptional response element (HRE)-mediated signaling, and significantly lowers HIF-1alpha levels. NF-kappaB signaling is also elevated during hypoxia by 1.2–1.6-fold (Haugen et al. 2012).

1.9 Taste Receptor Signaling

GPCRs are key transmembrane recognition molecules for regulatory signals such as light, odors, taste hormones, and neurotransmitters. In addition to activating G proteins, GPCRs associate with a variety of GPCR-interacting proteins (GIPs) (Bockaert et al. 2010). GIPs influence the targeting, trafficking, and signal transduction properties of serotonin receptors (Marin et al. 2012). Three currently recognized types of taste bud cells exhibit distinct morphological features and cellular functions: nucleoside triphosphate diphosphohydrolases (NTPDase)2 and glial glutamate/aspartate transporter (GLAST) co-localized type I cells (Bartel et al. 2006), the taste-specific G-protein α-gustducin expressed type II cells (Yang et al. 2000a), and serotonin, neuron-specific enolase, ubiquitin carboxyl terminal hydrolase, and neural cell adhesion molecule expressed type III taste cells (Yee et al. 2001). Adenosine triphosphate (ATP) activated presynaptic (type III) cells release serotonin and norepinephrine following ATP secretion from receptor (type II) taste bud cells during taste stimulation. Subsequently, serotonin released from presynaptic (type III) cells provides a negative paracrine feedback onto receptor cells by activating 5-HT1A receptors. Finally, taste-evoked Ca^{2+} mobilization is

inhibited from receptor cells (Huang et al. 2009). Salts and acids utilize apically located ion channels for transduction, while bitter, sweet, and umami (L-glutamate and 5′-ribonucleotides) stimuli utilize GPCRs and second messenger signaling pathways (Kinnamon 2012). Two classes of taste GPCRs have been identified: the first group type 1 taste receptors (T1Rs) for sweet and umami (L-glutamate and 5′-ribonucleotides) stimuli and the second group T2Rs for bitter stimuli (Bachmanov and Beauchamp 2007). Transient receptor potential cation channel subfamily M member 5 (melastatin 5 or TRPM5) depolarizes taste cells. TRPM5 leads to the release of ATP, which activates ionotropic purinergic receptors on gustatory afferent nerve fibers (Finger et al. 2005).

Cells expressing alpha-gustducin and phospholipase C isoform beta2 (PLC-beta 2) localize at multiple cardiorespiratory and CO_2/H^+ chemosensory sites. Especially in the medullary raphe, alpha-gustducin and PLC-beta2 are co-localized with TrpH-immunoreactive serotonergic neurons. It has been shown that different bitter-responsive T2Rs associate with G-protein alpha-gustducin, PLC-beta2, and TRPM5 in the brain stem of rats (Dehkordi et al. 2012). Mammalian taste cells normally contain serotonin, and taste cells can take up 5-hydroxytryptophan and convert it to serotonin. Subsequently serotonin functions as a neuromodulator or neurotransmitter in vertebrate taste buds. Diffuse, cytoplasmic syntaxin-1-like immunoreactivity is present in type III cells, and taste cell synapses use syntaxin-1 for neurotransmitter release (Kim and Roper 1995; Yang et al. 2000b; Yang et al. 2007). Serotonin-like immunoreactivity cells resemble syntaxin-1-like immunoreactivity cells in both shape and structure and have been shown to co-localize with a subset of syntaxin-1-like immunoreactive type III cells. Synapses are only observed from type III taste cells onto ionotropic ligand-gated ion channel receptors (P2X2)-like immunoreactivity nerve processes (Yang et al. 2012).

Synapses between gustatory receptor cells and primary sensory afferent fibers transmit the output signal from taste buds to the central nervous system. Actually several transmitter candidates have been proposed for these synapses, including serotonin, glutamate, acetylcholine, ATP, and peptides. Serotonin is one of the important neurotransmitters released by taste cells in response to gustatory stimulation (Huang et al. 2005). However, only serotonin and ATP are secreted by separate classes of taste cells. While presynaptic (type III) taste cells release serotonin upon stimulation (Huang et al. 2007), receptor (type II) taste bud cells secrete ATP during taste stimulation. In turn, ATP activates adjacent presynaptic (type III) cells to release serotonin and norepinephrine. Serotonin released from presynaptic (type III) cells provides negative paracrine feedback onto receptor cells by activating 5-HT(1A) receptors, inhibiting taste-evoked Ca^{2+} mobilization in receptor cells, and reducing ATP secretion (Huang et al. 2009). Majority of or all presynaptic (type III) taste cells secrete serotonin upon stimulation, but approximately one-third of them co-release norepinephrine with serotonin. In other words there are three to five times as many serotonergic presynaptic cells as there are norepinephrine/serotonin-secreting cells (Huang et al. 2008).

1.10 Conclusion

The Kyn pathway is the principle route of L-Trp metabolism and involves several mechanisms which trigger various metabolic pathways and transcription factors. Kyn produces neurotoxic and neuroprotective metabolic precursors before complete oxidation to NAD^+. Particularly QA-induced excitation and neurotoxicity are mediated by the overactivation of NMDA receptors, whereas KA is an antagonist of NMDA and alpha7nACh receptors and, thus, a potential neuroprotectant. While Kyn to Trp ratio reflects IDO activity, the Kyn to KA ratio indicates the neurotoxic challenge. Through the catabolic cascade of Trp metabolism, monoaminergic neurotransmission involves functional interactions between serotonin, norepinephrine, and dopamine systems. Serotonin not only affects neuronal excitability through activating postsynaptic receptors but also affects presynaptic excitatory or inhibitory neurotransmission in the central nervous system. However human SERT reuptakes biogenic amine neurotransmitters following release in the nervous systems and terminates the action of serotonin. OCT contributes to serotonin clearance if the expression of the SERT is low or absent. OCTs and the plasma membrane monoamine transporters are capable of clearing biogenic amines from extracellular fluid and may serve to buffer the effects of SSRIs. Desensitization and re-sensitization depend on the availability of functional receptors at the cell surface and on their mode of activation. Reviewing the dynamic aspects of Trp signaling intermediates helps to explain the mutual interaction of Kyn, serotonin, and melatonin pathways and opens up new vistas regarding the mechanism of diseases.

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Chapter 2
Tryptophan and Cell Death

Atilla Engin and Ayse Basak Engin

Abstract Cell death attributed to the tryptophan (Trp) metabolites is dependent on the exposure time and intracellular concentrations of cytotoxic Trp derivatives such as 3-hydroxykynurenine (3HK), 3-hydroxyanthranilic acid (3HAA), 5-hydroxyanthranilic acid (5HAA), and quinolinic acid (QA). However, 3HAA, 3HK, and QA at low concentrations may also serve as a precursor for nicotinamide adenine dinucleotide [NAD^+] which has vital importance to maintain cell viability. Inhibition of indoleamine 2,3-dioxygenase (IDO) activity results in a dose-dependent decrease in intracellular [NAD^+] levels. Mitochondrial permeability transition occurs in several forms of necrotic cell death. Disturbances in the normal function of the mitochondria are associated with the alterations in the balance of Trp metabolism. While kynurenic acid (KA) has proven to be neuroprotective with the potential endogenous antioxidant properties, QA is a specific agonist at the N-methyl-D-aspartate (NMDA) receptors and a potent neurotoxin with the marked free radical-producing property. QA-induced cytotoxic effects are mediated by overactivation of NMDA-like receptors and overexpression of inducible nitric oxide synthase (iNOS). L-Kynurenine-derived neurotoxin-induced apoptosis occurs through reactive oxygen species (ROS)-mediated pathways and is blocked by antioxidants. Unlike the kynurenine pathway, the methoxyindole metabolites of Trp metabolism protect cells against oxidative stress-induced apoptosis. Furthermore, deprivation of Trp triggers autophagy in a mammalian target of rapamycin (mTOR)-dependent manner. mTOR inhibition can suppress the activation of cyclin-dependent kinases and then inhibits the cell cycle progress, suppresses cell proliferation, and finally results in cell apoptosis.

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2.1 Cell Death

Cell death has been subdivided into the following categories: apoptosis (type I), autophagic cell death (type II), and necrosis (type III) (Lockshin and Zakeri 2004). Cell death patterns and their underlying molecular mechanisms may be different in various diseases and even in different progression stages of the same disease (Wang 2012). Death-associated protein kinases (DAPKs) regulate many signaling events of the cell death pathways, including apoptosis, autophagy, and membrane blebbing (Bovellan et al. 2010). Actually, death-associated protein kinase 1 (DAPK1) constitutes a critical integration point in both endoplasmic reticulum stress signaling and cell death pathway that transmit these signals into two different directions, caspase activation and autophagy (Gozuacik et al. 2008). Although functional studies indicated that DAPK may direct autophagy specifically toward autophagic cell death (Bialik and Kimchi 2010), DAPK1 is an interferon-gamma (IFN-gamma)-induced enzyme that controls cell cycle, apoptosis, and autophagy (Gade et al. 2012). Both tumor necrosis factor-alpha (TNF-alpha) and IFN-gamma significantly induce DAPK1 activities. Subsequently, DAPK1 can mediate the pro-apoptotic activity of TNF-alpha and IFN-gamma via the nuclear factor kappaB (NF-kappaB) signaling pathways (Yoo et al. 2012).

Apoptotic cell death begins with autophagy and autophagy progresses with apoptosis. Actually, caspases are central effectors of apoptosis. Apoptotic cell death is generally classified into two distinct pathways as extrinsic apoptotic pathway and intrinsic apoptotic pathway. By a majority of released molecules, caspase-dependent apoptosis is initiated, but some can activate cell death in a caspase-independent way (Lockshin and Zakeri 2004; Jin and El-Deiry 2005). The Nomenclature Committee on Cell Death (NCCD) proposed unified criteria for the definition of cell death and of its different morphologies. Suppressive effect of broad-spectrum caspase inhibitors suggested that cell death is frequently considered to be caspase dependent (Kroemer et al. 2009). Caspase inhibition simply induces a shift from an apoptotic to mixed cell death morphology or even to full-blown features of necrosis or autophagic cell death (Golstein and Kroemer 2005). Indeed two distinct pathways lead to nuclear apoptosis. One of these involves caspases, caspase-activated DNAse (CAD), and inhibitor of CAD (ICAD) and results in oligonucleosomal DNA fragmentation and advanced chromatin condensation. The second, caspase-independent pathway involves apoptosis-inducing factor (AIF) and leads to large-scale DNA fragmentation and peripheral chromatin condensation. Consequently, nuclear apoptosis is only prevented when both CAD and AIF are inhibited (Susin et al. 2000). If apoptotic cell death is induced by extracellular stress signals, it is defined as extrinsic apoptosis. Extracellular stress signals are sensed and propagated by specific transmembrane

receptors. Extrinsic apoptosis can be initiated by the binding of lethal ligands to various death receptors (Wajant 2002), whereas in both caspase-dependent and caspase-independent "intrinsic apoptosis," the apoptotic demise of cells can be triggered by intracellular stress conditions, including DNA damage, oxidative stress, cytosolic calcium overload, excitotoxicity, and endoplasmic reticulum stress (Galluzzi et al. 2012).

"Autophagic cell death" occurs in the absence of chromatin condensation but accompanied by massive autophagic vacuolization of the cytoplasm. Thereby, autophagy is a lysosome-dependent degradation pathway and activated by stressful situations such as starvation and oxidative stress (Vessoni et al. 2013). However, "necrotic cell death" or "necrosis" is morphologically characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture, and subsequent loss of intracellular contents (Galluzzi et al. 2012). Previously, necrosis has been considered as an accidental uncontrolled form of cell death, but evidences accumulated over time showed that necrotic cell death is a well-controlled and programmed process as caspase-dependent apoptosis and a consequence of extensive cross talk between several biochemical and molecular events at different cellular levels (Festjens et al. 2006). The receptor-interacting protein kinase 3 (RIP-3 kinase) and poly(ADP-ribose) polymerase-1 (PARP-1) are emerged as critical regulators of programmed necrosis/necroptosis (Moriwaki and Chan 2013; Jog and Caricchio 2013). The initiation of programmed necrosis, "necroptosis," by the death receptors requires the receptor-interacting protein kinase 1 (RIP-1 kinase) and RIP-3 kinase activity (Fig. 2.1) (Vandenabeele et al. 2010). Thus necroptosis displays signs of controlled processes such as mitochondrial dysfunction, enhanced generation of reactive oxygen species (ROS), adenosine triphosphate (ATP) depletion, proteolysis by calpains and cathepsins, and early plasma membrane rupture (Golstein and Kroemer 2007).

In this chapter, underlying mechanisms of cell death patterns attributed to the pathological accumulation of tryptophan (Trp) by-products have been discussed.

2.2 Toxic Versus Protective Effect of Tryptophan Metabolites

The essential amino acid Trp is primarily metabolized through the kynurenine (Kyn) pathway, some components of which may be neurotoxic. 3-Hydroxykynurenine (3HK), 3-hydroxyanthranilic acid (3HAA), and 5-hydroxyanthranilic acid (5HAA) induce cell death which increases with the exposure time and intracellular concentration compounds (Smith et al. 2009). Actually, the precursor of 3HAA, 3HK, is a potential endogenous neurotoxin. Cortical and striatal neurons are much more vulnerable to 3HK toxicity than cerebellar neurons. 3HK-induced neuronal cell death is dependent on the rate of cellular 3HK uptake and on the amount of intracellular ROS following exposure to 3HK (Fig. 2.1) (Okuda et al. 1998). Thus 3HK-induced neurotoxicity is mediated by the generation of hydrogen peroxide and hydroxyl radicals. In addition to nonenzymatic auto-oxidation of 3HK in extracellular

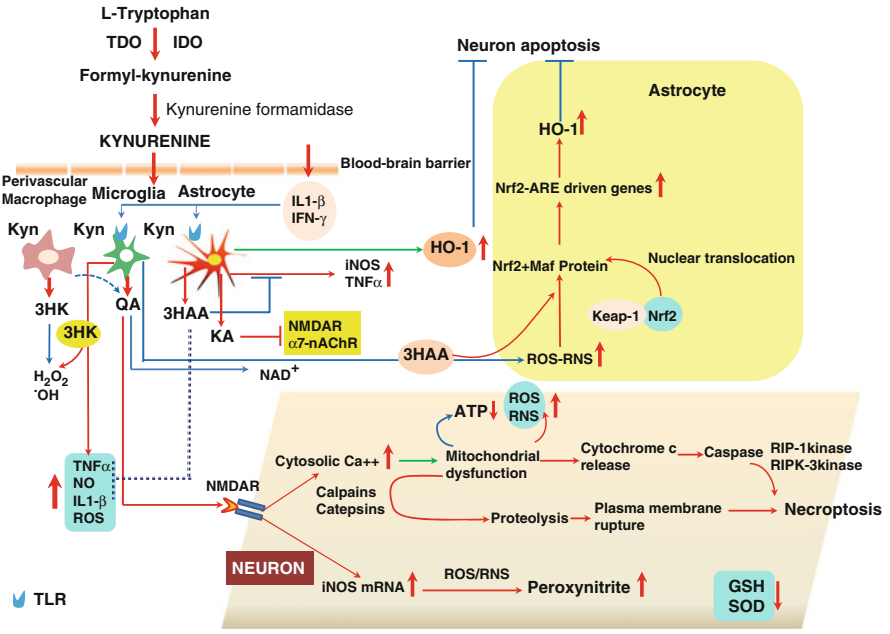

Fig. 2.1 Kynurenine pathway is the major route for tryptophan catabolism which generates QA, an agonist at NMDA receptor and a potent neurotoxin, as well as kynurenic acid which is an antagonist at glutamate and nicotinic receptors and, thus, a potential neuroprotectant. Other redox-active compounds, 3-hydroxykynurenine and 3-hydroxyanthranilic acid, are able to generate free radicals and can also damage neurons under many physiological and pathological conditions. *TDO* tryptophan-2,3-dioxygenase, *IDO* indoleamine 2,3-dioxygenase, *Kyn* kynurenine, *IL-1beta* interleukin-1beta, *IFN-γ* interferon-gamma, *3HK* 3-hydroxykynurenine, *QA* quinolinic acid, *3HAA* 3-hydroxyanthranilic acid, *KA* kynurenic acid, *NMDAR* N-methyl-D-aspartate receptor, *α7-nAChR* alpha-7 nicotinic acetylcholine receptor, *iNOSmRNA* inducible nitric oxide synthase mRNA, *TNFα* tumor necrosis factor-alpha, *HO-1* heme oxygenase-1, *ROS* reactive oxygen species, *RNS* reactive nitrogen species, *Keap-1* kelch-like ECH-associated protein-1, *Nrf2* nuclear factor erythroid-2-related factor 2, *Maf* transcription factor Maf, *ARE* antioxidant response element, NAD^+ nicotinamide adenine dinucleotide, H_2O_2 hydrogen peroxide, $^{\cdot}OH$ hydroxyl radical, *NO* nitric oxide, *TLR* Toll-like receptor, *RIP-1 kinase* receptor-interacting protein kinase-1, *RIP-3 kinase* receptor-interacting protein kinase-3, *GSH* glutathione, *SOD* superoxide dismutase

compartments, endogenous xanthine oxidase activity is involved in peroxide production. Hence the peroxide accumulation and cell death caused by 3HK are blocked by allopurinol or attenuated by catalase (Okuda et al. 1996; Wei et al. 2000). Furthermore, 3HAA and anthranilic acid interact at the level of 3-hydroxyanthranilic acid oxidase. The ratio of 3HAA to anthranilic acid levels represents a novel marker for the assessment of inflammation and its progression. Anthranilic acid concentrations normally equal or exceed 3HAA levels by up to fivefold. Decrease in the ratio of 3HAA to anthranilic acid levels represents a compensatory mechanism to reduce cell toxicity (Darlington et al. 2010). Anthranilic

acid inhibits 3-hydroxyanthranilic acid oxidase. Eventual conversion of 3HAA to quinolinic acid (QA) and picolinic acid is reduced (Guillemin et al. 2007).

Trp degradation through the Kyn pathway plays an important role in the pathogenesis of inflammatory processes. Likewise, in cortical and striatal neurons, microglial 3HAA and QA act as neurotoxins while kynurenic acid (KA) is a neuroprotectant. KA is an astrocyte-derived noncompetitive antagonist of the α7 nicotinic acetylcholine receptor (α7nAChR) and inhibits N-methyl-D-aspartate (NMDA) receptor competitively (Fig. 2.1). Changes in endogenous KA levels, by modulating α7nAChR function, control extracellular gamma-aminobutyric acid (GABA) levels and bidirectionally influence cortical glutamate concentrations. Glutamate receptor agonists, QA and 3HK, can contribute to or exacerbate neuronal damage by generating free radicals (Stone et al. 2003; Beggiato et al. 2013; Wu et al. 2010). Actually, both glutamate receptor-mediated excitotoxicity and free radical formation have been correlated with decreased levels of the neuroprotective Trp metabolite, KA (Zwilling et al. 2011). Even in minor changes that exceed the physiological concentrations of 3HK and QA, they may cause neuronal death. QA causes necrosis, whereas 3HK-exposed neurons primarily die by apoptosis (Chiarugi et al. 2001).

On the other hand, interleukin-1 (IL-1) is a critical cytokine for neurotoxicity. Following exposure of astrocytes to interleukin-1beta (IL-1beta)/IFN-gamma, inducible nitric oxide synthase (iNOS) and TNF-alpha are overexpressed. Neuronal cell death occurs at least within 48 h following cytokine stimulation. In this case, endogenous TNF-alpha has a crucial role in mediating neurotoxicity (Downen et al. 1999). These cytokines can stimulate astrocytes instead of microglia to express iNOS in humans (Liu et al. 1996). Actually, IL-1-induced neurotoxicity cannot affect the viability of pure cortical neurons. However, IL-1 treatment of co-cultures of neurons with glia or purified astrocytes induces caspase activation leading to neuronal death. Caspase-dependent neuronal death is also associated with the release of free radicals. Furthermore, IL-1-induced neuronal cell death is prevented by pretreatment with the IL-1 receptor antagonist (Thornton et al. 2006). Surprisingly, 3HAA suppresses cytokine and chemokine productions as well as neurotoxicity which are induced by IL-1/IFN- and Toll-like receptor (TLR) ligands. These effects are partly mediated by the capability of 3HAA to induce heme oxygenase-1 (HO-1) in human glial cells (Fig. 2.1) (Krause et al. 2011). Indeed it was demonstrated that 3HAA protects human neurons against cytokine- or TLR ligand-induced death. The neurotoxic effect of 3HAA can eventuate within the inflammatory environment. In these conditions, microglial HO-1 activity is suppressed by TLR ligands but is enhanced by the anti-inflammatory cytokine, interleukin-10 (IL-10) (Krause et al. 2011). Actually, HO-1, iNOS, and indoleamine 2,3-dioxygenase (IDO) are simultaneously expressed in murine macrophages subsequent to IFN-gamma stimulation. While nitric oxide (NO) overproduction by iNOS decreases IDO expression, HO-1 expression is increased (Oh et al. 2004). Inhibition of IDO expression by NO has occurred in the IFN-gamma-primed macrophages but not in microglial cells. Thereby, nitric oxide synthase (NOS) inhibitors increase the levels of IDO mRNA in MT2 macrophages; no changes are detected in IDO mRNA levels of microglial cells (Alberati-Giani et al. 1997).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an essential transcription factor that regulates expression of several antioxidant and phase II detoxification enzyme genes such as HO-1, glutamate-cysteine ligase (GCL), and peroxiredoxin-1 (Prx1), via binding to the antioxidant response element (ARE) under conditions of oxidative stress (Fig. 2.1). Activation of Nrf2 plays a crucial role in cellular defense against oxidative stress (Huang et al. 2014; Kim et al. 2012).

Kelch-like ECH-associated protein (Keap)-1 is a kind of stress sensor protein that plays mandatory roles not only as a sensor of oxidative and electrophilic stresses but also as a regulator of Nrf2 degradation. Thereby, the rapid degradation of Nrf2 requires direct association with Keap-1 (Kobayashi et al. 2004). Actually, Keap-1 together with Nrf2 composes a nuclear shuttling mechanism. As mentioned above, Keap-1 and Nrf2 constitute a crucial cellular sensor for oxidative stress and together mediate a key step in the signaling pathway that leads to transcriptional activation by the Nrf2 (Itoh et al. 1999a). In the absence of stress stimuli, the cytoplasmic protein Keap-1 binds Nrf2 and prevents its translocation to the nucleus (Itoh et al. 1999b). However, during inflammation or infection, 3HAA induces HO-1 expression and stimulates nuclear translocation of Nrf2 in human endothelial cells. In this case firstly, 3HAA induces Nrf2-dependent HO-1 expression. Later on, HO-1 inhibits monocyte chemoattractant protein (MCP)-1 secretion, vascular cell adhesion molecule (VCAM)-1 expression, and NF-kappaB activation, which are all associated with vascular injury (Pae et al. 2006).

Exogenous 3HAA dose-dependently suppresses iNOS expression and coincidently enhances HO-1 expression. This suppressive effect of 3HAA on iNOS expression is reversed by blocking HO-1 activity (Oh et al. 2004). The ability of 3HAA to induce HO-1 is most certainly related to its free radical-generating properties because ROS provide necessary signals for Nrf2 activation (Dykens et al. 1987; Opitz et al. 2007).

One of the well-known toxic metabolites of Kyn pathway, 3HK may cause cell death by inducing oxidative damage. However, different 3HK levels are detected in patients suffering from several diseases. In some of these patients, concentration-dependent antioxidant or scavenging features of 3HK have been attributed to the dual actions of this molecule (Colín-González et al. 2013).

Another endogenous toxic metabolite of the Kyn pathway is QA. Pathological accumulation of this by-product involves several mechanisms which trigger various metabolic pathways and transcription factors. The primary mechanism exerted by the QA in the central nervous system has been largely related to the overactivation of NMDA receptors and increased cytosolic Ca^{2+} concentrations. This initial phase is followed by the mitochondrial dysfunction, cytochrome c release, ATP exhaustion, free radical formation, and oxidative damage (Pérez-De La Cruz et al. 2012). Virtually, QA may also serve as a precursor for nicotinamide adenine dinucleotide [NAD^+]. Continuous biosynthesis of NAD^+ has vital importance to maintain cell viability (Ying 2006). In this respect, the Kyn pathway constitutes the major metabolic pathway for the synthesis NAD^+ which is an important enzymatic cofactor for the DNA repair protein, PARP-1. Thereby, increasing IDO activity and Kyn metabolism in astroglial cells during inflammation maintain NAD^+ levels through de novo

synthesis from Trp (Grant and Kapoor 2003). It is proposed that extracellular Kyn pathway metabolites at pathophysiological concentrations may contribute to astroglial dysfunction and cell death. Although 3HAA, 3HK, and QA at low concentrations significantly increase intracellular NAD^+ levels, at concentrations exceeding 100 nM, they cause a dose-dependent decrease in intracellular NAD^+ levels. Likewise to NAD^+ depletion, higher concentrations of anthranilic acid may also cause cell death (Braidy et al. 2009a). Competitive inhibition of IDO activity with 1-methyl-L-tryptophan results in a dose-dependent decrease in intracellular NAD^+ levels and sirtuin deacetylase-1 (silent mating type information regulation 2 homolog-1, SIRT1) activity. Consequently, a decrease in intracellular NAD^+ due to inhibition of IDO activity is correlated with reduced cell viability (Braidy et al. 2011). In this context, SIRT, a family of NAD^+-dependent deacetylases, is implicated in energy metabolism and life span (Sundaresan et al. 2008). PARP-1 is a major NAD^+-metabolizing enzyme. Overstimulation of NMDA receptors induces a decrease in cytoplasmic NAD^+ and an increase in ROS in neurons through PARP-1 activation (Yu et al. 2002). However, PARP-1-mediated NAD^+ depletion simultaneously increases mitochondrial SIRT3. SIRT3 acts as a prosurvival factor and protects neurons which are under the excitotoxic stress (Kim et al. 2011).

2.3 Redox-Active Molecules, Mitochondria, and Tryptophan Metabolites

Programmed necrosis is a recently recognized entity which is an important mechanism of neuronal damage following hypoxia-ischemia. RIP-1 kinase activity is essential in order to progress the well-described forms of apoptosis-necrosis cell death "continuum" (Northington et al. 2007). RIP-1 kinase-dependent apoptosis-necrosis cell death is associated with the increased ROS production, decreased ATP production, and decreased mitochondrial membrane potential (Irrinki et al. 2011). Actually, RIP-1 is also one of the key components of the TNF-alpha-tumor necrosis factor receptor 1 (TNFR1) signaling complex. RIP-1 kinase activity mediates the formation of the necrosome (RIP-1/RIP-3 complex) which induces ROS production via effects on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)-1 (Kim et al. 2007). On the other hand, iNOS expression and NO accumulation increase in hypoxic brain. Physiological amounts of superoxide which is produced by intact mitochondria react with NO to generate peroxynitrite. Peroxynitrite-mediated elevation of cytoplasmic calcium levels results in the accumulation of intracellular calcium into the mitochondria. Thus peroxynitrite-induced elevation of cytosolic calcium collapses the mitochondrial membrane potential and may cause peroxynitrite-induced cell death (Whiteman et al. 2004). Actually, nitrosative and oxidative stress cause the formation of protein nitrosamines. Meanwhile Trp residues of proteins may most likely be exposed to nitrosative and oxidative stress. Xanthine oxidase decomposes nitrosated Trp through superoxide and uric

acid pathways. Uric acid-induced decomposition of N-nitroso-tryptophan generates NO (Viles et al. 2013). Although NO and peroxynitrite inhibit enzymes that depend on oxidizable amino acids for activity, in the presence of more readily oxidized substrates, xanthine oxidase could not be inhibited by either NO or peroxynitrite (Houston et al. 1998).

Disturbances in the normal function of the mitochondria are associated with the alterations in the balance of Trp metabolism (Szalardy et al. 2012). The enhanced production of free radicals worsens the mitochondrial functions by causing oxidative damage to macromolecules and by opening the mitochondrial permeability transition pores thereby inducing apoptosis. In this process, Kyn functions as a deleterious substance which can be metabolized in two separate ways: one branch produces KA and the other 3HK and QA, the precursors of NAD^+ (Sas et al. 2007). An enzyme in the Kyn pathway, kynurenine 3-monooxygenase, is a flavin adenine dinucleotide (FAD)-dependent monooxygenase and is located in the outer mitochondrial membrane where it converts L-kynurenine to 3-HK (Amaral et al. 2013). Mitochondrial integrity is constantly threatened by the production of ROS within the membrane. 3HK has dual effect in redox balance depending on the metabolic demands of body cells, prooxidants or antioxidants. During the mitochondrial dysfunction, 3HK causes intracellular accumulation of peroxide and subsequent cell death (Okuda et al. 1996; Tan et al. 2012), whereas 5-hydroxyindoles, a class of Trp metabolites, protect cells by attenuating oxidative stress and consequently keeping them from mitochondrial dysfunction (Bae et al. 2010). Actually, 5-hydroxyindoles consist of three different molecules: 5-hydroxytryptamine (serotonin), 5-hydroxytryptophan (5HTP), and 5-hydroxyindoleacetic acid (5HIAA). Tryptophan hydroxylase (TPH) 2, the rate-limiting enzyme in the serotonin biosynthesis, is a phenotypic marker for serotonin neurons and is known to be extremely labile to oxidation. Oxidation of TPH2 inhibits its activity and leads to the formation of high molecular weight aggregates in a dithiothreitol-reversible manner. Oxidation shifts TPH2 from the soluble compartment into membrane fractions and large inclusion bodies (Kuhn et al. 2011).

Otherwise, L-Kyn can also induce cell death via generating ROS in natural killer cells. In this instance, treatment with the antioxidant N-acetylcysteine (NAC) inhibits cytochrome c release and activation of caspase-3 and discontinues apoptotic process (Song et al. 2011). Furthermore, IDO-induced T-cell death is an important mechanism in IDO-mediated T-cell suppression. In this respect, 3HAA-mediated depletion of intracellular glutathione (GSH) is the major mechanism in cell death. When cellular GSH levels are maintained by addition of NAC, 3HAA-mediated T-cell death is completely inhibited. This means that depletion of GSH from activated T cells takes place without increasing ROS (Lee et al. 2010). IFN-gamma stimulates the IDO expression through the JAK (janus kinase)/STAT1 (signal transducer and activator of transcription) signaling pathway in a dose-dependent manner especially in human lens epithelial cells. In these cells, 3HK concentration is higher than that of the other Trp metabolites and can cause higher rate of peroxide formation and apoptosis. Trp depletion as a cause of apoptosis is ruled out by blocking of

apoptosis through the inhibition of kynurenine 3-hydroxylase (Mailankot and Nagaraj 2010). Both 3HK and 3HAA generate superoxide and hydrogen peroxide in a copper-dependent manner. Thus 3HK and 3HAA may be cofactors in the oxidative damage of proteins through interactions with redox-active copper (Goldstein et al. 2000). While KA has proven to be neuroprotective, QA is a specific agonist at the NMDA receptors and a potent neurotoxin with the marked free radical-producing property (Sas et al. 2007). QA-induced cytotoxic effects on neurons and astrocytes are mediated by an overactivation of NMDA-like receptors. In these cells, QA enhances mRNA and protein expression of iNOS and may cause NO-mediated free radical damage (Braidy et al. 2009b). Neurotoxicity of 3HK is mediated by production of hydrogen peroxide and its subsequent decomposition to hydroxyl radical and these can cause cellular damage and induce apoptosis (Okuda et al. 1996).

2.4 Tryptophan and Programmed Cell Death

IDO has immunoregulatory function against antigenic stimulation. Treatments of human natural killer (NK) cell lines with L-Kyn result in dose-dependent growth inhibition and apoptosis. L-Kyn-induced apoptosis in NK cells occurs through an ROS-mediated pathway and is blocked by antioxidants (Song et al. 2011). IDO activation in cytokine-stimulated mesenchymal stromal cells mediates a marked sensitivity of myeloma cells to Trp depletion in the microenvironment and subsequently inhibits myeloma cell growth (Pfeifer et al. 2012). However, IDO-high expression within the tumor microenvironment creates an immunosuppressive network and avoids immune attack and defeats the invasion of T cells via production of pro-apoptotic Trp catabolites (Brandacher et al. 2006). The inhibition of IDO may efficiently reverse enhancement of T-cell apoptosis and amplification of Treg-mediated immunosuppression (Sun et al. 2011).

ROS may cause the initiation of DNA single-strand breakage, with subsequent activation of the nuclear enzyme poly(ADP-ribose) synthetase (PARS). Increase in PARS activity leads to a necrotic-type cell death through the energy depletion of the cells. Melatonin inhibits the activation of PARS and prevents the organ injury (Cuzzocrea and Reiter 2001). Moreover, antioxidant and anti-inflammatory effect of melatonin is correlated with the inhibition of peroxynitrite production in addition to PARS activation (Cuzzocrea et al. 1998). The enzymatic cofactor for the DNA repair protein, PARP, is activated at an intermediate stage of apoptosis and is then inactivated at a late stage by apoptotic proteases (Decker and Muller 2002). Inflammation increases the concentration of oxidative metabolites and causes NAD^+ depletion through increased PARP activity. However, the activity of IDO is also markedly increases in astrocytes during inflammation. Induction of IDO and subsequent NAD^+ synthesis may contribute to the maintenance of intracellular NAD^+ levels and cell viability under conditions of increased oxidative stress (Grant et al. 2000). Briefly, in the first step of the Kyn pathway, IDO catalyzes the oxidation of

Trp to N-formylkynurenine which eventually forms NAD^+ through a series of reactions (King and Thomas 2007).

Unlike the Kyn pathway, the methoxyindole branch of Trp metabolism does not affect the indole ring of Trp. Although melatonin has been extensively studied, other methoxyindoles, such as N-methylserotonin, 5-methoxyindole acetic acid, and 5-methoxytryptamine, are less known. Nevertheless, methoxyindole and Kyn branches of Trp pathway have different regulation mechanisms (Zhu et al. 2013). Melatonin protects cells against oxidative stress-induced apoptosis due to its ability to scavenge mitochondrial ROS. Thus mitochondrial protective effects of melatonin are provided by two ways: first due to its primary antioxidative action and second its direct targeting of the mitochondrial permeability transition (Jou et al. 2010). Via its ability to reduce mitochondrial ROS generation, the subsequent mitochondrial calcium overload, mitochondrial membrane potential depolarization, opening of the mitochondrial permeability transition pore, mitochondrial permeability transition-dependent cytochrome c release, downstream activation of caspase 3, and apoptotic fragmentation of nuclear DNA are inhibited by melatonin (Jou et al. 2004). Actually, mitochondrial permeability transition occurs in several forms of necrotic cell death, including oxidative stress, pH-dependent ischemia/reperfusion injury, and Ca^{2+} ionophore toxicity. Initially, few mitochondria undergo the mitochondrial permeability transition which does not occur uniformly during apoptosis (Lemasters et al. 1998). The indole molecule significantly reduces mitochondrial ROS formation. Thus melatonin displays a protective effect against oxidative stress by inhibiting the mitochondrial depolarization and opening of the mitochondrial permeability transition pores. This is associated with the high amount of environmental GSH content and mitochondrial pyridine nucleotides (Hibaoui et al. 2009). Depressed nocturnal melatonin concentrations or nocturnal excretion of the main melatonin metabolite, 6-sulfatoxymelatonin, promotes apoptosis in most tumor cells, in contrast to the obvious inhibition of apoptotic processes in normal cells (Sánchez-Hidalgo et al. 2012). Furthermore, melatonin is correlated with a decrease in the oxidative phosphorylation at liver mitochondria. This effect of melatonin is associated with a gradual decrease in the respiratory control index and significant alterations in the membrane potential. During the mitochondrial calcium overload, melatonin can also induce substantial release of cytochrome c and apoptosis-inducing factor (Martinis et al. 2012).

Actually, anticancer effects of physiological blood concentrations of melatonin are exerted via inhibition of cell proliferation and a stimulation of differentiation and apoptosis. In this regard, melatonin receptor-mediated suppression of cyclic adenosine monophosphate (cAMP) levels causes the diminishing of tumor fatty acids transport by decreasing plasma membrane-associated fatty acid transport proteins. The inhibition of these signal transductions leads to melatonin-induced suppression of tumor linoleic acid uptake. Consequently, reduced amount of intracellular linoleic acid turns to the inhibition of mitogenic signaling molecule 13-hydroxyoctadecadienoic acid (13-HODE) production (Blask et al. 1999, 2005). 13-HODE amplifies the activity of the epidermal growth factor receptor/mitogen-activated protein kinase (EGF/MAPK) pathway leading to cell proliferation.

Melatonin effectively blocks the production of 13-HODE. Therefore, rotating night shift works enhance the risk of cancer progression by increasing light exposure during night and decreasing nocturnal melatonin signal (Blask et al. 2002). Furthermore, dietary melatonin supplementation supports the endogenous melatonin signal to optimize the host/cancer balance in favor of host survival (Blask et al. 2005).

Glial cells and neurons are in constant reciprocal signaling both under physiological and neuropathological conditions. Microglia perceives the microenvironment like macrophages. Thus neuronal stress or injury may cause a deleterious type of microglial activation which is associated with neurotoxicity and neuronal cell death. Necrotic neurons induce subsequent microglial reactivation which results in the upregulation of co-stimulatory molecules, beta2 integrin CD11b, pro-inflammatory cytokines, iNOS, IDO, and cyclooxygenase-2 (COX-2) (Pais et al. 2008). Although microglial activation is often associated with neuronal death during inflammation, it also displays a neuroprotective role by saving neurons from QA-mediated toxicity. In this case, fibroblast growth factor-2 (FGF-2) has a fundamental role in the protection against QA toxicity. After releasing from neurons, FGF-2 activates c-Jun N-terminal kinase 1 and 2 pathway which contributes to neuronal survival (Figueiredo et al. 2008).

On the other hand, synaptic glutamate receptors are located on the membranes of neuronal cells. Astrocytes are responsible for most of the glutamate uptake in synaptic as well as nonsynaptic areas and, consequently, are the major regulators of glutamate homeostasis. Microglia in turn may secrete cytokines, which can impair glutamate uptake and reduce the expression of glutamate transporters. Finally, oligodendrocytes, the myelinating cells of the central nervous system, are very sensitive to excessive glutamate signaling, which can lead to the apoptosis or necrosis of these cells (Matute et al. 2006). The Kyn pathway of Trp metabolism includes an agonist, QA, and an antagonist, KA, at glutamate receptors. Glutamate receptors are also sensitive to NMDA. Necrotic neurons can induce pro-inflammatory markers of microglial activation. Overactivated microglia enhances NMDA-receptor-mediated neurotoxicity. However, NMDA-receptor-mediated cell death most likely depends on increased production of glutamate (Pais et al. 2008).

During cerebral hypoxia, as a consequence of mitochondrial dysfunction, free radical generation and cell death occurs. Simultaneous production of QA and KA is eventuated through the Kyn pathway activation. Actually, KA can modulate NMDA receptors and displays neuroprotective effect with preference for the glycine site of the NMDA receptors. However, NMDA receptor-dependent toxicity of mitochondrial inhibitors is independent of the glycine site of action of KA (Fatokun et al. 2008). Indeed KA reduces the increase in striatal superoxide anion and peroxynitrite production and lipid peroxidation in the forebrain and cerebellum in a concentration-dependent manner. These effects of KA are attributed to the potential endogenous antioxidant properties in addition to the antagonist actions on alpha-7nACh and NMDA receptors (Lugo-Huitrón et al. 2011). It is usually assumed that KA antagonizes the glycine site of the NMDA receptors and/or the neuronal cholinergic alpha-7nACh receptors. However, it is not obvious whether the KA interacts with these targets (Moroni et al. 2012).

Signaling proteins, MAPKs, regulate the cell proliferation and differentiation. Three members of MAPK, namely, the extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases, and p38 MAPK, are activated in vulnerable neurons and cause neuronal injury (Harper and Wilkie 2003; Zhu et al. 2002). ERK1/2 activation also takes place in the neurons of the neonatal rat brain after hypoxia-ischemia. In this case, phosphorylation of ERK1/2-positive neurons indicates DNA damage (Wang et al. 2003). The ERK-MAPK pathway may contribute to neuronal injury through the upregulation of matrix metalloproteinase-9 (MMP-9). Decreasing trauma-induced MMP-9 levels significantly attenuate tissue damage (Mori et al. 2002). Moreover, activated microglia exhibits a transient expression in iNOS, COX-2, and several pro-inflammatory cytokines, such as IL-1beta, interleukin-6 (IL-6), and TNF-alpha. Microglial ERK1/2 and p38 MAPK activation in the substantia nigra may involve dopaminergic neuronal cell death. However, by reducing iNOS and COX-2 mRNA expression, inhibition of ERK1/2 and p38 MAPK rescues neurons (Choi et al. 2003).

Thus, during the 3HK-induced apoptotic neuronal cell death, ERK phosphorylation may occur. Cell death is preceded by mitochondrial dysfunction and cytochrome c release from mitochondria to the cytosol. Mitochondrial dysfunction and subsequent caspase activation are dramatically provoked by inhibition of MAPK/ERK1/2, resulting in enhanced neuronal cell death (Lee et al. 2004). Apoptosis can be initiated via an intrinsic pathway through the mitochondria-mediated death signaling cascade. Damaged mitochondria release more ROS. A functional link is defined between ROS and the caspase cascade involving caspase 2 and cleavage of anti-apoptotic protein BclXL (Prasad et al. 2006). 3-HK toxicity depends on the intracellular generation of ROS. Subsequent neuronal apoptosis takes place as a result of transporter-related cellular uptake of 3HK. Inhibition of 3HK uptake prevents its toxicity (Okuda et al. 1998). Similarly, L-Kyn-induced apoptosis in natural killer cells occurs through an ROS-mediated pathway. Antioxidants block cytochrome c release and activation of caspase-3 during L-Kyn-induced apoptosis (Song et al. 2011). Likewise, 5-hydroxyindole (5HI), a metabolite of Trp, markedly inhibits cytochrome c release and caspase-3 activation but not caspase-9 activation by attenuating oxidative stress (Bae et al. 2010).

Some recent conflicting findings show that the activation of ERK1/2 contributes to cell death and inhibition of the ERK pathway blocks apoptosis. However, ERK can participate in cell death through the suppression of the anti-apoptotic signaling molecule protein kinase B (Akt) (Zhuang and Schnellmann 2006). Indeed Akt pathway has a critical role in mediating signaling transductions for cell survival. Protein serine/threonine phosphatase-1 is a major phosphatase that directly dephosphorylates Akt to modulate its activation. Dephosphorylated Akt significantly modulates its functions in promoting cell survival (Xiao et al. 2010). IL-27 activates ERK and p38 MAPKs as well as Akt, STAT1, STAT3, and STAT6 in intestinal epithelial cells. Actually, the activation of the antibacterial gene IDO1 is dependent on STAT1 signal transduction. IL-27-induced IDO1 activity leads to growth inhibition of intestinal bacteria by causing local Trp depletion (Diegelmann et al. 2012). Oxidative stress-induced injuries in neurons cause a biphasic or permanent ERK1/2

activation. Potential targets of ROS and reactive nitrogen species such as cell surface receptors, G proteins, upstream kinases, protein phosphatases, and proteasome components modulate the duration and magnitude of ERK1/2 activation and affect the subcellular localization of activated ERK1/2 (Chu et al. 2004). Thus L-tryptophan-derived 3HAA following induction of IDO acts as an endogenous inducer of monocyte/macrophage apoptosis. In these cells, catalase, superoxide dismutase, and manganese ions markedly enhance apoptosis in the presence of 3HAA (Morita et al. 1999). Monocytes and liver cells can directly convert L-Trp into QA following immune stimulation (Saito et al. 1993). 3HAA and QA induce the selective apoptosis of murine thymocytes and of T helper (Th) 1 but not Th2 cells. T-cell apoptosis is associated with the activation of caspase-8 and release of cytochrome c from mitochondria. However, for induction of T-cell apoptosis, tenfold lower concentrations of 3HAA and QA are observed than those required for neurotoxicity or for apoptosis of macrophages and dendritic cells (Fallarino et al. 2002).

2.5 Hypertryptophanemia

Hypertryptophanemia is a rare inherited metabolic disorder probably caused by a blockage in the conversion of Trp to Kyn, resulting in the accumulation of Trp and some of its metabolites in plasma and tissues of affected patients. The patients present mild-to-moderate mental retardation with exaggerated affective responses, periodic mood swings, and apparent hypersexual behavior. Usually creatine kinase plays a critical role in energy metabolism of tissues with intermittently high and fluctuating energy requirements. However, Trp inhibits creatine kinase in vitro and in vivo. In this case, inhibitory effect of Trp on creatine kinase activity is achieved by oxidation of essential thiol groups of the enzyme (Cornelio et al. 2004). In human hypertryptophanemia, Trp accumulates in the brain and significantly decreases the overall content of brain antioxidant defenses. Therefore, the Trp-induced increase in thiobarbituric acid-reactive substances is prevented by GSH and by combination of catalase plus superoxide dismutase (Feksa et al. 2006). Furthermore, oxidative stress due to Trp loading can also be prevented by the pretreatment with antioxidants. Thus the hypothesis of Trp-induced oxidative stress in brain cortex has been elucidated by giving taurine or alpha-tocopherol plus ascorbic acid (Feksa et al. 2008). Nonspecific NOS inhibitors decrease the homocysteine-induced lipid peroxidation than does the selective neuronal NOS inhibitor. Homocysteine can induce oxidative injury to nerve terminals, and this effect involves the NMDA receptor stimulation in addition to NO overproduction and associated free radical formation (Jara-Prado et al. 2003).

QA induces concentration-dependent increases in ROS formation in all synaptosomes, but increased production of peroxidized lipids is only estimated in the striatum and the hippocampus. These findings suggest that the excitotoxic action of QA involves regional selectivity in the oxidative status of brain synaptosomes

(Santamaría et al. 2001). The NMDA receptor antagonist completely abolishes the increase of QA-induced lipid peroxidation (Santamaría and Ríos 1993).

2.6 Hypoxic-Ischemic Brain Damage and Tryptophan

Perinatal hypoxia-ischemia (HI) of neonates may cause hypoxic-ischemic brain damage (HIBD). Actually, neural cells are selectively more susceptible to hypoxic-ischemic injury; in this respect, programmed cell death such as apoptosis or autophagy is the usual form of neural degeneration in HIBD (Northington et al. 2011). The principal mechanisms leading to neuronal death after hypoxia-ischemia/reperfusion are initiated by energy depletion and accumulation of extracellular excitotoxic amino acids and glutamate with the subsequent activation of glutamate receptors (Volpe 2001). It was shown that disruption of membrane integrity in hypoxic conditions by phospholipases plays a role in the excitotoxic amino acid release from neuronal cells. Thus brain extracellular levels of glutamate, aspartate, gamma-aminobutyric acid (GABA), and glycine increase rapidly following the onset of ischemia (Phillis and O'Regan 2003). Excessive phospholipase activation, along with a decreased ability to resynthesize membrane phospholipids, can lead to the generation of free radicals, excitotoxicity, mitochondrial dysfunction, and apoptosis/necrosis (Phillis and O'Regan 2004). Additionally, excess amounts of glutamate become toxic to the brain (Yager et al. 2002). Recent findings indicate that cells employ different signaling pathways to monitor the depletion or sufficiency of essential amino acids such as Trp. This information is integrated into cells as either growth or autophagy decisions (Metz et al. 2012). Autophagy is the process by which cells consume their own proteins and organelles to maintain levels of essential building blocks and promote survival under nutrient-poor conditions (Levine and Kroemer 2008; Mizushima et al. 2008). Hence autophagy can precede apoptosis and play a protective role in neuronal death (Carloni et al. 2008). However, apoptosis plays a predominant role in the pathological progress of HIBD (Li et al. 2007). Both are mainly controlled by the master metabolic regulator, the mammalian target of rapamycin (mTOR). mTOR consists of two different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 regulates some essential cellular processes including translation, transcription, and autophagy (Inoki et al. 2012). Adenosine monophosphate-activated protein kinase (AMPK) maintains the balance between ATP production and consumption (Hardie 2007). Actually, AMPK as a sensor of cellular energy status is activated under the conditions of intracellular ATP deprivation. mTOR and AMPK have opposite effects on the control of metabolic functions. Furthermore, AMPK effectively suppress mTORC1 signaling in mammalian cells. Successful downregulation of mTOR is required for AMPK activation (Gwinn et al. 2008). mTOR activation may be necessary to prevent apoptotic neuronal cell death during oxidative stress (Chong et al. 2012). In contrast, loss of mTOR activity during oxidative stress leads to apoptotic neuronal death (Chen et al. 2010).

Hypoxia or ischemia can also regulate hypoxia-inducible factor-1alpha (HIF-1alpha) and its target gene vascular endothelial growth factor (VEGF) expression to exert neuroprotection against to the HIBD. In this case, HIF-1alpha is a vital molecule in maintaining cellular oxygen balance (Li et al. 2008a). mTOR signaling pathway involves in the regulation of HIF-1alpha and VEGF and thus contributes to the survival mechanisms of HIBD through regulating apoptosis. Indeed mTOR is not only a positive regulator of HIF-1alpha-dependent gene transcription but also participates in the mechanism of hypoxia-promoted angiogenesis during the hypoxia (Humar et al. 2002). Actually, the protein expression of HIF-1alpha and its target gene VEGF is regulated through phosphatidylinositol 3-kinase/protein kinase B (PI3-K/Akt) signaling pathway, which is primarily involved in the survival process after HIBD (Li et al. 2008b). mTOR is a main downstream molecule of the PI3-K/Akt signaling pathway. Subsequent to activation of PI3-K, phosphoinositide-dependent kinase 1 (PDK1) is translocated to the membrane and activates p70 ribosomal S6 kinase (ribosomal serine/threonine kinase) (Kang et al. 2008; Nakamura et al. 2006; Gunn and Hailes 2008). Thus downstream of the nutrient-sensitive mTORC1 complex has two well-characterized substrates: eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) and the p70 ribosomal protein S6 kinases 1 (p70S6K). Phosphorylation of 4EBP-1 by mTORC1 suppresses its ability to bind and inhibit the translation initiation factor 4E (eIF4E) (Shaw 2009). As a principle hypoxia sensor, mTOR can also integrate the signals and transmit them to the nucleus and then activates 4EBP1 and p70S6K (Park et al. 2010). Eventually, mTOR may acquire the capability of limiting the ischemic neuronal death and promoting the neurological recovery by preventing neuronal apoptosis, inhibiting autophagic cell death (Chong et al. 2013).

Deprivation of an essential amino acid triggers autophagy in an mTOR-dependent manner. Induction of autophagy is reversed by restoring that essential amino acid. Thus, the IDO-mediated Trp deprivation would trigger autophagy. Substitution of Trp reverses autophagic response, based on their common ability to restore Trp sufficiency signaling in the mTOR pathway. When amino acids are sufficient and the Akt pathway is active, the mTORC1 becomes active and phosphorylates the translational regulators p70S6K and 4EBP1, stimulating their activity (Metz et al. 2012). Trp depletion as caused by IDO overactivation leads to an accumulation of uncharged Trp transfer RNA (tRNA) in cells. The integrated stress response kinase GCN2 (general control nonderepressible 2, a serine/threonine-protein kinase), a sensor of uncharged tRNA, is activated by amino acid deprivation. Uncharged tRNA is recognized as an important effector of the IDO pathway. This activates the GCN2, which then phosphorylates and inhibits the translation initiation factor 2alpha (eIF2alpha), blocking protein synthesis and arresting cell growth (Munn et al. 2005). Thus, in cells experiencing Trp limitation due to the activation of IDO, both GCN2 and mTOR should be affected. The potent IDO inducer IFN-gamma depletes Trp and represses mTOR activity (Metz et al. 2012). The cellular responses to IFN-gamma are complex, and emerging evidence suggests that IFN-gamma may regulate autophagic functions. Conversely, autophagy modulates innate and adaptive immune functions in various contexts. IFN-gamma promotes Trp depletion, activates the eIF2α kinase, GCN2, and leads to an increase in the autophagic flux.

Further, Trp supplementation and RNA interference directed against GCN2 inhibits IFN-gamma-induced autophagy (Fougeray et al. 2012). mTOR can participate in the regulation of neuronal death. mTOR inhibition by rapamycin can suppress the activation of cyclin-dependent kinases and then inhibits the cell cycle progress, suppresses cell proliferation, and finally results in cell apoptosis (Gu et al. 2008).

2.7 Conclusion

The Kyn pathway intermediates of Trp metabolism, 3HK, 3HAA, and 5HAA, may induce cell death depending on exposure time and intracellular concentrations of these compounds. A decrease in the ratio of 3HAA to anthranilic acid levels reduces cell toxicity. While microglial glutamate receptor agonist QA acts as a neurotoxin, KA which is an astrocyte-derived α7nAChR and NMDA receptor antagonist, is a neuroprotectants. Actually, astrocytes are responsible for most glutamate uptake in synaptic as well as nonsynaptic areas and, consequently, are the major regulators of glutamate homeostasis. Even in minor changes that exceed the physiological concentrations of QA and 3HK, they can contribute to neuronal damage by generating free radicals. On the other hand, 3HAA-generated free radicals induce Nrf2-ARE which is the necessary transcription factor for the expression of HO-1. 3HAA suppresses IL-1/IFN- and TLR ligands associated with neurotoxicity through HO-1.

Competitive inhibition of IDO activity or overstimulation of NMDA receptors decreases intracellular NAD^+ levels and reduces cell viability. Contrarily, IDO induction and subsequent increase in NAD^+ synthesis may contribute to the maintenance of intracellular NAD^+ levels and cell viability under conditions of increased oxidative stress. 3HK has dual effect in redox balance; in case of mitochondrial dysfunction, 3HK causes intracellular accumulation of ROS/reactive nitrogen species and subsequent cell death. In human hypertryptophanemia, Trp accumulates in the brain and significantly decreases the overall content of brain antioxidant defenses. Trp deficiency due to the overactivation of IDO leads to the suppression of mTOR activity which inhibits cell cycle progress. However, melatonin protects cells against oxidative stress-induced apoptosis due to its ability to scavenge mitochondrial ROS. Finally, different signaling informations about the depletion or sufficiency of cellular Trp create new decisions considering either cellular growth or cell death.

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Chapter 3
Tryptophan and Nitric Oxide in Allergy

Kathrin Becker, Giorgio Ciprandi, Johanna Gostner, Heinz Kofler, and Dietmar Fuchs

Abstract An immune deviation toward Th2-type immunity is involved in the pathogenesis of allergic asthma and rhinitis. Allergic inflammation is characterized by upregulation of Th2-type cytokines (the so-called Th2 polarization), whereas there is a downregulation of Th1-type immune response and related cytokines like interferon-γ (IFN-γ). The latter is a strong inducer of enzyme indoleamine 2,3-dioxygenase (IDO), which degrades the essential amino acid tryptophan as part of an antiproliferative strategy of immunocompetent cells to halt the growth of infected and malignant cells. Tryptophan metabolism may also play a relevant role in the pathophysiology of allergic disorders.

In patients with pollen allergy, raised serum tryptophan concentrations were observed compared to healthy blood donors. Moreover, the higher baseline tryptophan concentrations were associated with poor response to specific immunotherapy. It turned out that the increase of tryptophan concentrations in patients with pollen allergy only exists outside pollen season, but not in season. Interestingly, there was only a minor alteration of the kynurenine to tryptophan ratio (Kyn/Trp, an index of tryptophan breakdown), which is used as an estimate of IDO activity.

The reason for the higher tryptophan concentrations in patients with pollen allergy outside season remains obscure. With this respect, specific interaction of nitric oxide (NO**.**) with IDO could be important, because an enhanced formation of

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NO**.** has been reported in patients with asthma and allergic rhinitis: exhaled breath NO**.** is increased in asthma versus healthy controls, and serum nitrite concentrations were found to be higher in allergic patients out of pollen season than in season. Importantly, NO**.** slows down the expression and activity of the heme enzyme IDO. So, the higher tryptophan levels could be explained when IDO activity was suppressed by NO**.**. As a consequence, inhibitors of inducible NO**.** synthase (iNOS) should be reconsidered as candidates for antiallergic therapy out of season that may decrease the production of NO**.** and thus abrogate the arrest of IDO.

Keywords Allergy • Atopy • Cross-regulation • Indoleamine 2,3-dioxygenase • Interferon-gamma • Kynurenine to tryptophan ratio • Neopterin • Nitric oxide • Th2-type immunity • Tryptophan 2,3-dioxygenase

3.1 Tryptophan

L-Tryptophan is an essential amino acid that is required for protein biosynthesis and also serves as precursor of several metabolites in humans. Absorbed tryptophan circulates in its free form or bound to albumin in the peripheral blood stream. Only in its free form, it can cross the blood-brain barrier. There are three different biosynthetic pathways in which tryptophan is metabolized: (i) the formation of kynurenine derivates, which represents the major route; (ii) the generation of serotonin, a neurotransmitter and precursor of melatonin (Schroecksnadel et al. 2006; Chen and Guillemin 2009); and (iii) the biosynthesis of proteins (Fig. 3.1).

To generate kynurenine, tryptophan is oxidized by a cleavage of the indole ring moiety, which is achieved either by tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase 1 (IDO-1), or IDO-2. TDO is primarily expressed in the liver and is inducible by tryptophan or corticosteroids (Badawy 2013). IDO-1 is induced by various inflammatory cytokines, with this respect the most prominent one being interferon-γ (IFN-γ), and is expressed in numerous cells as macrophages, microglia, neurons, and astrocytes, but also epithelial cells and fibroblasts (Guillemin et al. 2007). The recently discovered IDO-2 possesses similar activities to IDO-1 but differs in its expression pattern, substrate specificity, and signaling pathways (Chen and Guillemin 2009).

IDO-1 plays an essential role within the immune response and could even serve as a biomarker for the inflammation status in human. It has been discovered that IDO-1 inhibits immune cell and pathogen proliferation by the depletion of tryptophan and/or by the production of bioactive catabolites (Samelson-Jones and Yeh 2006). In addition, tryptophan breakdown products such as kynurenine, 3-hydroxyanthranilic acid, and quinolinic acid may negatively affect neurological functions, while other metabolites such as kynurenic acid can be neuroprotective (Heyes et al. 1992; Klein et al. 2013; Sas et al. 2007).

The second metabolic pathway is the generation of the neurotransmitter 5-hydroxytryptamine (serotonin) via the enzyme tryptophan 5-hydroxylase (T5H).

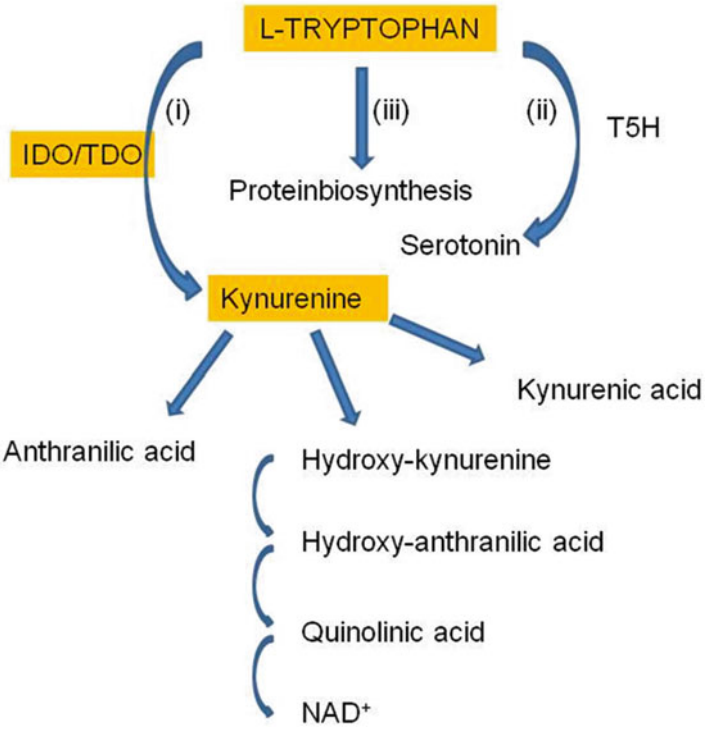

Fig. 3.1 The three different ways of tryptophan usage. (*i*) The first one represents the major route of tryptophan breakdown. The rate-limiting enzymes indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) convert the essential amino acid tryptophan into kynurenine. This metabolite is precursor of several metabolites. (*ii*) The second pathway is the conversion via tryptophan 5-hydroxylase (T5H) and followed by decarboxylation to the neurotransmitter serotonin (5-hydroxytryptamine). Furthermore, tryptophan is required for protein biosynthesis (*iii*)

In a first step, 5-hydroxytryptophan is formed, which is converted to serotonin under the influence of 5-hydroxytryptophan decarboxylases that require pyridoxal phosphate as a cofactor. In the case of insufficient tryptophan availability, serotonin production is diminished, which may cause neuropsychiatric symptoms like depression or other mood disorders (Widner et al. 2002). The third pathway represents tryptophan as a component of proteins.

3.2 Tryptophan and Its Influence on the Immune System

Significant alterations of serum tryptophan concentrations were observed in pregnant women (Schröcksnadel et al. 1996). Tryptophan concentrations declined with the duration of pregnancy and correlated inversely with neopterin concentrations. Data indicated that IDO activity was involved in the tryptophan metabolism.

Thereafter, it was found that IDO activation is an important aspect in the establishment of immunotolerance against the fetus (Munn et al. 1998) and thus that tryptophan metabolism is strongly involved in immunomodulation (Mellor and Munn 2004). Great attention was paid to the estimation of tryptophan breakdown as a biomarker in various immune pathologies such as infections, autoimmune and neurodegenerative disorders, and allergy (Schroecksnadel et al. 2006; Widner et al. 2000a, b; Raitala et al. 2006; Kositz et al. 2008). In the human immune system, various cell types play an important role to protect the integrity of the organism from invaders. The efficient host defense against pathogens is achieved through the thorough coordination of the innate and adaptive immune system. Once an antigen is present in the body, it has to be recognized by T cells, which identify the antigen in cooperation with antigen-presenting cells. The recognition sites include the T-cell receptor and the major histocompatibility complex. Furthermore, the signal cascades involve several binding proteins such as the protein ligand B7 and the cluster of differentiation 28 (CD28), which provide co-stimulatory signals to T cells (Balakrishnan and Adams 1995). The activation of different subsets of T-helper (Th) cells characterizes different immune responses. T cells can differentiate into a variety of effector subsets, including the classical Th1- and Th2-type cells, the recently defined Th17-type cells, the Th9 subset that control the growth and activation of mast cells, the follicular helper T (Tfh) cells that are responsible for the B-cell maturation responses (Zhou et al. 2009), and the regulatory T cell (Treg). The decision for differentiation is mostly driven by cytokines that are expressed in the microenvironment. Also, the interaction strength between the T-cell antigen receptor and the antigen can influence the direction of differentiation (Zhou et al. 2009).

Signaling by the arylhydrocarbon receptor (AHR) is thought to be involved in T-cell differentiation. AHR is a cytosolic receptor, which translocates into the nucleus after ligand binding and dimerizes with the AHR nuclear translocator (ARNT) to act as a transcription factor for various genes including the cytochrome P450 (CYP) enzymes (Van Voorhis et al. 2013). The AHR is known as a sensor to the outside environment that modulates the immune system in response to toxins. However, AHR signaling is activated not only after toxic exposures but also by endogenous compounds like the tryptophan catabolite kynurenine, which leads to the activation of several CYP isoenzymes and other metabolizing enzymes such as glutathione S-transferase Ya (GSTYa) or aldehyde-3-dehydrogenase (ALDH-3) (Van Voorhis et al. 2013). Via AHR, a toxin can also elicit an inflammatory response with the induction of Treg, where the IDO pathway and its metabolites are involved (Van Voorhis et al. 2013).

3.3 Types of Immune Response

Th1-type immune reaction is crucial in the pathogenesis of several inflammatory disorders like cardiovascular diseases, autoimmune syndromes, malignant tumor diseases, and neurodegenerative disorders (Asehnoune et al. 2004; Romagnani

2004; Schroecksnadel et al. 2007). Th1-type cells are characterized by the production of typical Th1-type cytokines like IFN-γ and are involved in the cellular immune response against pathogens and malignant cells. In the opposite, Th2-type cells predominate in allergic reactions and asthma (Romagnani 2004), interleukin-4 (IL-4), interleukin-5, and interleukin-13 representing prominent cytokines released from Th2-type cells. They mediate antibody responses, especially immunoglobulin E (IgE) production (Barth et al. 2003), and control helminthes infections (Zhou et al. 2009). Th17-type cells represent another type of T-helper cells, which modulate immune responses. They are supposed to combine innate and adaptive immunity (Yu and Gaffen 2008); produce IL-17A, IL-17F, and IL-22; and play important roles against extracellular bacteria or fungi. Regulatory T cells (Tregs) are involved in the maintenance of immunological self-tolerance (Hori et al. 2003) and limit potential collateral tissue damage (Zhou et al. 2009). Tregs are characterized by expression of forkhead transcription factor box p3 (Foxp3). There are two subgroups of Tregs: the naturally occurring Tregs (nTreg) and the induced Tregs (iTreg). Both cell types play a role in the maintenance of self-tolerance and the prevention of autoimmunity.

During the Th1-type immune reaction, the most prominent immune inductor IFN-γ is secreted by activated T lymphocytes and natural killer (NK) cells to initiate antimicrobial and antitumoral defense mechanisms (Romagnani 2006). Thereby, IFN-γ induces various biochemical pathways such as the activation of GTP-cyclohydrolase I (GTP-CH1) and IDO. This includes also the high output of reactive oxygen species (ROS) by human macrophages or monocytes (Nathan et al. 1983) and the induction of the inducible nitric oxide synthase (iNOS) and of several other immune effector pathways (Widner et al. 2000a; Werner et al. 1991).

The activation of GTP-CH1 by IFN-γ leads to the production of the pteridine derivatives neopterin and 5,6,7,8-tetrahydrobiopterin (BH_4). BH_4 is the essential cofactor for several monooxygenases including iNOS and is formed in various cells of several species upon exposure to proinflammatory stimuli. Upon stimulation, these cells produce NO**.** in a high rate. However, the production of BH_4 involves 6-pyruvoyltetrahydropterin (PTPS), an enzyme, which is of low activity in human and primate macrophages and dendritic cells. As a result of this biochemical peculiarity, human and primate monocyte-derived cells produce high amounts of neopterin at the expense of BH_4. In the absence of sufficient amounts of BH_4, also the proper function of enzyme iNOS and in this way NO**.** output are diminished (Werner et al. 1990; Andrew and Mayer 1999). In contrast, human fibroblasts or endothelial cells preferentially produce BH_4, and thus, also NO**.** is formed.

Neopterin is a stable biomarker of immune activation, which can be easily determined in body fluids like blood, urine, and cerebrospinal fluid (CSF) (Fuchs et al. 1992; Murr et al. 2002). Because of the common immunostimulatory background, neopterin production and tryptophan breakdown are not only induced in parallel in vitro (Weiss et al. 1999) but also in patients (Schroecksnadel et al. 2006). Several in vivo studies confirm the association between altered neopterin concentrations and tryptophan breakdown rates, as detected in serum samples of patients with infectious diseases, like HIV, gynecological cancer, malignant tumors, cardiovascular disease,

neurodegenerative disorders, or diseases associated with normal aging processes (Schroecksnadel et al. 2005a, 2006; Fuchs et al. 1988, 1990, 1991, 2009; De Rosa et al. 2011; Pedersen et al. 2011; Wirleitner et al. 2003). Both pathways turned out to represent robust and strongly predictive immune activation biomarkers.

Neopterin concentrations can be measured with commercially available ELISA. Usually, tryptophan and kynurenine concentrations are measured with high-performance liquid chromatography (HPLC), and IDO activity can be estimated by the ratio of kynurenine to tryptophan (Kyn/Trp) concentrations.

In vitro, high neopterin output by activated human monocyte-derived macrophages has been shown to be associated with a strong release of hydrogen peroxide (H_2O_2) (Nathan 1986). In line with this observation, higher neopterin concentrations in, e.g., patients with coronary artery disease were found to concur with low concentrations of serum antioxidants (Murr et al. 2009). This fact implies that neopterin concentrations can also serve as sensitive indirect marker of oxidative stress during immune activation (Murr et al. 1999).

3.3.1 Allergy

In the past few years, the incidence of allergy and asthma has increased drastically. Allergy and asthma are among the most common chronic diseases in the world. Currently, more than 130 million people are affected by asthma and the numbers are steadily growing. Interestingly, in developing countries, there is a lower prevalence of allergic diseases. Environmental factors, for example, more indoor allergens, pollution, changes in diet, or breastfeeding, could be the reason for these increasing atopic diseases. However, there are still little relations and evidences, which demonstrate definitive risk factors. A link between lifestyle, habits, and the development of allergy might exist, but the connection is still heavily discussed (Fuchs 2012). However, childhood infections seem to have a protective effect for the development of atopy and allergic diseases in the later life. A higher allergic sensitization occurs often in newborn, but less in children from large families and those who attend daily day care (Strachan 1989; Krämer et al. 1999; Yazdanbakhsh et al. 2002). These results suggest that a frequent contact with infections could have protective effects on the children (Strachan 1989).

The main explanatory theories for the increase of atopic diseases are altered hygienic conditions (Strachan 1989) and human nutrition. Nowadays, there exist improved sanitation and living conditions, vaccinations, and antimicrobial therapies, and most people have less contact to microbes. Immune stimulations by microbes are considered to be necessary to hinder the consolidation of the atopic responder type, as was concluded from the hygiene hypothesis (Liu and Murphy 2003). Furthermore, human nutrition has considerably changed. Food preservation and sterilization reduces the microbial exposure, and pasteurization has replaced drying and fermentation (Fuchs 2012; Yazdanbakhsh et al. 2002; Isolauri et al. 2004). The food preservatives have become more and more popular because of the globalization, as food is shipped and offered all over the world and needs to be conserved over a long

time. Many of the commonly used preservatives are antioxidative substances, which can inhibit the oxidation of the food components (Gostner et al. 2014).

Recently, major attention is payed to the role of the human innate immune system as it was shown to be strongly activated during allergic responses. Antigen-presenting cells can absorb the allergen and initiate the signal transduction for T-cell development within the Th2-type immune response direction. Th2-type cell activation leads to IL-4, IL-5, and IL-13 cytokine expression. These cytokines can interact with their receptors to stimulate allergen-specific IgE production. Furthermore, this cytokine production leads to the accumulation of a high number of eosinophils and mast cells and boosts inflammation in the body (Holt et al. 1999). Immune cells start to produce large amounts of cytokines, chemotactic factors, or free radicals, which leads in the end to enhanced vascular permeability and persistent inflammation (Ciprandi et al. 2011a). High amounts of IgE circulate in the blood and bind to the high affinity IgE receptor (FcεRI) of mast cells or basophiles to activate histamine release, which is the main inductor of an allergic disease (Brown et al. 2008). At this time point, the sensitization to a specific allergen is stored. If this antigen is present at another time, it can bind to the IgE of mast cells and activate several cascades like vasodilation, mucous secretion, and nerve stimulation of muscle contraction (Zaknun et al. 2012). However, not every Th2-type response is characterized by IgE production.

It has been argued that a decreased exposure to pathogens in early childhood may result in an insufficient stimulation of Th1-type cells, which leads to a diminished capability to counterbalance the expansion of Th2-type cells and thus results in a predisposition to allergy (Yazdanbakhsh et al. 2002). High IgE levels may indicate atopy, which underlies allergic diseases as asthma, rhinoconjunctivitis, and eczema. It is well accepted that Th1- and Th2-type cytokines cross-regulate each other (Romagnani 2004). Allergic inflammation is characterized by the upregulation of Th2-type cytokines and downregulation of Th1-type cytokines such as IFN-γ. Von Bubnoff and Bieber described the IDO pathway as one of the central pathways in allergy development. IDO activity not only is crucial during pregnancy, chronic inflammation, tumorigenesis, and infections but also influences the inflammatory state of atopy or allergy (von Bubnoff and Bieber 2012).

An in vitro study in human peripheral blood mononuclear cells (PBMC) further confirmed this observation that typical Th2-type cytokines, IL-4 and IL-10, can counteract IFN-γ- and Th1-mediated pathways, when the effects of the different cytokines on neopterin formation and tryptophan breakdown were compared (Weiss et al. 1999). After IL-4 or IL-10 exposure, a lower stimulatory effect of IFN-γ was observed, which resulted in a diminished tryptophan breakdown rate and lower neopterin levels, whereas Th1-type cytokine IL-12 had the opposite effect of co-stimulating both biochemical pathways. Thus, exposure of PBMC to Th2-type cytokines was reflected by higher tryptophan concentrations in culture supernatants, because the breakdown of the amino acid was suppressed.

Allergic rhinitis is associated with the dysfunction of T-cell responses, where the antigen induces mast cell activation by allergen-specific IgE. Severe allergic rhinitis has a huge impact on the health-related quality of life and/or work, which can result in a significant individual burden. Furthermore, allergic rhinitis and asthma are

often comorbid diseases. Frequent treatments with aspirin, anti-inflammatory agents, or antibiotics can inhibit Th1-type immune response and strengthen the development of Th2-type responses and cause allergic symptoms in the case of a concomitant phenomenon (Kuo et al. 2013). The activated cells release large amounts of proinflammatory cytokines, which induce inflammatory cell enhancement. It may lead to a persistent inflammation of the nasal mucosa, which is the main relevant pathophysiological feature in allergic rhinitis (Ciprandi et al. 2011a). Interestingly, treatment of PBMC with aspirin or salicylic acid had a similar effect on neopterin production and tryptophan breakdown as compared with Th2-type cytokines, where both were suppressed (Schroecksnadel et al. 2005b).

These results are in line with the hypothesis that allergy results from a shift of Th1- toward Th2-type immunity. Inhibition of IFN-γ and as a result also of IDO decreases the Th1-type immune response.

Besides typical nasal symptoms like itching, sneezing, rhinorrhea, or obstruction (Bousquet et al. 2008), many allergic rhinitis patients exhibit also nonnasal symptoms as behavioral changes like tiredness, somnolence, depression, apathy, and impaired attention, which can reduce the quality of life (Juniper and Guyatt 1991; Ciprandi et al. 2011b). This fact supports the hypothesis that the tryptophan pathway and as a result also serotonin production play an important role in allergy.

Allergen-specific immunotherapy (AIT) is widely used to treat asthma and allergic rhinitis and to modify the disease development. AIT is typically used, when medication or environmental changes cannot control asthma or allergic rhinitis symptoms. There are two ways of desensitization procedures, the subcutaneous immunotherapy (SCIT), where the allergens are injected subcutaneous to the patients, also known as "allergy shots." In contrast, the sublingual immunotherapy (SLIT) provides the allergen as drops to the sublingual area for local absorption. The outcome of both treatments seems to be equal (Saporta 2012), although some studies claimed that SCIT might have better results (Mungan et al. 1999). SCIT is a well-established method, which has been used for many decades, and furthermore, it is well tolerated (Saporta 2012). SLIT is also a very old method, which is not well established in the USA, but in Europe it is still a commonly used treatment. SLIT seems to be a safer method for treatment of children (André et al. 2000).

3.4 Tryptophan in Allergy

Induction of IFN-γ leads to the activation of downstream biochemical pathways like tryptophan breakdown by IDO. IDO activity is drastically enhanced during the proinflammatory Th1-type immune response and contributes to the pathogen defense by deprivation of the essential amino acid tryptophan. Furthermore, ROS and reactive nitrogen species (RNS) are produced in high amounts, and these are commonly known to interference with target cells or pathogens by oxidation and/or nitration of vital cellular structures. For the balance of immune responses, Th1 and Th2 responses can cross-regulate each other (Romagnani 2004, 2006). This can be achieved by the activation of redox-sensitive signaling cascades, where oxidative

conditions support Th1-type development, while excess of antioxidant compounds "antioxidant stress" can lead to a shift toward allergic Th2-type immune responses (Murr et al. 2005; Poljsak and Milisav 2012). IDO is widely accepted for its role in infection, pregnancy, autoimmunity, and neoplasia, but also the control of allergic inflammation was attributed to the enzyme (von Bubnoff and Bieber 2012).

Higher serum tryptophan concentrations were observed in adult patients with pollen allergy compared to healthy blood donors (Kositz et al. 2008). In this study, 44 patients with allergic rhinitis were examined before and after SCIT and compared to 38 healthy controls. In atopics, higher tryptophan levels in comparison to healthy blood donors were noted, but there were no differences in kynurenine concentrations. Also Kyn/Trp was only slightly, but not significantly, lower in atopics, and serum neopterin levels tended to be at the upper limit of the normal levels. Interestingly, higher levels of tryptophan were preferentially observed in nonresponders to SCIT. Thus, tryptophan concentrations could help to predict the outcome of SCIT.

A further study confirmed the higher tryptophan levels in patients with pollen allergy, but this observation was made only off pollen season but not in pollen season (Ciprandi et al. 2010). Notably, also the higher tryptophan levels observed in the first study (Kositz et al. 2008) were measured in patients before they received desensitization therapy and were thus off pollen season. Patients with pollen allergy seem to have a distinct IDO activity pattern with higher tryptophan levels due to a less breakdown out of season. However, tryptophan levels decline closer to normal values in spring; when under allergen exposure, tryptophan breakdown becomes initiated. In regard to this observation, the higher tryptophan levels during winter could represent a consequence of the chronic Th2-type immune response in summer due to counter-regulation.

However, any possible (primary or additional) role of TDO activation should not be disregarded.

Another substrate of IDO, serotonin, was found to be higher in patients with pollen allergy compared to outside of pollen season (Ciprandi et al. 2011b). Interestingly, low serotonin levels in allergic rhinitis patients in season upon pollen allergen exposure were strongly related with behavioral impairment, as was assessed by quality of life questionnaires, and thus, serotonin can serve as a biomarker of behavioral symptoms during allergic response (Ciprandi et al. 2011b). As in other clinical inflammatory conditions, tryptophan availability is strongly involved in the pathogenesis of mood disorders and depression (Widner et al. 2002). Abnormal tryptophan concentrations may be involved in the development of neuropsychiatric symptoms, while serotonin production is decreased (Widner et al. 2000a) or may be also above normal.

3.4.1 Nitric Oxide: Nitrite

The reason for the higher tryptophan concentrations in patients outside of pollen season still remains obscure. Specific interactions of NO**.** with IDO could be very important in this circumstance (Ciprandi and Fuchs 2013). There are several reports

in the literature that exhaled breath from patients with allergic rhinitis or asthma contained higher NO**.** levels compared to healthy controls (Stewart and Katial 2012). Likewise, serum nitrite concentrations were found to be higher in allergic rhinitis patients compared to healthy controls, and again, this was apparent off pollen season rather than during pollen season (Ciprandi et al. 2011a).

These observations seem to provide a link between tryptophan breakdown and the formation of NO**.**, which has been demonstrated earlier to inhibit the expression and function of IDO (Thomas et al. 1994) (Fig. 3.2). Thus, when NO**.** formation is increased, an inhibition of IDO becomes more likely, and as a consequence, tryptophan concentration would increase (Ciprandi et al. 2010). The increase of tryptophan in atopics out of season can be explained by a suppression of IDO activity through the enhanced availability of NO**.** (Gostner et al. 2014). Importantly, no inhibitory activity on GTP-CH1, the key enzyme for neopterin production, is known for NO**.**.This would agree with the independent development of tryptophan and neopterin concentration in patients with allergic rhinitis, e.g., mast cells can produce IFN-γ and stimulate the production of neopterin in monocyte-derived macrophages or dendritic cells, while NO**.** formation starts in endothelial cells and IDO activity becomes arrested by the presence of NO**.** (Ciprandi and Fuchs 2013).

iNOS inhibitors have already been considered as candidates for an antiallergic therapy (Hesslinger et al. 2009) without considering their influence on IDO. iNOS

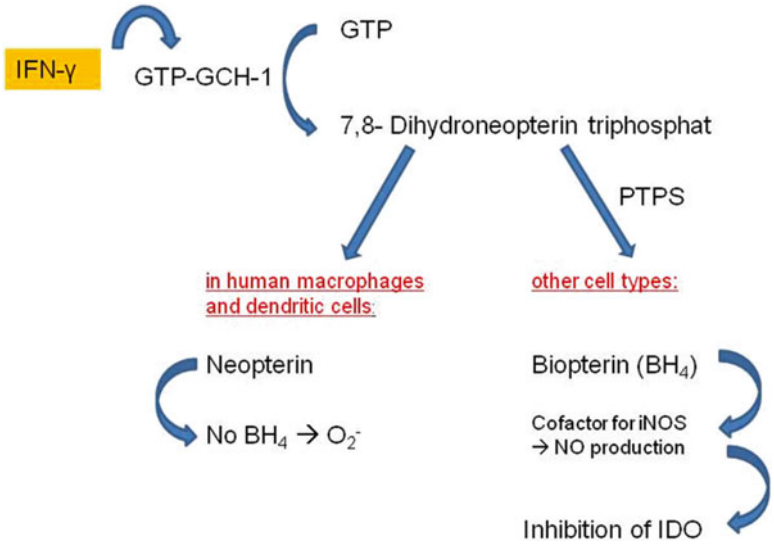

Fig. 3.2 Interferon-γ (IFN-γ) expression leads to the induction of GTP-cyclohydrolase I (GTP-CH1), which produces out of guanosine triphosphate (GTP) 7,8-dihydroneopterin triphosphate. In human macrophages and dendritic cells, the enzyme 6-pyruvoyltetrahydropterin (PTPS) is lacking, and neopterin is produced. In all other cell types PTPS forms 5,6,7,8-tetrahydrobiopterin (BH_4). BH_4 serves as a cofactor for inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO**.**). High levels of NO**.** can inhibit IDO activation and as a result inhibit tryptophan breakdown. If BH_4 is not available, iNOS produces superoxide ($O_2^{\cdot -}$) instead of NO**.**.

inhibitors may abrogate the IDO arrest, by diminishing NO**.** production. Still, treatment with iNOS inhibitors should be more effective outside of pollen season, than during season (Ciprandi and Fuchs 2013). IDO and iNOS are both induced by IFN-γ. iNOS inhibitors block NO**.** production and thereby can promote IDO activity. However, the interference of NO**.** and IDO could be cell specific. In stimulated monocyte-derived cells, ROS are concomitantly produced with NO**.** and give rise to the cell-toxic peroxynitrite ($ONOO^-$), whereas in other cells, because of the absence of superoxide anion ($O_2.^-$), NO**.** is not oxidized and exerts its inhibitory effect on IDO. This can be explained with an excess of antioxidants, which can stabilize the iNOS cofactor BH_4 to guarantee high NO**.** production. Furthermore, other NOS enzymes that are not induced by IFN-γ can continue to produce NO**.** and inhibit IDO.

3.4.2 Nitric Oxide and Tryptophan Metabolism

NO**.** is a classical messenger for several biological processes, which include vasodilatation (Allen et al. 2009), neurotransmission (Bult et al. 1990; Garthwaite 2008), macrophage-mediated cytotoxicity (Marletta et al. 1988), gastrointestinal smooth muscle relaxation (Bult et al. 1990), and bronchodilation (Lindeman et al. 1995). NO**.** synthases produce NO**.** by the oxidation of L-arginine and formation of the by-product, L-citrulline (McNeill and Channon 2012). There are three isoforms of NOS: (1) neuronal NO**.** synthase (nNOS) is involved in the regulation of autonomic functions in cardiovascular diseases; (2) iNOS has effects on vascular functions under conditions of sepsis and is a potent mediator of inflammation; (3) endothelial NO**.** synthase (eNOS) acts in vascular diseases such as atherosclerosis, hypertension, and ischaemia-reperfusion. All of them can be responsible for abnormalities in endothelial functions. nNOS and eNOS are both calcium dependent, while iNOS works calcium independently (Moncada 1999). Furthermore, the first two NOS isoforms are constitutively expressed, while iNOS seems to be active only during immune responses (Tsutsui et al. 2009).

NO**.** synthesis is commonly not only cell specific, but also the environment in which the cells, organs, or the whole organisms that are experienced at the time of the production site is important for the activity of the three different NOS (Villanueva and Giulivi 2010). Several cross talks have been described for NO**.** and IDO. For example, tryptophan and the tryptophan-kynurenine pathway metabolite 3-hydroxyanthranilic acid can inhibit iNOS at the expression and catalytic level (Samelson-Jones and Yeh 2006). Furthermore, during the immune response, NO**.** is an important regulator of the enzyme IDO. NO**.** inhibits IDO by preventing both the expression and the activity of IDO, by binding to the catalytic domain (Samelson-Jones and Yeh 2006). In turn, IDO inhibition then leads to higher tryptophan levels.

As described above, BH_4 is required for a proper function of NO**.** synthesis. However, in human macrophages, there is a lack of PTPS to produce BH_4, and instead, neopterin accumulates. When BH_4 levels become deficient, the oxygenase

domain of NOS enzymes produces $O_2^{\bullet -}$ instead of NO•. The produced $O_2^{\bullet -}$ can promote further reactions to form other ROS/RNS such as $ONOO^-$ or H_2O_2, which can disturb the redox balance and in the end lead to cellular injury and inflammation. Toxic ROS products like H_2O_2, $O_2^{\bullet -}$, or $ONOO^-$ can suppress the growth of target cells and pathogens (Schroecksnadel et al. 2010; Wink et al. 2011) but also lead to the dysfunction of protective cellular antioxidant mechanisms in inflamed tissue, which can result in a high oxidative stress milieu (Hesslinger et al. 2009; Bowler and Crapo 2002). In turn, a high degree of oxidative stress can activate signaling cascades such as mitogen-activated protein kinase (MAPK), transcription factor nuclear factor-κB (NF-κB), and activator protein (AP) pathways and initiate the expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1, chemokines, and adhesion molecules (Aggarwal 2004).

The increased ROS production can further limit BH_4 availability through the oxidation of the oxidation-sensitive molecule BH_4 itself (Lindeman et al. 1995). Oxidative stress is probably involved in a wide range of clinical pathologies like cardiovascular or neurodegenerative disorders (Halliwell 1996, 2006). To counteract ROS effects, different strategies have evolved. For example, some small molecules function as antioxidants or enzymes, which can neutralize ROS, like catalase, glutathione peroxidase (EC 1.11.1.9), and superoxide dismutase (Sies 1997; Halliwell 1999). Antioxidants may be synthesized in the body or can be obtained from the diet. An intake of dietary antioxidants can counteract oxidation processes by scavenging ROS and other redox-sensitive molecules and therefore protect against cellular damage (Schroecksnadel et al. 2007; Jenny et al. 2011). However, if antioxidants are present in a normal milieu without inflammation, an excess can shift the Th1-type immune response to Th2-type immunity, which can promote or accelerate allergic reactions when an allergen is met (Zaknun et al. 2012).

3.5 Food Antioxidants and Tryptophan Metabolism

In the last decades, antioxidant exposure and uptake have extremely increased. Food and beverages are supplemented with vitamins such as A, C, or E, and this is done because of the conviction that this should be healthy. However, it is still unclear whether supplemented antioxidant vitamins and other compounds have a benefit comparable to that of their natural counterparts. Moreover, meta-analyses demonstrated that supplemented antioxidant vitamins like vitamin A, C, and E may even increase mortality rather than reduce it (Bjelakovic et al. 2007); especially, vitamins E and A and ß-carotene seem to exert also adverse effects.

Today, when antioxidants are supplemented to almost every food or beverage, it is not easy to avoid overexposure. Thereby, extra vitamins are usually advertised. This is not the case for food preservatives and colorants. These are usually declared only as fine print. Food preservatives like sodium sulfite or benzoate but also colorants like curcumin or betalain are widely known for their antioxidant activity (Zaknun et al. 2012). However, a well-functioning human organism does not need extra antioxidant supplementation; the content in the normal Western diet is sufficient.

An excess of food preservatives or antioxidants may increase allergy risks. They can suppress Th1-type immune responses and cytokine expression, and consequently, due to the cross-regulation of Th1- and Th2-type immune responses, Th2-type cytokines are upregulated, and the development of asthma and allergic diseases might be favored (Murr et al. 2005). If an allergen is presented, Th2-type cytokines are produced in a high concentration, and this condition can strengthen the Th2-type immune response. Under antioxidant-loaded conditions, allergic response already initially becomes stronger when an allergen is met, than in conditions with normal antioxidant levels (Zaknun et al. 2012).

It is well documented that antioxidants can stabilize BH_4 and promote NO**.** production, which leads in the end to the inhibition of IDO and an increase of tryptophan and higher serotonin levels. In parallel, high serotonin levels could even precipitate the serotonin syndrome, which is a life-threatening disease, characterized by a clinical triad of mental-status changes, autonomic hyperactivity, and neuromuscular abnormalities. It is known to be induced either by adverse drug reaction; from therapeutic drug use, intentional self-poisoning, or inadvertent interaction between drugs; or by an excess of antioxidants (Boyer and Shannon 2005).

Physical exercise is the most convenient way to escape from reductive caused by excess intake of antioxidative compounds. Sports help not only in burning fat; it especially oxidizes the even stronger antioxidants like vitamins, spices, and food preservatives. This interaction may help to understand the findings that supplementation with antioxidant vitamins was found to slow down the antioxidant defense response induced by physical exercise and sports (Ristow et al. 2009; Peternelj and Coombes 2011). Thus, moderate sports and physical exercise can be recommended to combat allergic responses.

Unfortunately, in the current generation, every negative effect of food is first denominated by the public as an allergy. However, food allergy is often not an allergy in its sense. In its strict sense, food allergy is an adverse reaction to the food itself, and the classical immune mechanism is specific, for it being indicated by the presence of IgE antibodies is typical. The diagnosis will be taken after a case history, the demonstration of IgE sensitization by a skin-prick test on an in vitro test, and will be confirmed by a positive oral provocation (Wüthrich 2009). By contrast, food intolerance is considered as a "nonimmune"-mediated adverse reaction to the food. There are enzymatic (e.g., lactose intolerance, lactase deficiency), pharmacological (reactions against biogenic amines, histamine intolerance), or undefined food intolerances (against food additives). Interestingly, under such conditions, huge amounts of H_2 are produced and exhaled, H_2 under certain circumstances being a strong antioxidative compound. Still, it has to be kept in mind that not every sign of sickness after food intake has to be an allergy.

Recently, performed studies reported an association between fast-food consumption and the prevalence of asthma, rhinoconjunctivitis, and eczema in children and adolescents (Ellwood et al. 2013). In addition, antioxidants or additives may disturb the endogenous appetite and satiation regulatory circuits. On the one hand, antioxidants may suppress tryptophan breakdown by IDO (Jenny et al. 2011) and thus increase the availability of tryptophan for serotonin production and as a consequence contribute to mood improvement. Tryptophan metabolic changes may also

contribute to the weight gain after a calorie-restricted diet (Berger et al. 2013), when under starvation conditions tryptophan levels decline, which increases carbohydrate craving as a substitute for brain serotonin (Wurtman and Wurtman 1995) followed by weight gain. This sequence of events can explain the often observed yo-yo effect, also known as weight cycling when people rapidly gain weight after a diet.

Also, the histamine content of beverages and food has to be taken in consideration. As mentioned above, histamine is known to trigger acute symptoms like acute rhinitis, bronchoconstriction, diarrhea, or cutaneous wheal. It has a strong activity on endothelium and bronchial or smooth muscle cells and modulates also chronic inflammatory events (Jutel et al. 2002). Histamine is important in the early and late phase response to soluble antigens. It increases the vascular permeability and is involved in the recruitment, adherence, and activation of inflammatory cells (Andersson et al. 1994). Histamine content is increased in preserved food and thus could play an important role in the precipitation of allergic symptoms, if too high histamine uptake can trigger allergy development. Moreover in vitro, an inhibitory effect of histamine on neopterin formation in myelomonocytic cells has been described (Gruber et al. 2000).

Also air pollution can be responsible for increasing allergy appearance. An important compound is carbon monoxide (CO), which accumulates in the blood or is inhaled during cigarette smoke. CO can downregulate Th1-type immune responses via inhibition of IFN-γ and inhibition of IDO activity and thus activate Th2-type immunity (Naito et al. 2012). An excess of antioxidants can explain the connection of obesity, smoking or pollution, and their association with the increase of allergies (Hosick and Stec 2012), when CO, a gas with well-known antioxidant properties, exerts its effect to counteract Th1-type immune activation. As another consequence, tryptophan availability will increase, when IDO is suppressed. In turn, the higher tryptophan and thus serotonin availability may enhance mood and thus support addiction to tobacco smoking.

3.6 Conclusion

Significant alterations of tryptophan metabolism have been described in patients suffering from allergy. Allergy development is characterized by Th2-type immune activation that is related to Th2-type cytokine expression like IL-4, IL-5, and IL-10 by Th2-type cells. The immoderate increase of allergies in the past decades posed the question of the underlying trigger. Various approaches were taken into consideration, like the hygiene hypothesis or the pollution of the air. Still the aspect of antioxidants and allergy development has to be investigated in more detail. The enormous presence of antioxidants as food additives, preservatives, or colorants is indispensable in our lifestyle. Antioxidants can inhibit Th1-type immune response and thus can result in an insufficient clearance of infectious pathogens. The inhibition can be mediated by downregulation of IFN-γ and/or by the inhibition of IDO leading to higher tryptophan levels. The radical scavenging property of antioxidants

can stabilize BH_4, the cofactor for iNOS, and thus promote high NO**.** output. The high NO**.** level inhibits IDO activity by binding to the catalytic domain. For the allergy diagnosis, high levels of NO**.** and tryptophan can be good biomarkers that may be of value for the judgment of treatment response. The excess of antioxidants can represent the missing link between the constant growing number of allergy and obesity patients. The downregulation of Th1-type immune response and expression of Th2-type cytokines can be attributed to the inhibiting nature of anti-inflammatory agents like antioxidants. The impression is emerging that in otherwise healthy people, stress due to overwhelming exposure to antioxidants is more relevant than oxidative stress, which is critical in clinical conditions related with Th1-type immunity and excess IFN-γ and thus ROS production. A right balance between cellular produced ROS and antioxidant uptake via nutrition is essential to support human health.

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Chapter 4
Tryptophan Metabolites: A Microbial Perspective

Evren Doruk Engin

Abstract The discovery of the regulation of tryptophan biosynthetic pathway by means of a repressible operon has been recognised as a milestone in genetics. This long multistep pathway is energetically so expensive that numerous allosteric control loops exist in addition to tight genetic regulation. That's why essential amino acid tryptophan is a valuable product for food industry and animal breeding. Traditionally, vitamin auxotrophs of soil microorganisms have been used for amino acid manufacturing. However, in the age of synthetic biology, metabolic engineering has recently become the method of choice to construct the producer strains.

Additionally, in contrast to mammalian cells, bacterial cells are able to produce tryptophan starting at central metabolic intermediates from pentose phosphate pathway and glycolysis. This metabolic divergence provides an excellent target for the development of novel antimicrobials which interfere with the biosynthesis of tryptophan.

Tryptophan metabolism makes at least two contributions to microbial quorum-sensing pathways, which may have implications in antimicrobial chemotherapy. The autoinducers of renowned *Pseudomonas* quinolone signalling system originate either from kynurenine or shikimate pathways. Fluorinated 4-quinolone derivative antimicrobial drugs exhibit antipathogenic effects at subinhibitory concentration, presumably by interfering with *Pseudomonas* quinolone signalling. Another recently recognised bacterial signal molecule is indole, a degradation product of tryptophan. Unconventional stationary phase signal molecule indole is unique, as no receptor/response regulator protein has been identified to date. Instead, the effects of indole have been attributed to its physicochemical interaction with the cell membrane.

Keywords Tryptophan • Quorum sensing • *Pseudomonas* quinolone signalling • Indole • Antimicrobial

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4.1 Introduction

Tryptophan (Trp, W) is one of the 22 standard amino acids, which has aromatic indole ring as functional group. Mainly L-stereoisomer is found in natural polypeptide chains, whereas D-form is quite rare to occur in proteins. Apart from being incorporated into proteins, various metabolites of tryptophan act as intercellular signal molecules, pheromones/hormones in organisms from all three kingdoms, both intra- and interspecies manner (Tryptophan 2014).

4.2 Tryptophan Biosynthesis

Mammalian cells lack the capability to synthesise this amino acid; thus exogenous L-tryptophan supplementation is considered essential, and as a result, all mammalian organisms must rely on dietary consumption of protein-rich food. On the contrary, prokaryotes, eukaryotic microorganisms and higher plants usually have biosynthetic pathways for this amino acid (Gibson and Pittard 1968).

The synthesis of tryptophan from glucose covers an energetically expensive long metabolic pathway (Figs. 4.1 and 4.2). Therefore, L-tryptophan synthesis rate is

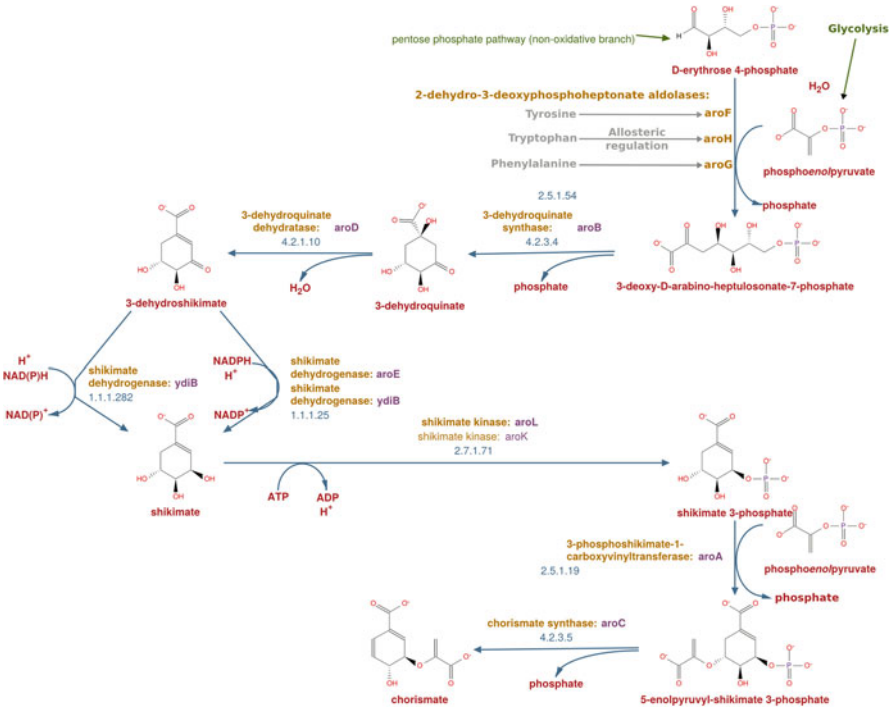

Fig. 4.1 Chorismate pathway in *Escherichia coli* (Adapted from EcoCyc Pathways, publicly available at http://ecocyc.org/ (Caspi et al. 2014))

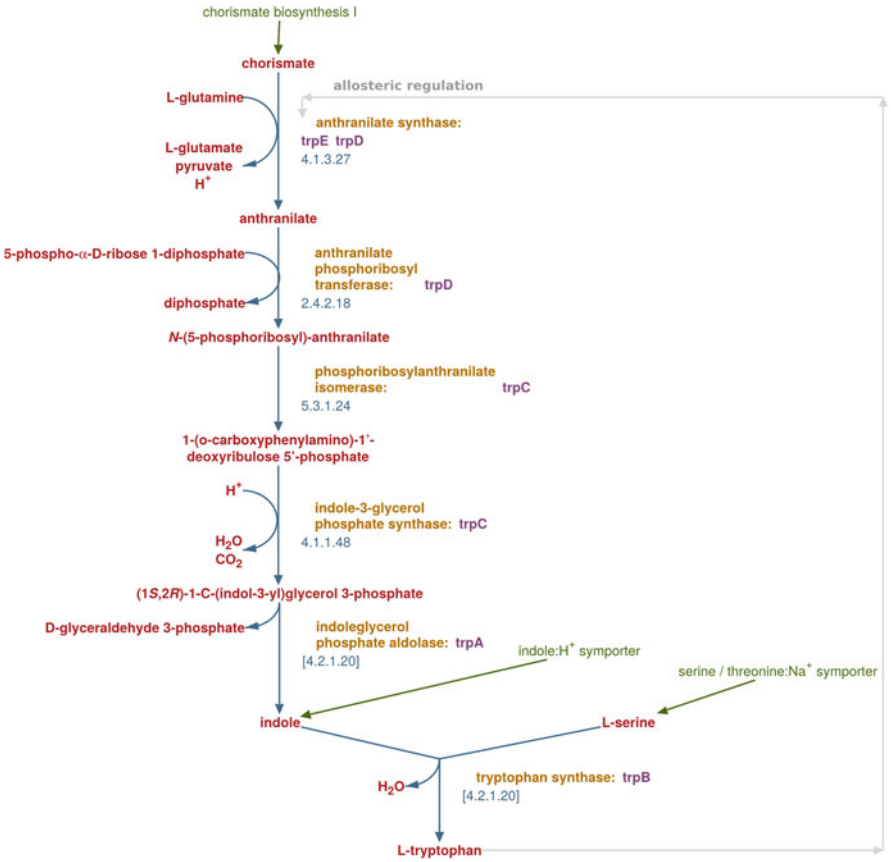

Fig. 4.2 Tryptophan biosynthesis in *Escherichia coli* (Adapted from EcoCyc Pathways, publicly available at http://ecocyc.org/ (Caspi et al. 2014))

tightly controlled in multiple steps, by means of transcriptional repression, attenuation and feedback inhibition. Plants and microorganisms utilise shikimic acid or anthranilate as starting compound for L-tryptophan synthesis (Jacob and Monod 1961; Yanofsky et al. 1981).

The shikimate pathway draws D-erythrose 4-phosphate from the non-oxidative branch of pentose phosphate pathway and phosphoenolpyruvate from glycolytic pathway to yield chorismate as end product, which further proceeds to aromatic amino acid biosynthesis (Fig. 4.1). The aldol condensation reaction catalysed by 2-dehydro-3-deoxyphosphopentonate aldose (EC 2.5.1.54, DAHP synthase) catalyses the first committed step of aromatic amino acid biosynthesis. In *Escherichia coli* K-12 substrain MG1655, the genome contains *aro*F, *aro*G and *aro*H which encode three allosterically regulated isozymes of DAHP synthase for tyrosine, phenylalanine and tryptophan biosynthesis, respectively. These three isozymes are all homodimeric or homotetrameric metalloenzymes that require divalent iron as cofactor – with the exception of AroF, which may couple with various divalent metal cations such

as Mn^{+2} – and share about 40 % of sequence identity. In minimal medium, *E. coli* cells mainly express phenylalanine-regulated $[AroG]_4$ isoform of DAHP synthase and, to a smaller extent, tyrosine-regulated $[AroF]_2$ form. Tryptophan-sensitive $[AroH]_2$ constitutes only 1 % of the total DAHP synthase activity of the cell (Tabaka 2009). The allosteric sites of DAHP synthases are located at N-terminal domain. Certain missense mutations in the N-terminal of tyrosine-sensitive DAHP synthase were shown to abolish the negative feedback regulation (Jossek et al. 2001). The enzyme levels in the cell are transcriptionally controlled by tryptophan repressor *trp*R for *aro*H and tyrosine repressor *tyr*R for *aro*G and *aro*F, respectively (Wallace and Pittard 1969). Once chorismate has formed, anthranilate synthase (EC 4.1.3.27) catalyses the transfer of one amine group from L-glutamine, yielding anthranilate, L-glutamate and pyruvate. This reaction constitutes the first committed step of L-tryptophan biosynthesis and is subjected to allosteric regulation by the end product, L-tryptophan.

Tryptophan operon was the first repressible operon discovered, which had a deep impact in our understanding of the genetic regulatory mechanisms in protein synthesis (Fig. 4.3) (Jacob and Monod 1961). The operon consists of a regulatory region followed by structural genes. The regulatory region encodes the constituently expressed repressor protein, promoter, operator and *trp*L (leader peptide–attenuator complex). The helix–turn–helix DNA-binding motifs of *trp*R-encoded tryptophan repressor are exposed to interact with the operator region of the *trp* promoter, whenever tryptophan is available in the cytoplasm in adequate concentrations. Hence, transcription cannot pass through operator-bound trp repressor, and the operon is *repressed* in abundant supply of tryptophan (Jayaraman 2011; Faghfuri et al. 2013). The enzyme expression economy of tryptophan operon is further fine-tuned by a mechanism called *attenuation*. As the availability of tryptophan decreases, the operon gets transcribed. In the bacterial cell, no distinct nuclear barrier exists to separate the translation and transcription processes. Thus, ribosomes begin translation on mRNAs while transcription from DNA continues. In case of tryptophan operon transcript, mRNA begins with *trp*L leader sequence which includes four functional sequence segments. Segment 1 encodes the leader peptide, which contains tandem tryptophan codons. The following segments 2, 3 and 4 do not code protein. Instead, segment 2 is able to form stem loop structure with segment 3, and segment 3 is able to form stem loop structure with segment 4. In case there is no tryptophan shortage in the cell, ribosome rapidly processes segment 1 and advances to segment 2. Ribosomal blockade of segment 2 before segment 3 transcription permits stem loop structure formation between segments 3 and 4, which in turn attenuates transcription of the remaining operon. When tryptophan becomes limiting for the cell, the ribosome gets stalled while translating the leader peptide, leaving segment 2 available to form stem loop structure with segment 3. In this case, segment 3 is no longer available to form attenuator stem loop structure. The transcription and translation

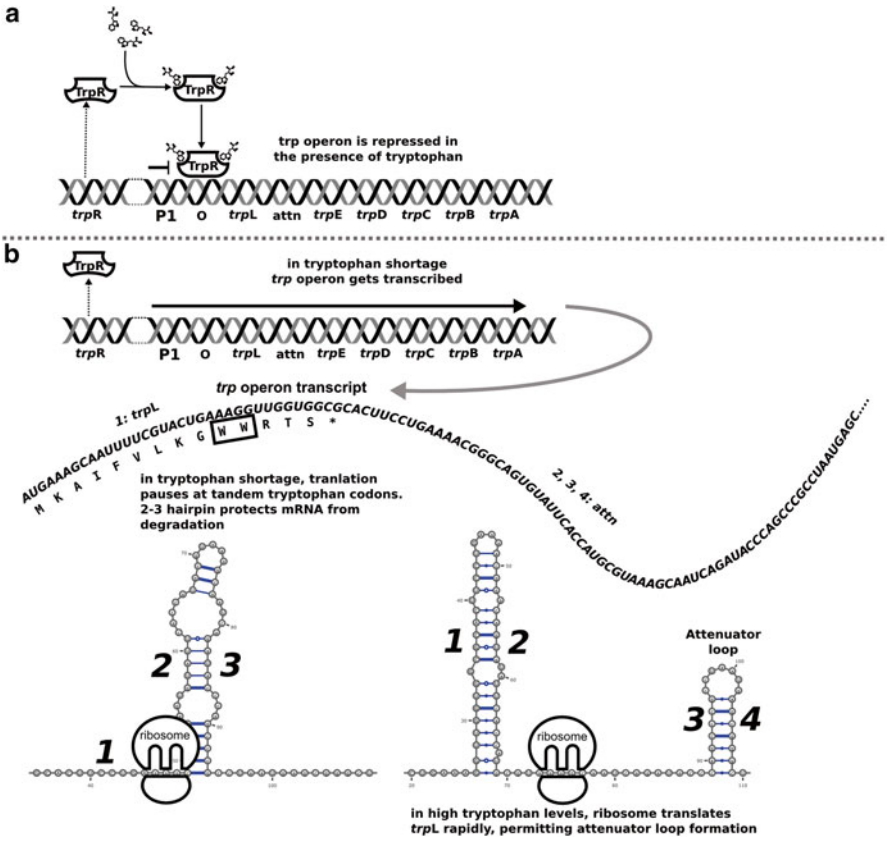

Fig. 4.3 The regulation of tryptophan operon in high (**a**) and low (**b**) cytoplasmic tryptophan concentrations

of trp operon proceed. In *E. coli* cells, translating ribosome acts as tryptophan sensor (Nudler and Mironov 2004).

4.2.1 Tryptophan Production in Bacterial Cells

The demand for commercial sources of L-tryptophan is constantly increasing, as this amino acid has been realised to be important in animal nutrition, treatment of certain psychological diseases and synthesis of antimicrobials. However, tryptophan synthesis involves energetically expensive multistep pathway, which complicates commercial manufacturing process. Moreover, the chemical synthesis of this essential amino acid for industrial purposes is not feasible, as it requires

nonrenewable toxic raw materials. Additionally, the end product is a racemic mixture of both stereoisomers of tryptophan (Aiba et al. 1982; Shen et al. 2012). Therefore, microbial production of tryptophan is still the most feasible way, yet it's not a trivial task to bypass the stringent control over tryptophan biosynthetic pathways to obtain overproducer strains. Generally, auxotrophic soil bacteria have been used in the production of various amino acids. In the case of tryptophan, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Brevibacterium lactofermentum*, *Citrobacter freundii*, *Escherichia coli*, *Pseudomonas hydrogenothermophila*, *Aureobacterium flavescens* and *Arthrobacter parafineus* have been engineered to overproduce this valuable product. One strategy involves to obtain phenylalanine and tyrosine auxotrophic mutants with the aid of toxic substrates p-fluorotryptophan and 5-methyltryptophan (Mukhopadhyay and Roy 2011). A more rational means of increasing tryptophan synthesis capacity of bacterial cells is to engineer the pathway around phosphoenolpyruvate and erythrose 4-phosphate condensation reaction, which provides aromatic metabolites. In one study, Shen et al. succeeded dramatic increases in tryptophan synthesis in *E. coli* cells by concomitantly overexpressing phosphoenolpyruvate synthase (a.k.a. pyruvate, water dikinase) and transketolase. These two enzymes catalyse the formation of phosphoenolpyruvate from pyruvate and D-erythrose 4-phosphate from β-D-fructose-6-phosphate, respectively, conveying them to shikimate pathway as substrates of AroF/G/H (Shen et al. 2012). Gu et al. have constructed metabolically engineered mutant *Escherichia coli* strains which accumulate 6,000 times more tryptophan compared to wild-type bacteria. The researchers first constructed a basic L-tryptophan synthetic strain by knocking out tryptophan repressor *trp*R, tryptophanase *tnaA* and major glucose transporter *pts*G, in order to derepress *trp* operon, prevent degradation of tryptophan to indole and convey phosphoenolpyruvate molecules to shikimate pathway, respectively. The strain also included feedback inhibition-resistant mutants of DAHP synthase and anthranilate synthase to bypass the regulation on the initial committed steps of aromatic amino acid synthesis and tryptophan synthesis branches. In addition to these modifications, the researchers swapped the repressible *trp* promoter with a relatively strong 5CP*tacs* promoter, in order to elevate the expression of *trp* operon (Gu et al. 2012).

4.2.2 Tryptophan Synthesis Inhibitors: Promising Antimicrobials

The famous futurist and science fiction writer Isaac Asimov had imagined the planet Aurora devoid of pathogenic microorganisms in his novel, *Robots of the Dawn* (Asimov 1994). Asimov must have realised the insidious role of nasty and ruthless microscopic organisms in altering the history, along with all prominent characters that you can come across in the pages of history books. The discovery of penicillin in 1929 and streptomycin in 1943 were followed by many others in the following two decades. This golden age of antibiotic discovery had witnessed dramatic

decreases in morbidity and mortality due to infectious diseases (Davies 2006). However, the misbelief of microbial geneticists that the emergence of resistant strains upon antibiotic exposure would be rather rare in 1950s might had a promoting impact on the more liberal use of these drugs. The controversial practice of nontherapeutic use of growth-promoting antibiotics in animal breeding has its roots in those years (Graham et al. 2007). Massive and unlimited use of antibiotics soon led to the emergence of resistant bacterial pathogens. The problem has recently become further complicated with the appearance of multiple drug-resistant strains. Besides, as the population ages, the intensive care of critically ill and/or immunosuppressed individuals (i.e. cancer patients) became a routine task, rather than an exception (Tanwar et al. 2014). Unfortunately, it appears that microorganisms have lapped humans in this race. In the last few decades, antimicrobial research has become an expensive and unprofitable business for pharmaceutical companies. As the companies abandon their research and development activities in the field, the innovation gap between introductions of new molecular classes is widening (Committee on New Directions in the Study of Antimicrobial Therapeutics: New Classes of Antimicrobials 2006).

Despite the fact that a vast number of antimicrobial molecules that belong to numerous classes exist, only four major metabolic pathways have been targeted for the sake of selective toxicity (Haag et al. 2012):

- Protein synthesis
- Nucleic acid synthesis
- Cell wall synthesis
- Folate synthesis

Novel systems biology-based approaches have started to emerge as cost-effective methods to discover brand new antimicrobial drug targets. With today's technology, high-throughput omics studies generate tremendous amount of heterogeneous big data from genome, transcriptome and metabolome. Efforts have been directed to combine all into a unified knowledge. Structural systems pharmacology framework is a drug discovery platform, which aims to be an integrative modelling framework for drug action (Xie et al. 2014). Chang et al. have recently used this approach to predict novel antimicrobial targets in *Escherichia coli* K12 MG1655 expanded genome-scale model (GEM-PRO) (Chang et al. 2013; Monk et al. 2013). Over 12,000 molecules that are presented with at least one PBD structure have been scanned as possible ligands of target proteins included in GEM-PRO. Notably, there were five molecular structures that were potential inhibitors of *trp*B-encoded tryptophan kinase β-subunit, which catalyses the irreversible condensation of indole and L-serine to form tryptophan in the presence of pyridoxal phosphate as cofactor (Chang et al. 2013). Tryptophan is an essential amino acid that mammalian cells cannot produce. Accordingly, biosynthetic pathway of this amino acid comprises a suitable antimicrobial target in terms of selective toxicity.

Shikimate pathway provides biosynthetic precursors for several pathways, which synthesise aromatic compounds including tryptophan, in plants and microorganisms, but not in mammals (Gibson and Pittard 1968; Kishore and Shah 1988).

Therefore, any of the seven enzymes that form shikimate pathway may be considered as possible targets for antimicrobials. The inhibitor of 3-phosphoshikimate-1-carboxyvinyltransferase, glyphosate, which is marketed as an herbicide, also exhibits inhibitory effect on gram-positive and gram-negative bacteria in vitro. In relatively higher concentrations, this compound also restricts the growth of apicomplexan parasites (Roberts et al. 2002). Recently, Pitchandi et al. have manually curated information on the analysis of chorismate synthase in 42 pathogenic bacterial species, along with 48 inhibitor substances with known IC50/Ki values, and compiled them into a database to enable ligand-based drug design strategies (Pitchandi et al. 2013). All these efforts are encouraging in that application of systems biology techniques in rational drug discovery and design will eventually accelerate the development cycles and cut off the expenses of pharmaceutical industry.

4.3 Tryptophan in Microbial Cell to Cell Signalling

4.3.1 Glow While Speaking

Since the seventeenth century, mariners have been reporting incidences of "milky sea", as mentioned by Jules Verne in his science fiction novel, *Twenty Thousand Leagues Under the Sea*, nearly 150 years ago. In Chapter 24 of the novel, Conseil – the faithful servant of Professor Pierre Aronnax – expresses his amazement at the glowing milky sea, while Nautilus is sailing half-immersed on the Bay of Bengal in a dark moonless night. Apparently, Verne rewarded his science-thirsty readers with gems of knowledge enclosed in the explanations of Professor Aronnax, who attributed the lactified ocean to the presence of "myriads of infusoria, a sort of luminous little worm" (Verne 1870). It was one century later that chronobiologists Nealson and Hastings described the glowing of "luminous little worms" – the marine *Vibrio* – depended on the presence of cellular mass (Nealson et al. 1970; Nealson and Hastings 1979). Later on, Miller et al. succeeded to capture the first satellite images of a milky sea in the northwestern Indian Ocean, in an area close to the route of Nautilus. With the aid of 833-km attitude polar-orbiting meteorological satellites, this truly massive event was documented to span over 17,000 km^2 with an estimated bacterial biomass of 4×10^{22} cells (Miller et al. 2005).

Bacteria were the first inhabitants of the biosphere. As being the first comers, they had to convert inorganic material into highly ordered biomolecules to form living cells. Though conforming a complex and dynamic environment is by no means a solitary endeavour for microbial cells, they further organise to form hierarchically structured colonies. This multicellular/communal lifestyle requires to achieve the proper balance between individuality and sociality and involves the production, liberation and detection of a variety of chemical signal molecules. These chemical signal molecules form the mediators of *quorum sensing* – the communication network (Jacob et al. 2004).

Quorum sensing is everywhere and can occur both within and between bacterial species. More interestingly, transkingdom signalling is also common between bacteria and eukaryotic organisms.

Communication of microorganisms over chemical signal networks is a widespread phenomenon. Both intra- and interspecies coordinated communal behaviour is called quorum sensing and involves the production, release and sensing of small signal/pheromone molecules called autoinducers. To date, N-acylated homoserine lactone derivatives, cyclic dipeptides, quinolones, furanosyl borate diesters and lactonised small peptides have been accredited to serve as autoinducers (Parsek and Greenberg 2005). Quorum sensing governs the control and regulation of all the metabolic activities of bacterial cell. The effects of these molecular signalling networks have been thoroughly studied and documented for a number of phenotypes, including biofilm formation, toxin production, motility and exopolysaccharide formation (Yang et al. 2014; Wang et al. 2014).

An oversimplified scheme of bacterial quorum-sensing system includes:

- An *autoinducer* (*pheromone*) *synthase* gene, which encodes the enzyme which catalyses the synthesis of autoinducer molecule, usually making use of intermediary metabolites provided from central metabolic pathways. In case of gram-positive bacteria, the autoinducer gene usually encodes the amino acid sequence of pre-pheromone molecule.
- The *autoinducer molecule*, a small organic molecule or a lactonised oligopeptide.
- A *sensor kinase* and a corresponding *response regulator*, which usually form a two-component regulatory system.
- *Quorum-sensing-responsive target genes*.

Marine bacterium *Vibrio fischeri* was the first microbial species described to use quorum sensing to monitor the population density and relaying a response accordingly. In 1970, Nealson and Hastings had observed that the cellular density of *Vibrio fischeri* cultures determined the bioluminescence phenotype of the cells. They hypothesised that small messenger molecules (or pheromones) travel between cells to stimulate luminescence. Later on, with works of Bassler and others, at least three signalling pathways have been discovered in this marine bacterium (Henke and Bassler 2004). Autoinducer synthases LuxM, LuxS and CqsA catalyse the synthesis of pheromone molecules N-(3-hydroxybutanoyl) homoserine lactone (autoinducer-1), (2S,4s)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate (autoinducer-2) and (S)-3-hydroxytridecan-4-one (cholera quorum-sensing autoinducer), respectively. In a growing culture, the concentration of these pheromone molecules increases with the increasing bacterial density (Defoirdt and Sorgeloos 2012). These signal molecules diffuse and bind the periplasmic autoinducer receptors of both secreting and nearby bacterial cells. All three autoinducers have their respective sensor kinases LuxN, LuxQ and CqsS. In low population densities and accordingly low autoinducer concentrations, these receptors act as *kinases* that phosphorylate LuxU, which in turn phosphorylates LuxO. Phospho-LuxO, together with σ54, activates the expression of regulatory small RNAs encoded by *qrr*1–5 (Lilley and Bassler 2000).

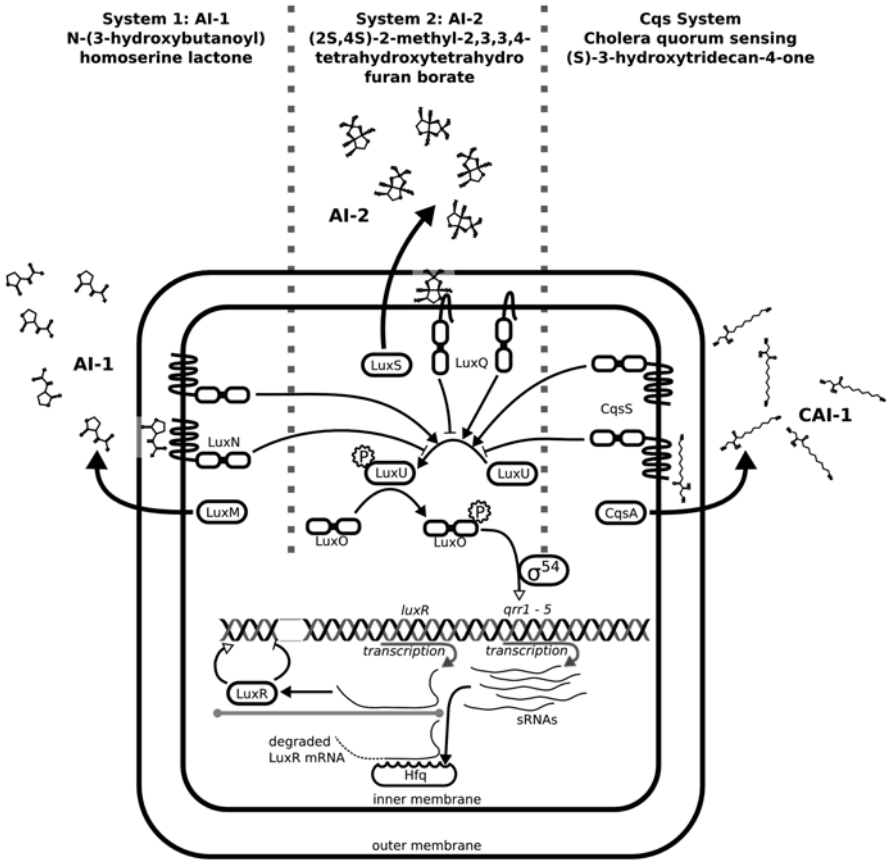

Fig. 4.4 Quorum-sensing systems in *Vibrio harveyi* (Henke and Bassler 2004; Federle and Bassler 2003; Mehta et al. 2009)

These sRNAs and RNA chaperone Hfq destabilise the *lux*R transcripts. In high cell densities, the autoinducer receptors turn into phosphatases that dephosphorylate phospho-LuxU. Consequently, no *qrr* expression occurs and *lux*R mRNAs survive. LuxR is the master quorum sensor in *V. harveyi*, which controls many aspects of the bacterial metabolism (Fig. 4.4) (Henke and Bassler 2004).

Notably, like many other bacteria, *V. harveyi* is able to respond to more than one type of signals received from the environment that later converge into a single downstream transmission and gene expression pathway. An important reason for this supposedly "wasteful" strategy is to socialise. In nature, it's rather an exception to find monospecies bacterial populations. Instead, microorganisms form complex communities. Recently, with the emergence of massively parallel DNA sequencing technologies, we have recognised that the bacterial diversity of a single human gut microbiota extends to more than 1,000 "species-level" phylotypes (Lozupone et al.

2012). It becomes an absolute necessity for a microorganism to perceive the chemical status of the environment to conform and survive in this enormous crowd of diversity. Accordingly, most of the *intraspecies* signalling molecules also act as *interspecies* communication pheromones (Ryan and Dow 2008). Even eukaryotic cells have been shown to respond to bacterial quorum sensing. Bacterial acyl homoserine lactones display immunomodulatory effects on macrophages and T cells and activate inflammatory responses in mice (Mathesius et al. 2003).

Many gram-positive and gram-negative bacteria have been shown to produce and sense autoinducer-2 molecules. Thus this pheromone is considered as a universal signalling molecule (Federle and Bassler 2003).

Most gram-negative bacteria are able to produce AI-1 class of molecules which have species-specific acyl side chains with variable lengths, oxidation and saturation properties. For instance, *Pseudomonas aeruginosa* has two AI-1 signalling circuits. The LuxI homologues LasI and RhlI catalyse the synthesis of N-(3-oxo-dodecanoyl)-L-homoserine lactone (oxoC12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL), respectively. These signalling molecules are also perceived by bacterial cells that belong to a related genus, *Burkholderia* (Nadal Jimenez et al. 2012).

In an ecological and evolutionary context, it must be emphasised that different bacterial species display niche-specific behaviours in response to various autoinducer molecules. Concurrently, not every class of autoinducers has been demonstrated to be synthesised in every bacterial species (Federle and Bassler 2003). Interestingly, the genuses *Escherichia*, *Salmonella* and *Klebsiella* genomes encode the LuxR homologue SdiA, without AI-1 synthase (LuxI) homologue. It has recently been shown that *E. coli* SdiA is responsive to C8-HSL, 3-oxo-C8-HSL and C6-HSL (Yao et al. 2006). A notable impact of N-AHL enabled function of SdiA on *Salmonella* metabolism is the efflux of indole, an important tryptophan metabolite, outside the cell (Ryan and Dow 2008).

4.3.2 Indole as a Signal Molecule

Indole is an aromatic heterocyclic organic compound which gives the intense faecal odour when present in high concentrations. Numerous organisms including gram-positive and gram-negative bacteria have the enzyme tryptophanase, which converts L-tryptophan to indole and pyruvic acid (Fig. 4.5). Copious amounts of indole are produced by bacterial cells to facilitate communication in microbial communities, especially in stationary phase cultures. It has been suggested that indole is a "global" signal molecule which affords/provides interkingdom communication. This kind of communication is indispensible in various ecological settings, especially in host–pathogen interactions. Indole performs its cell-wide actions in transition to stationary phase via the global regulator RpoS (Mueller et al. 2009). Indole production in *Escherichia coli* is affected by certain environmental factors. Extreme alkalinity or

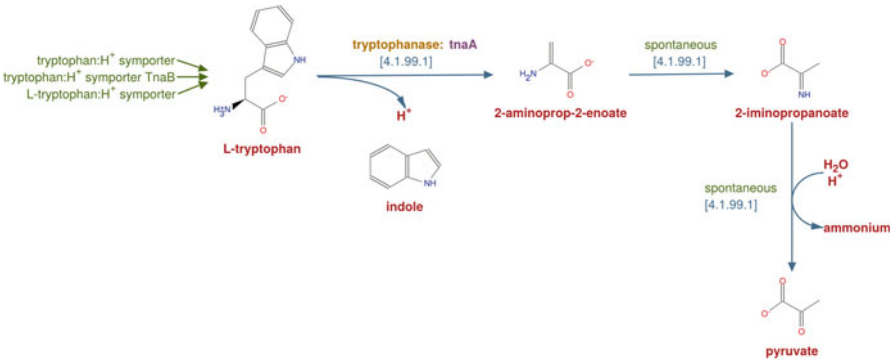

Fig. 4.5 Tryptophan degradation to indole and pyruvate (Adapted from EcoCyc Pathways, publicly available at http://ecocyc.org/ (Caspi et al. 2014))

acidity, the presence of antibiotics and high or low temperature promote indole production (Han et al. 2011; Hirakawa et al. 2010).

Enterohaemorrhagic *Escherichia coli* (EHEC) is an important human pathogen that causes food-borne diseases such as non-bloody diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome. EHEC adheres to the intestinal epithelium and causes attaching and effacing lesions, which is characterised by the destruction of brush border microvilli. The pathogenesis of EHEC depends on the 36-kbp chromosomal pathogenicity island which harbours 41 ORFs clustered in locus of enterocyte effacement (LEE) 1–5 operons. LEE4 operon encodes the key components of type III secretion machinery, which is responsible for the externalisation of bacterial adhesin called intimin. Hirakawa et al. showed that indole production enhances the expression of type III secretion system components, which in turn increases intimin secretion and lesion formation (Hirakawa et al. 2009).

Indole also enhances the production of enteropathogenic *Escherichia coli* (EPEC) toxin which paralyses and kills the roundworm *Caenorhabditis elegans*, in much similar way as in EHEC (Anyanful et al. 2005). Likewise, tryptophanase-deficient delta-tnaA mutants of both EHEC and EPEC display attenuated virulence. Interestingly, indole represses various virulence-related phenotypes such as motility, biofilm formation and attachment. These findings suggest a dual role for indole in disease processes (Lee et al. 2010).

Unlike other "classical" autoinducer molecules, to date, no response regulators have been shown for indole to exert its effects on the cellular metabolism. Indole has to enter the cell in order to exert it effects. The mode of indole entry into the cells has been a subject of dispute. Indole has been known for its capability to penetrate mammalian cellular membranes, owing to the hydrophobic nature of this small molecule. Yet, recent data suggests that indole transporter proteins play an important role in bacterial metabolism. Tryptophan/indole:H+ symporter Mtr has been implicated in

active uptake of indole compounds. Tryptophan auxotrophic *Escherichia coli* cells could be rescued by the addition of exogenous indole in tryptophan-free medium, where indole is converted to tryptophan by the action of tryptophanase. However, delta-mtr Trp auxotrophs are unable to survive (Piñero-Fernandez et al. 2011).

It has been recently shown that indole induces xenobiotic exporters and oxidative stress protective mechanisms, which in turn provide decreased susceptibility to antimicrobial drugs and toxins.

Physicochemical properties of indole drive it to interact with the lipid bilayer membrane. The most probable sites of indole localisation in lipid bilayer membranes depend on the hydrophobic and lipophilic effects along with hydrogen-bonding, cation-pi and electrostatic interactions. Indole molecules are strongly attracted to the relatively hydrated interfacial regions of the lipid bilayer membrane. The three sites where indole molecules are most probably localised are as follows (Norman and Nymeyer 2006; Gaede et al. 2005):

1. Near the glycerol moiety, localised in the interface
2. Near the choline moiety (weakly bound)
3. At the centre of the bilayer's hydrocarbon core (weakly bound)

The displacement of indole from water phase is aided by the hydrophobic effect, whereas the binding of indole to the interface is mainly an enthalpy-driven process (Norman and Nymeyer 2006; Gaede et al. 2005). Chimerel et al. have shown that indole acts as a proton ionophore and, at concentrations of 3–5 mM, significantly reduces the electrochemical potential (proton motive force; PMF) across the cytoplasmic membrane of *Escherichia coli* cells. This, in turn, deactivates MinCD oscillation and prevents FtsZ polymerisation to form cell division ring. In this respect, the cytoplasmic membrane itself behaves like a target of indole as a signal molecule that controls the cell cycle (Chimerel et al. 2012). To date, numerous biological ionophores have been characterised. Plasmid-encoded colicins A, E1, Ia, Ib and K and antibiotics nigericin, valinomycin, monensin, gramicidin S and many more are well known for their toxic pH and electrical potential dissipating effects on the target cells. The cell cycle arresting ionophore indole is regarded beneficial to the producer cells as it exerts its effects to prepare the cell to stationary phase (Chimerel et al. 2012).

4.3.3 Pseudomonas *Quinolone Signalling*

The investigation on quinolones as antimicrobials has started decades ago, with the discovery of 4-quinolones in the 1940s and nalidixic acid in the early 1960s (Emmerson and Jones 2003). Yet, the discovery of 4-quinolones as a new class of signalling molecules is rather new in quorum-sensing research. Pesci et al. have demonstrated that 4-quinolone family compounds 2-heptyl-3-hydroxy-4-quinolone, 2-hydroxy-3-heptyl-4-quinolone and 2-heptyl-4-hydroxy-quinolone are

released from bacterial cells and bind their cognate autoinducer-dependent transcriptional activator PqsR (response regulator) to express appropriate target genes in a cell density-dependent manner (Pesci et al. 1999). Pyocyanin production, lectin synthesis, reduced biofilm formation, swarming motility and increased drug resistance phenotypes have been linked to PQS (Nadal Jimenez et al. 2012) (Fig. 4.6).

Two distinct biosynthetic pathways have been defined for the synthesis of *Pseudomonas* quinolone signal molecules. These two pathways converge to form anthranilate and ultimately the HHQ and PQS signal molecules. Anthranilate is synthesised either directly by tryptophan degradation via kynurenine formation or by transformation from tryptophan precursor shikimate (Farrow and Pesci 2007; Miller et al. 1953). Detailed analyses of the pathway revealed that PqsABCD proteins are able to produce approximately 50 structurally related 4-quinolone compounds, most of which have no significant role in cell to cell signalling (Nadal Jimenez et al. 2012). Interestingly, the addition of hydroxyl group to HHQ to form PQS dramatically increases the iron affinity of the molecule. Membrane-bound PQS molecules enhance the accumulation of iron from the surrounding environment (Diggle et al. 2007).

4.3.4 Antipathogenics: Taming the Wild Bacteria

As discussed earlier in the text, the bottleneck in the antimicrobial discovery has insidiously become a real public health threat. With the emergence of multidrug-resistant bacteria, the infections that were once treatable have become impossible to treat. While trying to preserve the currently available drugs, we urgently need to discover new viable targets for antimicrobial chemotherapy.

Social intelligence is defined as the capability of an individual to perceive and understand the environment and mount appropriate responses. As more and more genomic data becomes available, we are starting to apprehend the extent of microbial social capabilities to communicate each other. Bacterial cells are equipped with complex communication capabilities which permit them to adapt to the environment and organise into highly structured colonies. Quorum-sensing signalling enables the bacterial population to make collective decisions and cooperative hierarchical organisations. In order to survive in a complex microbial community, it's a prerequisite to have the ability to sense the local environment for limiting nutrients, presence of toxins and signal molecules from other cells (Jacob et al. 2004). Likewise, an infectious disease process also involves the appropriate use of these communication capabilities, in order to determine when to hide from the immune system and when to express the full set of lytic enzymes. From the pathogen perspective, successful invasion of a host organism depends on excellent management of information processing and signal integration (Mehta et al. 2009).

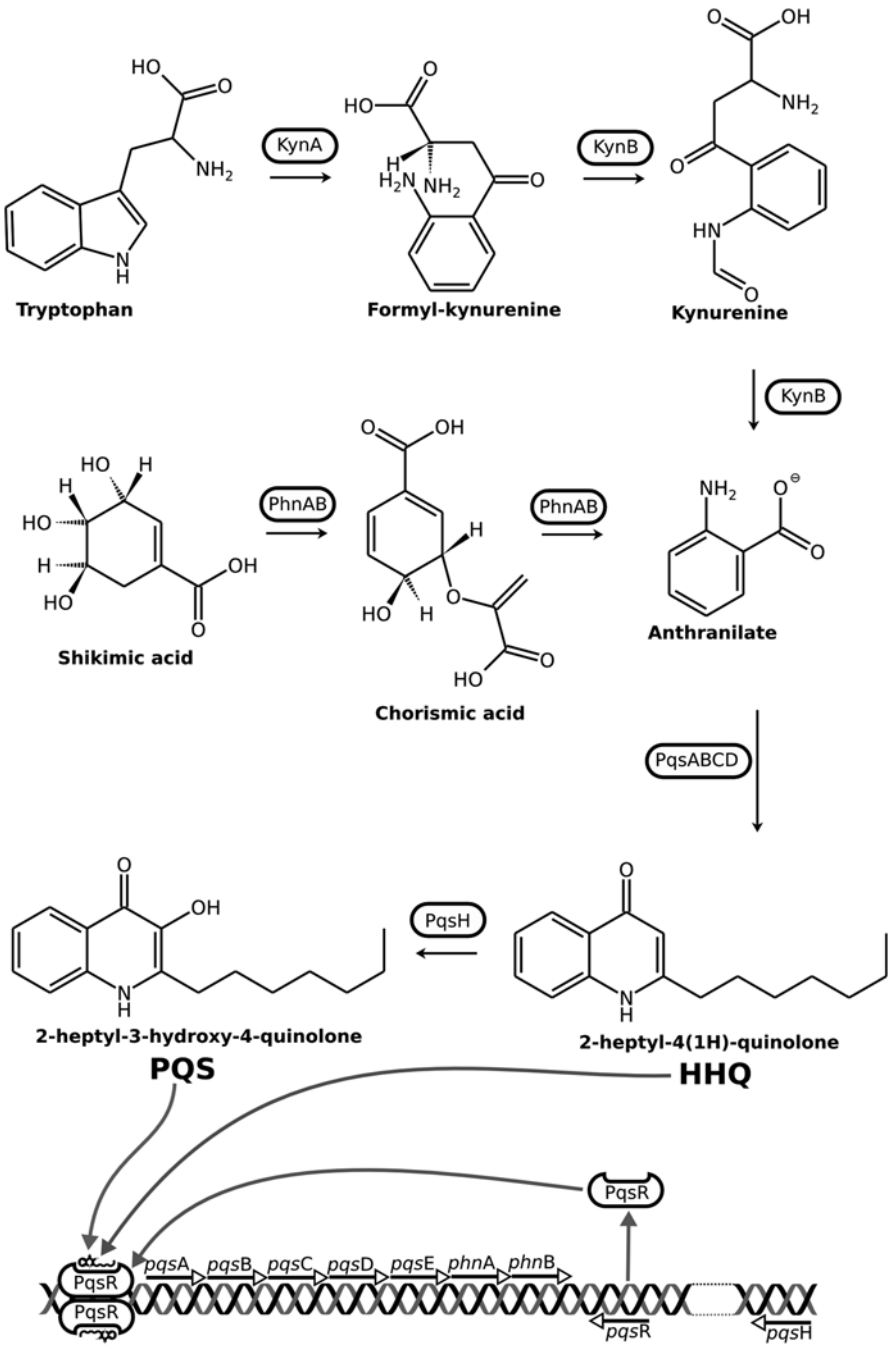

Fig. 4.6 *Pseudomonas* quinolone signalling (Nadal Jimenez et al. 2012; Heeb et al. 2011)

Sirota-Madi et al. have sequenced the genome of the pattern-forming bacterium *Paenibacillus vortex* and performed a detailed comparative analysis between *P. vortex* and a set of 500 complete bacterial genomes, in terms of social IQ distribution based on the number of genes directed to information processing and integration. They have concluded that "brilliant" *P. vortex* stays in a social IQ score range of three standard deviations above the "ordinary" *Bacillus subtilis* and *Escherichia coli* (Sirota-Madi et al. 2010). Unsurprisingly, *Pseudomonas aeruginosa* and *Bacillus anthracis* are ranked within the two standard deviations above normal. Both pathogens cause difficult to treat infections.

Interfering with multistep quorum-sensing circuits at any point may direct the bacterial community to make inappropriate decisions. This assumption comprises the basis of using therapeutic quorum-sensing inhibitors as antipathogenic drugs (Rasmussen and Givskov 2006).

Fluorinated 4-quinolone derivative antimicrobials have been in the market for a considerable period of time. In contrast to the ancestor compound nalidixic acid, fluoroquinolones carry a fluorine atom in the central ring system at C6 or C7 positions. The suggested cellular target for these broad-spectrum bactericidal antimicrobial compounds is the inhibition of relaxation of DNA supercoils in bacterial cells, which in turn impairs the replication and transcription of DNA. DNA gyrase and topoisomerase IV are the main enzymes which these compounds bind and inhibit (Hooper 1999a). In extended clinical use, it's not unusual to experience resistance against these compounds, due to the missense mutations in the DNA gyrase and topoisomerase IV genes, which hinders the binding of the drug to the target enzyme. To date, most of the resistance-bearing mutations were confined to the 83rd and 87th codons of *gyr*A and 80th and 84th codons of *par*C, which encode DNA gyrase and topoisomerase IV, respectively, most probably due to mechanistic reasons (Hooper 1999b, 2001).

Pseudomonas aeruginosa is an opportunistic gram-negative bacterium that causes severe infectious diseases in humans. It has a particular importance in cystic fibrosis that over 90 % of these patients develop chronic lung infection. Interestingly, certain members of fluoroquinolone antimicrobials may exhibit antipathogenic effect by suppressing the expression of virulence factors such as elastase, phospholipase C, exoenzyme S, exotoxin A and total protease activity in this bacterium (Grimwood et al. 1989b). In a rat *Pseudomonas aeruginosa* chronical lung infection model, animals were treated with subinhibitory concentrations of fluoroquinolones as low as 1/20 of minimum inhibitory concentration (MIC). In spite of similar bacterial counts with the control group, fluoroquinolone treatment group had less severe histopathological lung damage. Furthermore, the alleviation of the histopathological damage did not deteriorate with the increasing MIC values in the time course of treatment (Grimwood et al. 1989a). It's yet to be proven whether fluoroquinolone antimicrobials interfere with PQS to suppress the expression of these virulence factors. Still, the interference of quorum sensing is a promising target in antimicrobial chemotherapy (Pesci et al. 1999).

4.4 Conclusion

Tryptophan is an essential aromatic amino acid in human and animal nutrition. As mammalian cells are not able to produce tryptophan, they need to take it in food. Furthermore, tryptophan supplementation has been suggested for treatment of certain neurological conditions (Mukhopadhyay and Roy 2011). Therefore, diverting microbial metabolism to construct hyperproducer bacterial strains has become important. More than 50 years have passed after Jacob and Monod have demonstrated the genetic regulation of tryptophan biosynthesis as the first repressible operon discovered (Jacob and Monod 1961). Similar to other aromatic amino acids, biosynthetic pathway for tryptophan spans numerous enzymatic steps, including the allosterically regulated ones, which provide tight regulation of the synthesis of this metabolically "expensive" product (Shen et al. 2012). The data acquired by using systems biology techniques enabled us to engineer superior microbial factory cells with the aid of synthetic biology methods. Tryptophan production is no exception.

After the golden age of antimicrobial drug discovery, pharmaceutical companies have almost quitted antimicrobial development business to divert their resources to more profitable fields. Keeping in mind that mammalian cells are enable to produce tryptophan, it's worthwhile to "hack" the microbial tryptophan biosynthetic pathway in order to develop novel antimicrobial drugs with high therapeutic indexes. Using systems biology databases for rational drug design is coming into reality (Haag et al. 2012; Chang et al. 2013).

Another promising approach for prevention and treatment of infectious diseases is quorum-sensing interference. Microbial cells continuously monitor the biochemical state and population density of the environment. In the case of pathogenic microorganisms, the decision mechanisms for virulence factor expression are hardwired to quorum-sensing circuits. Indole and 4-quinolone derivatives constitute two important autoinducer classes, which take part in such decisions. Obviously, the modulation and interference of quorum sensing may also interfere with the expression of virulence factors and thus eliminate the pathogenetic mechanisms (Heeb et al. 2011; Lee and Lee 2010).

Acknowledgement Figures 4.1, 4.2 and 4.5 have been adapted from EcoCyc Pathways, publicly available at http://ecocyc.org/ (Caspi et al. 2014 Jan 1).

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Chapter 5
The Role of L-Tryptophan Kynurenine Pathway Metabolism in Various Infectious Diseases: Focus on Indoleamine 2,3-Dioxygenase 1

Yuki Murakami, Hiroyasu Ito, and Kuniaki Saito

Abstract The kynurenine (KYN) pathway is the major route of L-tryptophan (L-TRP) catabolism and an anabolic source of nicotinamide-containing nucleotide. To date, three enzymes that catalyze the first and rate-limiting step in the KYN pathway of TRP metabolism have been described: indoleamine 2,3-dioxygenase (IDO) 1, IDO2, and L-tryptophan 2,3-dioxygenase (TDO). In this chapter, we focus on the role of IDO1 in various infectious diseases. IDO1 has a much broader substrate profile for indoleamine-containing compounds and is induced by several pro-inflammatory cytokines. Substantial increases in the TRP-KYN pathway metabolites occur in human brain, blood, and systemic tissues during immune activation. This enzyme also plays a key role in the immunomodulatory effects on several types of immune cells. Originally known for its regulatory function during pregnancy and chronic inflammation in tumorigenesis, the activity of IDO1 seems to modify the inflammatory state of infectious diseases. Understanding the regulation of IDO1 and the subsequent biochemical reactions is essential for the design of therapeutic strategies in certain immune diseases. Therefore, we will discuss current knowledge about the role of IDO1 and its metabolites during various infectious diseases, e.g., infection by hepatitis virus, HIV, influenza virus, encephalomyocarditis virus, and parasites. Especially the regulation of type I interferon (IFN) production via IDO1 in these infectious diseases is discussed.

Keywords Hepatitis virus • Human immunodeficiency virus • Influenza virus • Encephalomyocarditis virus • *Toxoplasma gondii* • *Chlamydia* spp. • Fungi

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5.1 Regulatory Enzymes in L-Tryptophan-Kynurenine Pathway

L-tryptophan (L-TRP) is an essential starting point of two biochemical pathways: (1) the enzyme tryptophan 5-hydroxylase converts L-TRP into 5-hydroxytryptophan, which is subsequently decarboxylated to 5-hydroxytryptamine (5-HT, serotonin), an essential neurotransmitter, and (2) two atoms of oxygen are inserted into L-TRP to form N-formylkynurenine, the first and rate-limiting step in the kynurenine (KYN) pathway (Fig. 5.1). It is estimated that only 1 % of dietary L-TRP can be converted into 5-HT (Russo et al. 2003). The remaining 99 % of L-TRP is metabolized via the KYN pathway. L-TRP is catalyzed by three different enzymes: indoleamine 2,3-dioxygenase (IDO) 1, IDO2, and L-tryptophan 2,3-dioxygenase (TDO) (Table 5.1).

IDOs have broad substrate specificity and will accept several different indoleamines including D- and L-TRP, tryptamine, 5-hydroxytryptophan (5-HTP), 5-HT, and melatonin (Shimizu et al. 1978). IDO1 is widely expressed in different cell types in the central nervous system (CNS) and peripheral tissues, and TRP metabolism via IDO1 activity is related to many different functions, dependent on the tissues, cell types, and physiological context. IDO1 is induced by IFN-γ-mediated effects of the signal transducer and activator of transcription 1α (STAT1α) and interferon regulatory factor-1 (IRF-1). The IDO1 gene has two interferon-stimulated response elements (ISREs) and IFN-γ-activated site (GAS) element sequences in the 5′-flanking region (Hassanain et al. 1993; Chon et al. 1995; Konan and Taylor 1996). IDO1 induction is also mediated by an IFN-γ-independent mechanism under certain circumstances (Hissong and Carlin 1997; Fujigaki et al. 2001, 2006). Fujigaki et al. demonstrated that IDO1 induction by lipopolysaccharide (LPS) is not mediated by STAT1α or IRF-1 binding activities that induce IDO1 transcriptional activity by IFN-γ in many cells (Fujigaki et al. 2006). LPS stimulation of human monocytes and macrophages activates several intracellular signaling pathways, including the IkappaB kinase-nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. These pathways, in turn, activate a variety of transcription factors that include NF-κB and activator protein-1 (AP-1). A part of the induction of IDO1 by LPS is mediated by a signal from NF-κB or p38-MAPK pathways. A homology search of the 5′-flanking region of the IDO1 gene shows consensus sequences for transcriptional factors such as AP-1, NF-κB, and NF-IL-6, which are activated by LPS and other proinflammatory cytokines such as TNF-α, IL-6, and IL-1β. Therefore, the IDO1 gene could be upregulated by LPS or these cytokines in a synergistic manner.

While the expression and function of IDO2 has been well explored in the mouse model, there is a lack of knowledge about its expression and functional significance in human tissue. Studies combining mRNA and protein analysis suggest that mouse IDO2 is expressed in the liver, epididymis, and kidney (Ball et al. 2007; Fukunaga et al. 2012). Although it is difficult to assess the distribution of IDO2 in humans as very few studies have examined both protein and mRNA expression, IDO2 mRNA and protein expression has been detected in human pancreatic cancer cell lines

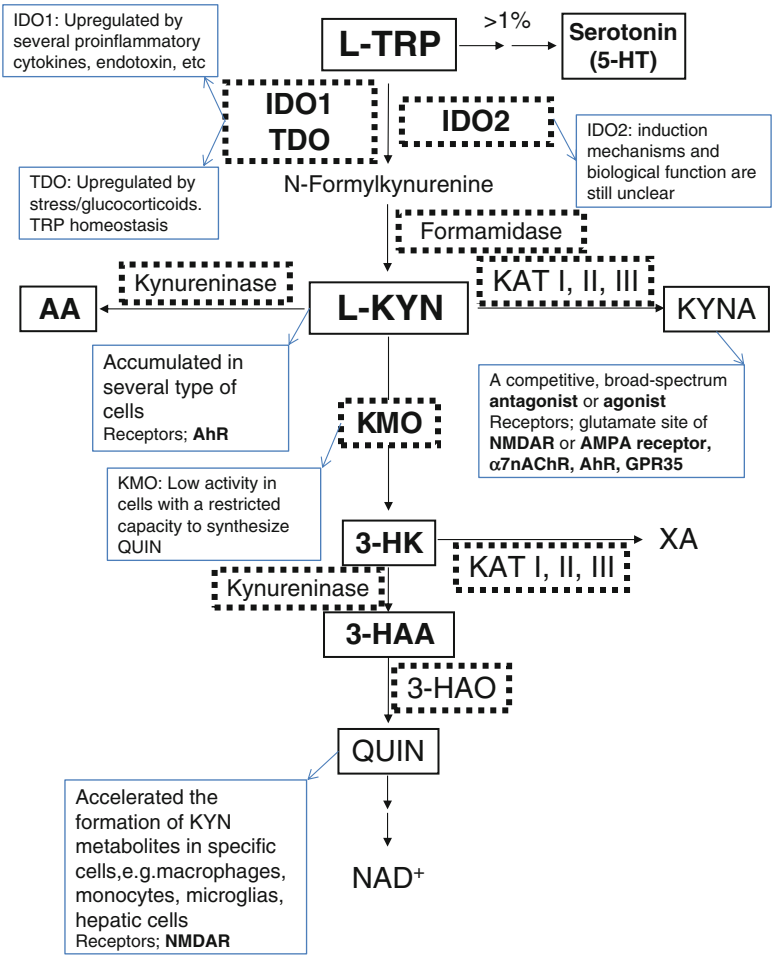

Fig. 5.1 Schematic overview of the kynurenine pathway. L-tryptophan (L-TRP) in the extracellular fluid is transported into cells by a high-affinity tryptophan transporter. The first rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO) 1 catalyzes the initial enzymatic step in the kynurenine (KYN) pathway leading to the synthesis of a number of KYN metabolites. IDO1 is induced by several proinflammatory cytokines; therefore, KYN metabolism is increased during many inflammatory conditions. By contrast, glucocorticoid hormones increase transcription of tryptophan 2,3-dioxygenase (TDO) and peripheral degradation of L-TRP via the KYN pathway. The biological function and induction mechanism of IDO2 are still unclear and controversial. IDO1 is an important regulatory enzyme in the production of L-KYN in a broad spectrum of cell types. Once synthesized, L-KYN can be further metabolized through three distinct pathways to form kynurenic acid (KYNA), 3-hydroxy-L-kynurenine (3-HK), and anthranilic acid (AA). Low activity of kynurenine 3-monooxygenase (KMO) in some cells restricts the capacity to synthesize quinolinic acid (QUIN) from L-TRP. *KAT I, II, III* kynurenine aminotransferase, *XA* xanthurenic acid, *3-HAA* 3-hydroxyanthranilic acid, *3-HAO* 3-hydroxyanthranilic acid oxidase, *NAD+* nicotinamide adenine dinucleotide+, *NMDAR* N-methyl-D-aspartate receptor, *AMPA* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, *α7nAChR* α7 nicotinic acetylcholine receptor, *AhR* aryl hydrocarbon receptor, *GPR35* G protein-coupled receptor 35

Table 5.1 First rate-limiting enzymes in the KYN pathway

Enzyme	Protein (kDa) (amino acid)	Active enzyme formation	Major expression tissues	Inducer	Ref
hIDO1	45.3 kDa (403)	Monomer	Brain, heart, lung, spleen, uterus, vascular endothelium, epididymis	Proinflammatory cytokines, LPS	Yamamoto and Hayaishi (1967), Fujigaki et al. (2001), (2006), Murakami et al. (2013) and Sugimoto et al. (2006)
hIDO2	47 kDa (420)	Monomer	Brain, liver, kidney, epididymis	Unknown	Ball et al. (2007), (2009), Fukunaga et al. (2012) and Yuasa et al. (2009)
hTDO	47.8 kDa (406)	Tetramer	Liver, brain	Glucocorticoid L-TRP	Forouhar et al. (2007), Schutz and Feigelson (1972), (1972) and Knox and Auerbach (1955)

(Witkiewicz et al. 2009), and the expression of IDO2 mRNA has been detected in gastric, colon, and renal tumors in humans (Lob et al. 2009). A recent report also suggested that response to a combination chloroquine/radiotherapy treatment for brain metastases was more pronounced in patients with an "active" IDO2 genotype (Eldredge et al. 2013). The current studies on IDO2 may suggest that human IDO2 is active under specific conditions. It is possible that IDO2 activity is determined by the presence of particular cofactors and is only evident in certain cell types or conditions.

In contrast to both IDOs, TDO is a highly substrate-specific dioxygenase and deoxygenates only L-TRP and some TRP derivatives. TDO is also the rate-limiting enzyme in the KYN pathway of TRP metabolism in the periphery (Botti et al. 1995), catalyzing the oxidative cleavage of TRP and regulating homeostatic plasma TRP concentrations. TDO expression can be induced, or its activity can be increased by L-TRP and its analogs via actions at a distinct allosteric activation site 2. In stress-related neuropsychiatric disorders, glucocorticoid hormones also increase TDO activity and peripheral degradation of TRP via the KYN pathway, limiting TRP availability for 5-HT synthesis. Although the major site of expression of TDO is the liver, mRNA and protein levels of TDO expression have been shown in astrocytes within the human frontal cortex (Miller et al. 2004), and several studies suggest that the enzyme and its activity are upregulated in the anterior cingulate cortex of patients with schizophrenia and psychiatric disorders (Miller et al. 2006; Kanai et al. 2009). Now it seems clear that peripheral TDO activity is inversely related to

brain L-TRP concentrations (Badawy et al. 1989) and that brain L-TRP concentrations can be dramatically increased by inhibiting TDO activity (Salter et al. 1995). Therefore, the expression and activity of peripheral and potentially brain TDO are also considered to understand TRP metabolism in the CNS during health and disease states.

5.2 TRP Metabolites in Peripheral Tissues and the CNS

Studies in vitro have shown that not all human cells are capable of directly synthesizing quinolinic acid (QUIN) from L-TRP. The activities of KYN pathway enzymes and the production of KYN metabolites depend on cell types (Heyes et al. 1997). IDO1 is not only the most important regulatory enzyme for KYN pathway, but also kynurenine 3-monooxygenase (KMO), kynureninase, and 3-hydroxyanthranilic acid oxidase (3-HAO) are important determinants of whether a cell can make QUIN. Indeed, stimulation by proinflammatory cytokines resulted in large increases of IDO1 activity in most cell types, although the accumulated amounts of QUIN are very different. It has been shown that blood macrophages and monocyte-derived cells produced the largest amount of QUIN in accordance with the highest activities of KMO and kynureninase compared to other cell types (Fig. 5.2) (Heyes et al. 1997). Previously, we demonstrated the activities of KYN pathway enzymes and the ability of different human cells to convert pathway intermediates into QUIN (Heyes et al. 1997). Stimulation with IFN-γ substantially increased IDO1 activity and L-KYN production in primary peripheral blood macrophages and fetal brains (astrocytes and neurons), as well as cell lines derived from macrophage/monocytes, astrocytoma, B lymphocyte, liver, and lung. High activities of KMO, kynureninase, or 3-HAO were found in IFN-γ-stimulated macrophages and monocyte- and liver-derived cells; these cells made large amounts of QUIN when supplied with L-TRP, L-KYN, 3-hydroxykynurenine (3-HK), or 3-hydroxyanthranilic acid (3-HAA). QUIN production by human fetal brain cultures and astrocytoma cells was restricted by the low activities of KMO, kynureninase, and 3-HAO, and only small amounts of QUIN were synthesized when cultures were supplied with L-TRP or 3-HAA. In lung-derived cells, QUIN was produced only from 3-HK and 3-HAA, consistent with their low KMO activity. The results are consistent with the notion that IDO1 is an important regulatory enzyme in the production of L-KYN and QUIN. KMO and, in some cells, kynureninase and 3-HAO are important determinants of whether a cell can make QUIN from L-TRP.

Under physiological conditions, KYN pathway enzymes in the mammalian brain are preferentially, although not exclusively, localized in nonneuronal cells (Heyes et al. 1992, 1993; Schwarcz et al. 2012). Metabolism of the pathway is driven by blood-derived L-TRP, L-KYN, and 3-HK or by locally formed metabolites (Fig. 5.2). Astrocytes express kynurenine aminotransferases (KATs) but do not contain KMO and therefore cannot produce 3-HK from KYN (Gramsbergen et al. 1997; Hodgkins and Schwarcz 1998; Hodgkins et al. 1999). 3-HK and its major

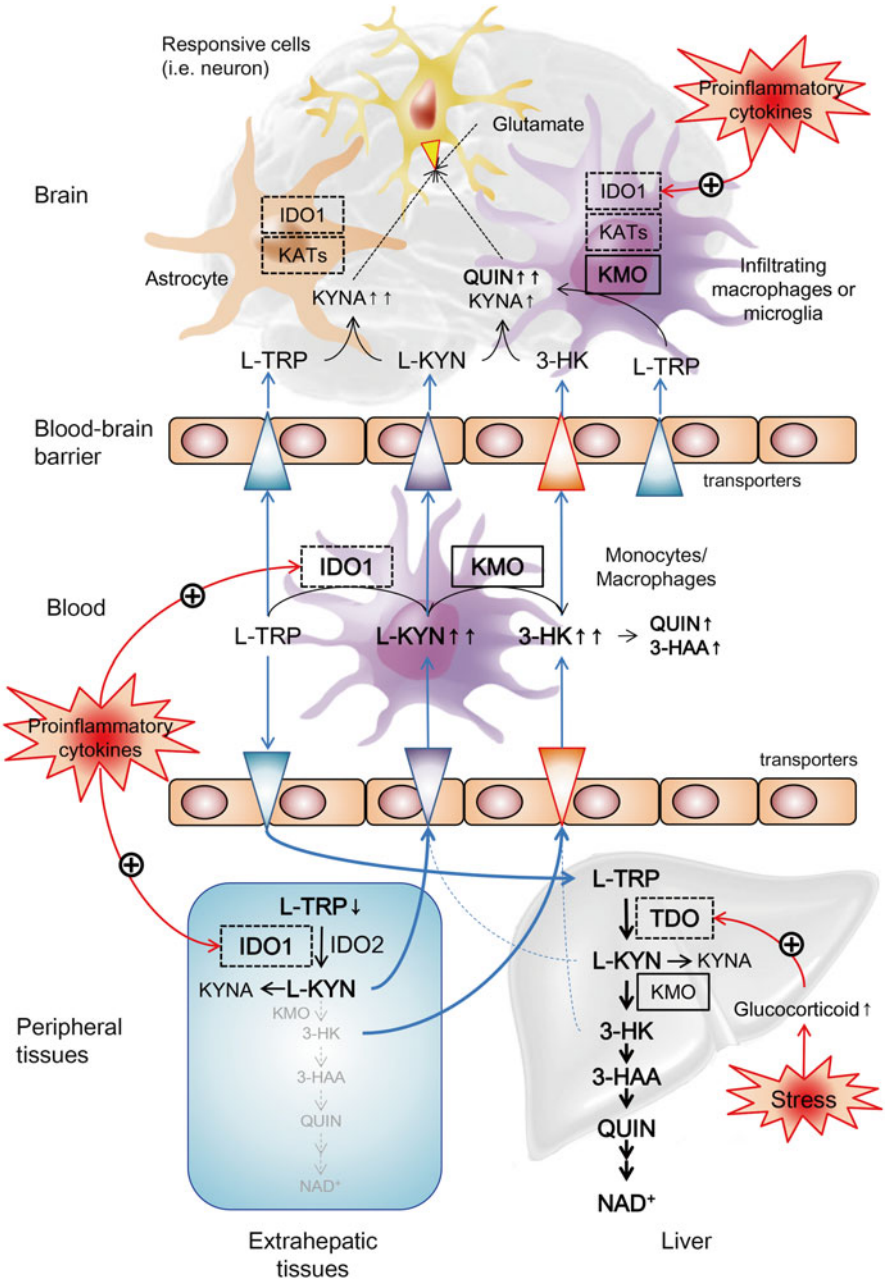

Fig. 5.2 Clinical conditions associated with altered TRP metabolism in the peripheral and the central nervous systems (CNS). It should be noted that the magnitude of substrate flux through the KYN pathway is influenced by individual tissue enzyme activities, tissue blood flow, blood metabolite concentrations, cell membrane permeability, and active transport mechanisms. Systemic immune stimuli induce IDO1 in extrahepatic tissues and increase L-KYN production. However,

downstream metabolites are synthesized in microglia and other monocyte-derived cells (Alberati-Giani et al. 1996; Heyes et al. 1996). Once synthesized within glial cells, QUIN and kynurenic acid (KYNA) are promptly released into the extracellular milieu to affect their pre- and postsynaptic neuronal targets.

Many peripheral tissues express IDO1/2, while the liver has a predominance of the unrelated and less substrate-selective enzyme TDO (Fig. 5.2). These enzymes lower TRP levels and increase KYN concentrations, with the latter leading to increased generation of the more distal metabolites such as QUIN and KYNA. About 90 % of total plasma TRP is bound to albumin, forming a complex that cannot across the blood-brain barrier (BBB), but free-form TRP is available for transport across the BBB into the brain (Madras et al. 1974). An important interface exists with the CNS since TRP, KYN, and 3-HK can cross the BBB quite readily (Eastman et al. 1992; Fukui et al. 1991; Speciale and Schwarcz 1990; Speciale et al. 1989). On the other hand, KYNA and 3-HAA do not cross the BBB, but their concentrations are elevated markedly after systemic administration of KYN (Miller et al. 1992; Nozaki and Beal 1992). QUIN also does not normally cross into the CNS, but when the barrier is compromised, it may do so, like many compounds (Vezzani et al. 1989). These results mean that altering the TRP-KYN ratio in the blood can produce significant secondary changes in the amount of KYNs in the CNS (Saito et al. 1992), contributing, no doubt, to the effects of immune activity and stress. The induction of IDO1 in endothelial cells by inflammatory mediators including IFN-γ and the resulting changes of local TRP and KYN concentrations are likely to alter the levels of both compounds and their metabolites in the CNS, especially since there is evidence that the KYN generated is secreted preferentially from the basolateral pole of the endothelial cells, gaining direct access to the cerebral aspect of the BBB (Owe-Young et al. 2008). This relationship may be of special significance for

Fig. 5.2 (continued) extrahepatic tissues have very low activity of KMO, while the stress-related hormone, glucocorticoid, increases TDO activity and peripheral degradation of L-TRP via the KYN pathway. Therefore, in the periphery, the degradation of L-TRP and the subsequent formation of circulating KYNs are normally regulated by steroid hormones (glucocorticoid) and proinflammatory cytokines (IFN-γ, TNF-α, IL-6, and IL-1β). Metabolites may enter the blood and be exchanged among different tissues for further metabolism. L-TRP, L-KYN, and 3-HK in the extracellular fluid are transported into cells by a high-affinity transporter, and they all cross the blood-brain barrier (BBB) using the large neutral amino acid transporter into the brain (Fukui et al. 1991). Increases in brain metabolite levels may reflect their direct diffusion into the brain from blood or by entry of precursors and subsequent metabolism in brain cells. Increased levels of metabolites within the CNS following immune stimulation may result from induction of IDO1 in brain cells, including monocyte infiltrates and glial cells. Astrocytes harbor KATs but do not contain KMO. 3-HK and its major downstream metabolites are synthesized in microglia and other cells of monocytic origin. Once synthesized within glial cells, QUIN and KYNA are promptly released into the extracellular milieu to affect the normal functions of responsive cells (e.g., neurons) by interfering with binding of glutamate and other excitatory amino acid to these receptors. KYNA is also a ligand of α7 nicotinic acetylcholine receptor (α7nAChR), which mediates inflammatory regulation. Together, peripheral and central events connect chronic inflammation and neuroinflammatory mechanisms with abnormal metabolism along the KYN pathway and pathological conditions in the body

understanding the cognitive and neurodegenerative effects of human immunodeficiency virus (HIV) infection (see details in later section) in which there is strong evidence for the role of QUIN neurotoxicity (Kandanearatchi and Brew 2012). Local changes in TRP and KYN metabolite levels will also affect immune tolerance which may further exacerbate susceptibility of the CNS in some individuals (Owe-Young et al. 2008).

5.3 Immune Regulation by IDO1

IDO1 was first isolated from rabbit intestine in 1967 (Yamamoto and Hayaishi 1967), and it became rapidly clear that its induction serves the mechanism of antimicrobial resistance. Infection by bacteria, parasites, or viruses induces a strong IFN-γ-dependent inflammatory response. IFN-γ-induced IDO1 degrades TRP, and the depletion of TRP results in the regulation of intracellular pathogens (Yoshida et al. 1979; Murray et al. 1989; Daubener et al. 1993; Nagineni et al. 1996; Pfefferkorn and Guyre 1984). On the other hand, Munn D.H. et al. provided evidence for a much broader immunoregulatory significance of TRP degradation by IDO1. They demonstrated that tolerance to allogeneic fetuses is regulated by IDO1-expressing cells in the mice placenta (Munn et al. 1999). Indeed, many studies also showed that a marked increase in IDO1 suppresses the immune response by locally depleting TRP and hence preventing T-lymphocyte proliferation using the IDO1 inhibitor, 1-methyl-DL-tryptophan (1-MT) (Heseler et al. 2008; Munn et al. 2004; Schmidt et al. 2009). These previous studies clearly showed that TRP degradation by IDO1 substantially contributes to immunoregulation, and therefore, IDO1 has been considered a strong immunoregulatory factor.

IDO1 is predominantly expressed in antigen-presenting cells (APCs) of the immune system – the dendritic cells (DCs), monocytes, and macrophages (Heitger 2011; Blaschitz et al. 2011; Murakami et al. 2013). As described previously, IDO1 can be introduced by soluble cytokines such as IFN-γ, type I IFNs, transforming growth factor-β (TGF-β), TNF-α, or toll-like receptor (TLR) ligands such as LPS (Fujigaki et al. 2001). In addition, KYN and 3-HK could be also involved in the exacerbation of TRP starvation in T cells. Kaper T. et al. have proposed the existence of a positive feedback between IDO1-mediated TRP metabolism in DCs and KYN-induced TRP depletion in CD98-expressing T cells (Kaper et al. 2007). CD98 is expressed on astrocytes and activated T cells. T cells are sensitive to low levels of TRP and TRP metabolites in vitro. TRP deficiency specifically activates the general control nonderepressing-2 (GCN2) kinase in murine and human T cells, which leads to a halt in the G2 phase of T-cell division and, consequently, T-cell suppression (Fig. 5.3a) (Munn et al. 2005). Moreover, a specific combination of TRP metabolites can inhibit anti-CD3 antibody-induced T-cell proliferation and induce T-cell apoptosis in vitro (Terness et al. 2002; Fallarino et al. 2002). The combination of low TRP concentration and specific TRP metabolites leads to the generation of regulatory T cells (Tregs) from naïve T cells in vitro (Fallarino et al. 2006; Belladonna et al. 2007). Tregs inhibit the activation, differentiation, and survival of

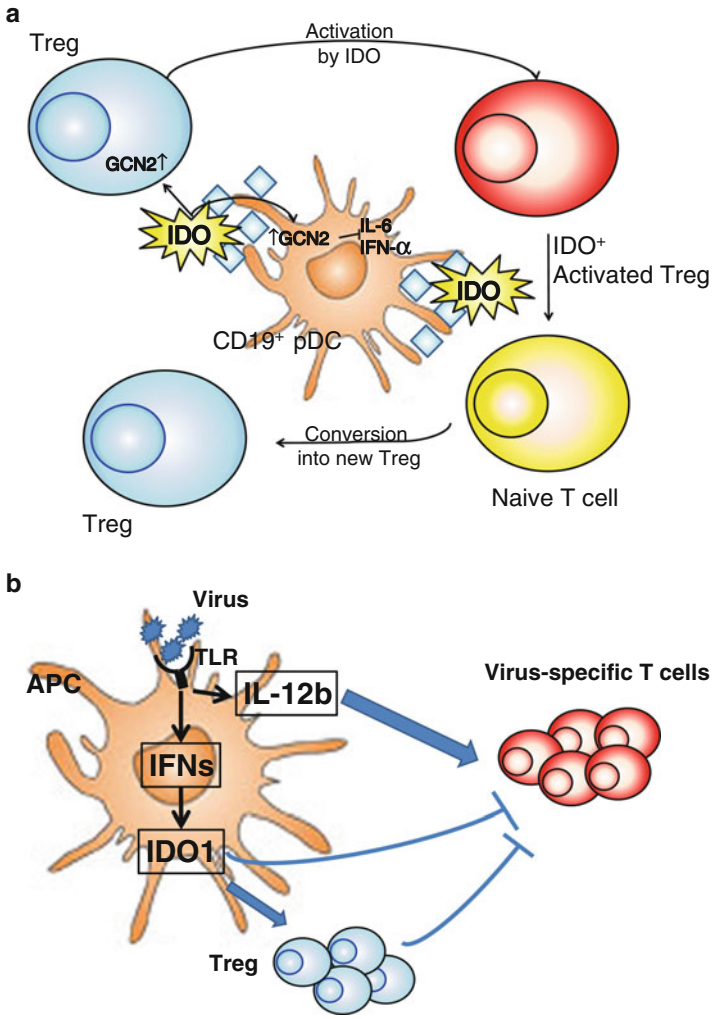

Fig. 5.3 Immune regulation by IDO1. (**a**) $CD19^+$ plasmacytoid DCs (pDCs) express high levels of IDO1, which can activate mature regulatory T (Treg) cells via activation of the protein kinase general control nonderepressing-2 (GCN2) pathway of protein synthesis inhibition (Sharma et al. 2007; Murakami et al. 2013). pDC-produced IDO1 and activated Treg can convert naïve T cells into new Treg. IDO1 acts in an autocrine manner to suppress pDC production of IL-6, which prevents the conversion of Treg into IL-17-producing Th17 proinflammatory cells (Sharma et al. 2009). IDO1 also downregulates type I IFN (IFN-α) production by pDC (Manlapat et al. 2007). (**b**) Virus-specific T-cell regulation by IDO1. Antigen-presenting cells produce IL-12b, which can induce virus-specific T cells, via TLR signaling activated by viral component. Simultaneously, the activation of TLR signaling enhances IDO1 expression via IFN-γ production. IDO1 impairs the induction and proliferation of virus-specific T cells directly and/or indirectly (via the induction of Treg)

effector T cells through the induction of IDO1 in APCs by ligation of inhibitory ligands and cytokines from Tregs (Fig. 5.3b) (Fallarino et al. 2004).

It is possibly the selective pressure by Tregs that drove the evolution of the IDO1 mechanism from one operating in innate and inflammatory responses to pathogens (Bozza et al. 2005; Fallarino et al. 2006) to an effector mechanism of Treg function (Grohmann and Puccetti 2003; Fallarino et al. 2003). Functional plasticity in DCs allows these cells to present antigens in an immunogenic or tolerogenic fashion, largely contingent on environmental factors (Grohmann and Puccetti 2003). Co-stimulatory and co-inhibitory interactions between DCs and T cells are pivotal in tipping the balance between immunity and tolerance in favor of either outcome. When CD80/CD86 molecules on DCs were engaged to T cells, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), widely expressed by Tregs, was later shown to behave as an activating ligand for CD80/CD86 receptors, resulting in intracellular signaling events. Through an unidentified signal cascade, DCs release type I and type II IFNs that act in an autocrine and paracrine fashion to induce strong IDO1 expression and function (Grohmann and Puccetti 2003). KYN-dependent T-cell differentiation would contribute to expand the pool of Tregs (Puccetti and Grohmann 2007). However, in the long-term control of immune homeostasis and tolerance to self, IDO1 relies on different regulatory stimuli and cytokines, providing a basal function amenable to regulation by abrupt environmental changes (Belladonna et al. 2008).

In a TGF-β-dominated environment and in the absence of IL-6, IDO1 activates a variety of downstream signaling effectors that sustain TGF-β production, production of type I IFNs, and a bias of plasmacytoid DCs (pDCs) toward a regulatory phenotype (Lande and Gilliet 2010; Matta et al. 2010). IDO1 enhances its own expression and stably tips the balance between proinflammatory and anti-inflammatory NF-κB activation.

5.4 The Role of IDO1 and Its Metabolites in Various Infectious Diseases

Infection caused by various microbes (bacteria, virus, fungus, and parasite) induces inflammation in various body organs and promotes the production of many cytokines related to inflammation. Innate immune systems are deeply involved in the infection-induced inflammation via TLR, RIG-I-like receptor (RLR), and NOD-like receptors (NLRs) (Meylan et al. 2006; Kawai and Akira 2011). These receptor signals increase the production of various cytokines, including type I and II IFN, and proinflammatory cytokines (IL-1β, IL-6, and TNF-α). It is well known that these cytokines could enhance the expression of IDO1 in dendritic cells, monocytes, and macrophages. A recent report demonstrated that TLR signaling could directly induce IDO1 expression without the stimulation of cytokines (Godin-Ethier et al. 2011). Thus, the expression of IDO1 in various host cells is enhanced via inflammatory molecules during infectious diseases.

Recently, many studies evaluated the induction and role of IDO1 during infections caused by various pathogens including bacteria, virus, fungus, and parasitic insects. These reports make it increasingly clear the significance of IDO1 in infectious diseases. In this section, we discuss the involvement of IDO1 and TRP metabolites in infectious diseases caused by some viruses (hepatitis virus, human immunodeficiency virus, influenza virus, encephalomyocarditis virus), *Toxoplasma gondii*, *Chlamydia* spp., and fungus.

5.4.1 Hepatitis Virus Infection

There are five types of hepatitis viruses (A, B, C, D, and E) which can infect humans either via oral or blood transmission. These viruses can cause acute or chronic hepatitis in humans. All types of human hepatitis viruses can induce acute hepatitis. Moreover, Epstein-Barr (EB) virus, cytomegalovirus, and herpes virus can also induce acute liver injury. Fulminant hepatitis is a clinical syndrome consisting of sudden and severe liver injury that results in hepatic encephalopathy and acute liver failure (Meyer and Duffy 1993; Wright and Lau 1993). The rate of mortality in fulminant hepatitis patients remains very high, although intensive medical care and implementation of the latest therapies, including liver transplantation, are available today. Chisari et al. have established acute hepatitis B model using human hepatitis B virus (HBV) transgenic (Tg) mice and hepatitis B surface (HBs) antigen (Ag)-specific cytotoxic T lymphocytes (CTLs) (Ando et al. 1993, 1994). A fulminant hepatitis model has been created by adoptive transfer with HBsAg-specific CTLs into HBV Tg mice. The mice develop a necroinflammatory liver disease that is histologically similar to acute viral hepatitis in man. In this acute hepatitis murine model, IDO1 expression was significantly increased in the liver of HBV Tg mice after HBsAg-specific CTL injection (Iwamoto et al. 2009). IFN-γ expression is markedly increased in the liver after the CTL injection, and enhancement of IFN-γ production is deeply involved in the induction of IDO1 in hepatocytes. However, the role of IDO1 is unclear in this acute hepatitis model. To evaluate the role of IDO1 during acute hepatitis, we backcrossed HBV Tg mice and IDO1 knockout (KO) mice to establish HBV Tg/IDO1 KO mice and evaluated liver injury using these established mice. Liver injury after administration of HBsAg-specific CTLs was attenuated in HBV Tg/IDO1 KO mice compared to that in HBV Tg/IDO1 wild-type (WT) mice (unpublished data). Moreover, coadministration of CTLs and KYN, which is a metabolite of L-TRP converted by IDO1, induced severe liver injury even in HBV Tg/IDO1 KO mice. These results indicated that KYN induced by IDO1 may be one of the aggravating factors for liver injury in HBV Tg mice during acute hepatitis induced by HBsAg-specific CTLs. Woodchuck hepatitis virus infects woodchucks and also causes acute hepatitis. IDO1 expression in the liver is upregulated during acute hepatitis caused by woodchuck hepatitis virus (Wang et al. 2004). IFN-γ is involved in the enhancement of IDO1 expression during this virus infection. In acute liver injury model induced by a chemical agent – carbon tetrachloride

(CCL4) or alpha-galactosylceramide (GalCer) – IDO1 expression in the liver was immediately enhanced after the injection of these chemical agents (Li et al. 2012; Ito et al. 2010). Thus, IDO1 expression was enhanced during acute hepatitis or liver injury in which IFN-γ or proinflammatory cytokine production was involved in the development of liver injury. In acute hepatitis caused by any virus, IFN-γ production by immune cells and Th1 response become dominant to eliminate intracellular pathogens (virus), and consequently, IDO1 expression and the metabolites of TRP in the liver increase during acute hepatitis caused by various viral infections. Inhibition of induction of IDO1 may lead to new therapies aiming at reducing tissue damage, because these TRP metabolites induced by IDO1 exacerbate liver injury cooperatively with antigen-specific CTLs.

Chronic hepatitis is generally caused by HBV or human hepatitis C virus (HCV) infection. Most cases of hepatocellular carcinoma (HCC) are associated with chronic hepatitis induced by HBV or HCV (Arzumanyan et al. 2013). Therefore, the elimination of HBV or HCV in the early phases of infection is critical for an improved prognosis for the patient. Recent reports demonstrated that the expression and activity of IDO1 are increased during chronic hepatitis caused by HBV infection (Chen et al. 2009). In patients with HBV infection, alanine aminotransferase (ALT) levels and HBV load correlated with IDO1 expression in the blood samples. Some reports have also demonstrated that IDO1 expression and activity are significantly enhanced in chronic hepatitis with HCV infection (Larrea et al. 2007; Zignego et al. 2007). For patients, the expression of IDO1 in the liver was upregulated and correlated with CTLA-4 directly. CTLA-4 is expressed on the surface of T cells and transmits an inhibitory signal to T cells. Therefore, the induction of IDO1 may inhibit T-cell reactivity to viral antigens in chronic HCV infection directly or via enhancement of CTLA-4 expression. In clinical practice, patients with HCV infection are generally treated with type I interferon (IFN) and guanosine analog (ribavirin) to eliminate the HCV. This IFN and ribavirin therapy sometimes induces depression in patients. Some reports indicated that IDO1 may be involved in the development of IFN-induced depression in HCV-infected patients (Zignego et al. 2007; Baranyi et al. 2013). Type I IFN can also upregulate the expression of IDO1 in various host immune cells (Huang et al. 2013; Von Bubnoff et al. 2011). Therefore, the measurement of IDO1 may be able to predict the onset of type I IFN-induced depression during therapy to HCV-infected patients (Raison et al. 2010; Smith et al. 2012). A recent study demonstrated that the addition of protease inhibitor to standard therapy with pegylated IFN and ribavirin, as compared with standard therapy alone, significantly increased the rates of sustained virologic response in previously untreated adults with chronic HCV genotype 1 infection (Poordad et al. 2011). Now, while we may completely eliminate HCV from the patient's system by the coadministration of IFN, ribavirin, and protease inhibitor, there is no treatment to completely eliminate HBV from patients with chronic HBV infection. In chronic hepatitis caused by HBV infection, patients are generally treated with IFNs and a nucleoside analog (lamivudine). However, this treatment only suppresses viral pro-

liferation and does not completely eliminate HBV. Therefore, patients with chronic hepatitis by HBV infection must be on continuous treatment with IFN and lamivudine. Lifelong viral persistence in chronic hepatitis B (CHB) carriers is due to liver-induced immune tolerance toward HBV, which is characterized by defective HBV-specific T-cell responses and undetectable anti-HB Ab levels (Rehermann and Nascimbeni 2005). One possible treatment to eliminate HBV completely from the patients with HBV may be immunological therapy using the activation of host immune cells. In chronic HBV infection state, the host immune system is tolerant to HBV itself. The enhancement of host immune response against HBV can induce seroconversion in HBV-infected patients; seroconversion seems to completely eliminate HBV. Induction of a powerful immune response to the HBV antigen may lead to complete elimination of HBV from the patient's system. Several reports state attempts to induce HBV-specific immune response in HBV transgenic mice in which host immune system is tolerant to HBV antigen; it was seen that general vaccination never induced HBV-specific immune response in the host animal (Ito et al. 2008; Zeng et al. 2013). IL-12-based vaccination therapy may reverse liver-induced immune tolerance toward HBV by restoring systemic HBV-specific $CD4^+$ T-cell responses, eliciting robust hepatic HBV-specific $CD8^+$ T-cell responses, and facilitating the generation of HBsAg-specific humoral immunity. We previously demonstrated that α-galactosylceramide (GalCer), which is a ligand specific to natural killer T (NKT) cells, powerfully induced HBsAg-specific CTLs with HBsAg vaccination (Ito et al. 2008). As previously indicated, the expression of IDO1 is enhanced in chronic hepatitis caused by HBV infection (Chen et al. 2009). Many reports demonstrated that IDO1 can suppress the activity and proliferation of several types of lymphocytes via depletion of tryptophan and/or the increase of some TRP metabolites (Li et al. 2012; Hoshi et al. 2010; Murakami et al. 2012). During chronic hepatitis, the enhancement of IDO1 activity may inhibit HBV-specific immune response by lymphocytes in the host, and HBV cannot be eliminated by host immune system completely. However, GalCer, which can strongly induce HBV-specific CTLs with HBsAg vaccination, also enhanced the expression of IDO1 in the liver and spleen and increased the concentration of KYN in the serum (Ito et al. 2010). In our unpublished results, HBsAg-specific immune response induced by the immunization with HBsAg and GalCer was strongly enhanced in IDO1 KO mice compared with WT mice. A competitive inhibitor of IDO1, 1-MT could also increase HBsAg-specific CTL response induced by HBsAg and GalCer immunization. These results indicated that IDO1 inhibition and activation of immune response by GalCer could extremely enhance HBV-specific cellular immune response during HBsAg vaccination. Coadministration of HBsAg, GalCer, and 1-MT may lead to a new therapy to completely remove HBV in patients with chronic hepatitis caused by HBV infection. Thus, IDO1 is enhanced and deeply involved in chronic hepatitis. The control of IDO1 induction may open a new treatment to purge the hepatitis virus completely even in immune-tolerant states such as during chronic hepatitis.

5.4.2 *Human Immunodeficiency Virus (HIV) Infection*

HIV is a lentivirus and causes acquired immunodeficiency syndrome (AIDS) in humans. The immune system of a patient infected with HIV allows life-threatening opportunistic infections and cancers. Patients show highly elevated serum/plasma concentrations of the proinflammatory cytokines, type I and type II IFN. These cytokines induce the production of reactive oxygen species and the degradation of TRP by IDO1. For two decades and counting, it was shown that sources of the neurotoxin QUIN in the brain of HIV-1-infected patients and retrovirus-infected macaques were synthesized locally within the brain, and these results demonstrate a role for induction of IDO1 in accelerating the local formation of QUIN within the brain tissue, particularly in areas of encephalitis, rather than entry of QUIN into the brain from the meninges or blood (Heyes et al. 1998). Indeed, IDO1 has powerful immunosuppressive activity, which could contribute to the immune dysfunction observed in HIV-infected patients. HIV infection induced the expression of IFN-β and IFN-γ in peripheral blood mononucleated cell (PBMC) from a healthy donor (Boasso et al. 2007). These cytokines enhanced IDO1 expression in PBMC after HIV infection. Moreover, a recent study demonstrated that HIV Tat protein directly induced IDO1 expression on DC (Planes and Bahraoui 2013). In this report, Tat induced IDO1 expression before the production of IFN-γ at the kinetic level, and IFN-γ pathway inhibitors had no effect on Tat-induced IDO1. Thus, during HIV infection, IDO1 expression is enhanced by HIV proteins and IFN induced by HIV infection in host immune cells. IDO1 inhibitor (1-MT) enhanced the elimination of HIV-infected macrophages in an animal model (Potula et al. 2005). The treatment with 1-MT increased the number of HIV-specific CTLs, leading to elimination of HIV-infected macrophages in the brain. As a therapy against simian immunodeficiency virus (SIV) infection, IDO1 inhibitor was used in macaques. Combination therapy with 1-MT and antiretroviral reagents (didanosine, stavudine, 9-(2-phosphonylmethoxypropyl) adenine) significantly reduced the virus levels in plasma and lymph nodes of SIV-infected animals (Boasso et al. 2009). 1-MT appeared to synergize with antiretroviral therapy (ART) for HIV in inhibiting viral replication and did not interfere with the beneficial immunologic effects of ART (increased frequency of total CD4 T cells, increase of CD8 T cells, and reduction of regulatory T cells). IDO1 activity is strictly associated with the function of regulatory T cells, in that regulatory T cells induce IDO1 expression in APC, and IDO1-expressing APC induce a regulatory T-cell phenotype in naïve T cells (Fallarino et al. 2003; Curti et al. 2007). Regulatory T cells accumulate in lymph nodes where IDO1 is upregulated during HIV infection (Nilsson et al. 2006; Estes et al. 2006). These regulatory T cells induced by IDO1 may inhibit the induction and proliferation of HIV-specific effector T cells to eliminate HIV from the patients. A previous study investigated the role of IDO1 in mice infected with LP-BM5 murine leukemia virus, which results in the development of a fatal immunodeficiency syndrome known as murine AIDS (Hoshi et al. 2010). The absence of IDO1 upregulated the production of type I IFNs and downregulated virus

replication in the animals with LP-BM5 infection. The survival rate of IDO1 KO mice or 1-MT-treated mice infected with LP-BM5 alone or with both LP-BM5 and *Toxoplasma gondii* was clearly greater than the survival rate of WT mice. In general, HIV impairs the host immune system via the destruction of $CD4^+$ T cells. Moreover, the enhancement of IDO1 expression induced by HIV infection is also one of the mechanisms to cause immunodeficiency in HIV patients.

5.4.3 Influenza Virus Infection

Influenza, commonly known as the flu, is an infectious disease caused by RNA viruses of the family Orthomyxoviridae, the influenza viruses. The most common symptoms are chills, fever, runny nose, sore throat, muscle pains, headache (often severe), coughing, weakness/fatigue, and general discomfort. Previous reports indicated that IDO1 activity in the lung and lung-draining mediastinal lymph nodes were enhanced after the infection with the influenza virus (Huang et al. 2013; Yoshida et al. 1979). Induction of IDO1 expression impaired influenza-specific effector CD8 T-cell responses and delayed the recovery after viral clearance (Huang et al. 2013). Moreover, treatment by IDO1 inhibitor increased the numbers of activated and functional $CD4^+$ T cells, influenza-specific $CD8^+$ T cells, and effector memory cells in the lung after influenza virus infection (Fox et al. 2013). However, the influenza-induced IDO1 activity did not affect virus clearance during influenza infection (Huang et al. 2013). Secondary bacterial pneumonia is a serious disease during or after influenza infection. A previous report demonstrated that viral infections and bacterial components in the airway synergize to produce huge proinflammatory mediators that contribute to the severe prognosis of secondary bacterial complications during or after influenza infection (Zhang et al. 1996). Upregulation of IDO1 expression induced by influenza virus infection enhanced bacterial outgrowth during secondary pneumococcal pneumonia (van der Sluijs et al. 2006). Treatment of 1-MT in secondary bacterial infection after influenza infection model significantly reduced the bacterial outgrowth and proinflammatory cytokine production in the infected lung. These results indicated that control of IDO1 expression leads to the treatment of influenza virus infection in itself and secondary pneumonia after influenza virus infection. Though the therapy to inhibit IDO1 expression can hardly eliminate influenza virus, it is useful to improve the prognosis in the patients infected with the virus.

5.4.4 Encephalomyocarditis Virus Infection

Encephalomyocarditis virus (EMCV), a member of the Picornaviridae family which includes the *Enterovirus* genus, causes acute myocarditis in animals. EMCV infection in mice is an established model for viral myocarditis, dilated cardiomyopathy,

and congestive heart failure (Topham et al. 1991). An earlier report demonstrated that type I IFN is upregulated by IDO1 knockdown or inhibition during experimental EMCV infection, resulting in suppressed EMCV replication (Hoshi et al. 2012). Moreover, the treatment of IDO1 KO mice with KYN metabolites eliminated the effects of IDO1 knockdown on the improved survival rates. Type I IFN is produced by macrophages or dendritic cells that recognize the virus via TLRs or RIG-I. KYN decreased the number of macrophages and suppressed the production of type I IFN. These results suggested that KYN metabolites regulate the production of type I IFNs by the suppression of immune cells during EMCV infection.

5.4.5 Toxoplasma gondii *Infection*

Toxoplasma gondii, an intracellular protozoon, is a major pathogen of the opportunistic infectious disease toxoplasmosis in infants, pregnant women, and immunocompromised hosts, such as patients with AIDS or those treated with immunosuppressive drugs. Previous studies have also indicated that IDO1 induction by IFN-γ resulted in the degradation of L-TRP and inhibited *T. gondii* growth in vitro and in vivo (Heseler et al. 2008; Silva et al. 2002). On the other hand, a recent study demonstrated that IDO1 activity inhibited by 1-MT attenuated *T. gondii* replication and inflammatory damage in the lung after infection (Murakami et al. 2012). Also, several reports indicated that marked increases in IDO1 may suppress immune responses by locally depleting L-TRP and hence preventing T-lymphocyte proliferation (Munn et al. 1999; Liu and Wang 2009). In other words, IDO1 may impair the host immune system's ability to eliminate *T. gondii* during infection. Thus, IDO1 has two opposite effects in the control of *T. gondii* infection. A recent report demonstrated that the minimum concentration of tryptophan required for bacterial growth is 10–40-fold higher than the minimum concentration necessary for T-cell activation (Muller et al. 2009). A balance between the antimicrobial effect and the inhibition of host lymphocytes may be important during *T. gondii* infection.

5.4.6 Chlamydia *spp. Infection*

Chlamydia is a genus of bacteria that are obligate intracellular parasites. *Chlamydia* infection is a common sexually transmitted infection (STI) in humans caused by the bacterium *Chlamydia trachomatis. Chlamydia trachomatis* is a gram-negative bacterium that causes severe diseases of the eye and the urogenital tract. It is recognized by host immune cells via TLR2 (Darville et al. 2003). The host immune response to *Chlamydia trachomatis* potently depends on IFN-γ, which is a strong inducer of IDO1 (Roshick et al. 2006). IDO1-mediated TRP starvation is thought to be the major innate immune mechanism to control *Chlamydia* growth. Since the addition of TRP reversed the effect of IFN-γ in *Chlamydia* growth, restriction of *Chlamydia*

spp. intracellular growth depended on tryptophan deprivation. Only a condition of severe IDO1-mediated TRP degradation would inhibit bacterial persistence and concomitantly reduce bacteria reactivation. However, at suboptimal concentrations of IFN-γ, tryptophan starvation could allow the infection to enter a persistent state (Beatty et al. 1994). Thus, chlamydial development is rigorously regulated by severe TRP depletion with high IDO1 activity.

5.4.7 Fungal Infection

Although human beings are continuously exposed to fungi, they rarely develop fungal infections. A variety of environmental and physiological conditions contribute to the development of fungal diseases. Fungal infections easily develop under conditions of primary or acquired immunodeficiency. A stable host and fungi interaction requires that the elicited host immune response be strong enough to allow host survival with or without pathogen depletion and to establish persistency without excessive inflammation. In an experimental fungal infection model, IDO1 activity was enhanced at sites of infection as well as in dendritic cells and effector neutrophils via IFN-γ- and CTLA-4-dependent mechanisms. IDO1 inhibition by the treatment of 1-MT greatly exacerbated *Candida* infection and associated inflammatory pathology as a result of deregulated innate and adaptive/regulatory immune responses (Bozza et al. 2005). 1-MT impaired the activation and functioning of suppressor CD4+CD25+ regulatory T cells producing IL-10 during *Candida* infection. Several types of regulatory host immune cells could influence the outcome of fungal infection. A good balance is required between regulatory T-cell responses, effector components, and the pathogen. A recent study demonstrated that the IDO1/regulatory T-cell axis had a protective effect on fungal allergy (Grohmann et al. 2007). Glucocorticoid-inducible TNF receptor (GITR) modulated tryptophan catabolism by IDO1 induction and inhibited host Th2 response during fungal allergic diseases. Thus, induction of IDO1 could be an important mechanism underlying the anti-inflammatory action of corticosteroids. The induction of IDO1 may be involved not only in the outcome of fungal infectious diseases by host immune system but also in the allergic diseases caused by fungal infection.

5.5 Future Strategy Targeting the TRP-KYN Pathway

An early study suggested that IDO1 participates in controlling fetal allograft rejection (Munn et al. 1998). Since then, a number of studies support the importance of IDO1 as a negative co-stimulatory molecule. IDO1 deficiency has been shown to promote T-cell response, downregulate Treg responses, and exacerbate autoimmune inflammatory diseases (Yan et al. 2010). The current study provides evidence that absence and inhibition of IDO1 are critical for suppressing virus replication with

upregulated type I IFN (Hoshi et al. 2010). Although the role of IDO1 may be complex and may depend on the difference of disease stages (e.g., acute/chronic disease) or the stimulus pathogens, current studies suggest that modulation of the IDO1 pathway may be an effective strategy for treatment of various infectious diseases.

On the other hand, recent studies provide interesting evidences. Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor. AhR is an important transcriptional regulator of drug-metabolizing enzymes, best known for mediating the toxicity of dioxin. AhR also has endogenous functions that include controlling cell cycle, immune responses, and cell differentiation. It has been recently reported that KYNA may be one of the endogenous ligands of AhR (Denison and Nagy 2003). Interestingly, it has been recently shown that KYN is also able to activate AhR responses (Mezrich et al. 2010) and IDO1 is induced by DCs in response to dioxin (Vogel et al. 2008). Possibly, AhR can interact with several KYNs and promote the generation of immunosuppressive T cells (Mezrich et al. 2010). It has been repeatedly shown that KYNA is a potent antagonist of the glycine allosteric site at the NMDA receptor complex, and for several years, it was assumed that interaction between KYNA and the NMDA receptor complex could have a physiological role in brain function (Stone 1993). However, KYNA affinity for the NMDA receptor complex is not sufficient in physiological conditions. It has also been demonstrated that KYNA antagonizes α7 nicotinic acetylcholine receptors (α7nAChRs) (Alkondon et al. 2004), which have been shown to mediate inflammatory regulation in a variety of inflammatory states, such as sepsis, endotoxic shock, and colitis (Pavlov 2008). Thus, targeting the KYN pathway for new drug development could be of value not only for treatment of various infectious diseases but also for preventing the development of neurodegenerative disorders. In addition, understanding the subsequent steps on the KYN pathway and physiological mechanisms responsible for regulation of KYN and its metabolite concentration in biological fluids may be important for future drug development.

5.6 Conclusion

TRP metabolism via KYN pathway is a good example of how metabolism of small molecules can impact the immune system. IDO1, an enzyme involved in the catabolism of TRP, is expressed in a variety of cells including immune cells, such as monocyte-derived macrophages and DCs. TRP depletion via IDO1 is part of the cytostatic and antiproliferative activity, and IDO1 activity participates in the regulation of T-cell responses and immune homeostasis. Therefore, induction of the KYN pathway and/or controlling the systemic TRP concentrations by stimulation of immune cells or by diet might be an effective strategy for treatment of virus infection and immune diseases. We believe that further findings on the mechanism of

immune regulation by IDO1 and KYN pathway might contribute to the development of a novel therapy protocol, which would target several immune disorders.

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Chapter 6
Evaluation of Tryptophan Metabolism in Chronic Immune Activation

Ayse Basak Engin

Abstract Chronic immune activation is encountered in different pathologies including granulomatous and functional bowel diseases, cancer, aging, atherosclerosis, and obesity. Persistence of chronic inflammatory stimuli over time creates a biologic background for immunosenescence and favors neopterin formation with the enhanced tryptophan (Trp) degradation in diseases concomitant with cellular immune activation. Trp degradation leads to the generation of several neuroactive compounds by three distinct pathways.

Indoleamine 2,3-dioxygenase (IDO) induction leads to many complex changes within the affected cells resulting in immunosuppression through breakdown of Trp. Thus, neopterin concentrations as well as IDO expression significantly increase in inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease.

However, irritable bowel syndrome (IBS) is linked with abnormal serotonin functioning and immune activation. In this case, enteric serotonin (5-HT) signaling may be defective and inactivated by the serotonin-selective reuptake transporter (SERT) in the enterocytes. A positive correlation is evident between IBS severity and kynurenine (Kyn) to Trp ratio which is significantly correlated with the rise of interferon (IFN)-gamma.

The dual host-protective and tumor-promoting actions of immunity are referred to as cancer immunoediting. IDO-reactive T cells are able to recognize and kill tumor cells as well as IDO-expressing dendritic cells (DCs). IDO activation leads to immunosuppression through breakdown of Trp in the tumor microenvironment and tumor-draining lymph nodes. C-C chemokine receptor type 4 (CCR4)+ forkhead boxp3(Foxp3)+ regulatory T (Treg) cells create a favorable environment for tumor escape from host immune responses. Thus, Foxp3+/IDO+ tumors are associated with more advanced disease.

Age-related changes in the immune system are known as immunosenescence. A causal relationship is evident between the Trp metabolism and immune deficiency in elderly. Eventually, the reduced serum Trp concentrations and increased Kyn levels indicate increased chronic low-grade inflammation in elderly. In this case,

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IDO-induced Trp degradation is associated with increase in neopterin and nitrite levels. The amounts of neopterin produced by activated macrophages correlate with their capacity to release reactive oxygen species (ROS). Melatonin not only improves the antioxidant potential of the cell by stimulating the synthesis of antioxidant enzymes but also reduces free radical generation. The decline in melatonin production in aged individuals is a primary contributing factor for the development of age-associated neuronal damage.

IDO activity also has a significant positive correlation in both sexes with carotid artery intima/media thickness as an early marker of atherosclerosis. Enhanced Trp degradation in patients with coronary heart disease correlates with enhanced neopterin formation. In addition to elevated Kyn to Trp ratio, neopterin concentrations correlate with the abdominal obesity and metabolic syndrome.

Keywords Tryptophan • Kynurenine • Neopterin • Ulcerative colitis • Crohn's disease • Irritable bowel syndrome • Immune escape mechanisms • Obesity • Aging

6.1 Introduction

Tryptophan (Trp) is an indispensable amino acid that should be supplied by dietary protein. L-Tryptophan metabolism is associated with numerous physiological functions and leads to the generation of several neuroactive compounds by three distinct pathways (Ruddick et al. 2006). First of all through the kynurenine (Kyn) pathway, while a large amount of Trp is oxidatively metabolized in the liver, simultaneously a small amount of Trp degradation can occur extrahepatically (Wirleitner et al. 2003). In this respect the conversion of Trp to Kyn is catalyzed by either the ubiquitous indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) which is localized in the liver (Ruddick et al. 2006). In the central compound of this pathway, Kyn can turn into free radical generator 3-hydroxykynurenine, kynurenic acid (KA), and quinolinic acid (QA). KA is an N-methyl-D-aspartate (NMDA) receptor and alpha7 nicotinic acetylcholine receptor (alpha7nAChR) antagonist at physiological concentrations through its competitive blockade of the glycine co-agonist site (Schwarcz and Pellicciari 2002). QA has excitotoxic properties due to potent activation of NR2A and NR2B; NMDA receptor subtypes and its ability to generate free radicals are independent of receptor activation (Schwarcz and Pellicciari 2002). The activity of TDO can be increased by L-Trp and its analogs via an allosteric binding site and is competitively inhibited by some common indoleamines including tryptamine (Ruddick et al. 2006). IDO is stimulated during cellular immune responses preferentially by T-helper (Th)1-type cytokine interferon-gamma (IFN-gamma). IDO induction has been correlated with the conversion of Trp to Kyn and simultaneously induction of guanosine triphosphate cyclohydrolase (GTPCH), which is the key enzyme in pteridine biosynthesis. Therefore, IDO is recognized as one of the prominent mediators of immune regulation by metabolic pathways. IDO activity is best characterized by the Kyn to Trp ratio which correlates with

concentrations of immune activation markers such as neopterin (Taylor and Feng 1991; Schröcksnadel et al. 2006). Thus, increased neopterin formation with the enhanced Trp degradation is only observed in diseases concomitant with cellular immune activation (Widner et al. 2002). In this context a significant correlation between Kyn-Trp ratio and neopterin concentrations indicates the involvement of IDO during the degradation of Trp (García-Lestón et al. 2012). Therefore, immunosuppressant substances are effective by inhibiting IDO activity and neopterin production simultaneously in a similar dose-dependent manner (Schroecksnadel et al. 2011).

In another pathway, a small portion of Trp is used for the synthesis of serotonin. Serotonin (5-hydroxytryptamine, 5-HT) is a key neurotransmitter that modulates a wide variety of functions in both peripheral organs and the central nervous system (CNS). The predominant site of 5-HT synthesis throughout the gastrointestinal tract is the enterochromaffin (EC) cells of the intestinal mucosa (Martel 2006). 5-HT is synthesized through the actions of two different tryptophan hydroxylases, tryptophan hydroxylase (TpH)-1 and TpH-2, which are found in EC cells and neurons, respectively (Gershon and Tack 2007). Tetrahydrobiopterin (BH4) is essential for the biosynthesis of serotonin, which serves as cofactor for tryptophan hydroxylase. GTPCH 1 is the first and rate-limiting enzyme for BH4 biosynthesis (Nagatsu and Ichinose 1999; Ichinose et al. 2013). The effects of 5-HT occur via seven distinct families of 5-HT receptors (5-HTRs). Six of them are G-protein coupled, whereas the remaining 5-HT3R is ionotropic (Hoyer et al. 2002; Hannon and Hoyer 2008). The synaptic concentration of released serotonin is regulated by the serotonin transporter (SERT) that removes serotonin from the synapse. Intestinal 5-HT is inactivated by metabolic degradation after SERT-mediated uptake into enterocytes or neurons. Furthermore, inhibition of SERT causes an increase in transmural transport of 5-HT in intestinal segments and augments the extracellular concentration of 5-HT (Martel 2006). Since Trp is known as the primary amino acid precursor of serotonin, systemic Trp depletion results in decreased serotonin synthesis (Bell et al. 2001; van Donkelaar et al. 2011). Consequently, the two metabolic pathways, Kyn and 5-HT, compete for their reciprocal precursor, Trp. Eventually, KA concentrations reduce and 5-HT synthesis increases. Although serotonergic metabolism in the intestinal mucosa is not affected by acute Trp depletion, profound effects on systemic concentrations of serotonergic metabolites are evident (Keszthelyi et al. 2012).

Another pathway that is activated in response to signals from the circadian clock and arylalkylamine N-acetyltransferase (AANAT; serotonin N-acetyltransferase) is the first rate-limiting enzyme in melatonin production and converts serotonin to N-acetyl serotonin. AANAT also constitutes a key interface between melatonin production and regulatory mechanisms (Coon et al. 2002). Actually, synthesis of melatonin starts with hydroxylation of L-Trp to 5-hydroxytryptophan. 5-Hydroxytryptophan is converted to serotonin. Serotonin is subsequently converted to N-acetylserotonin by the enzyme AANAT (Slominski et al. 2012). AANAT mRNA is uniformly distributed in the pineal gland but is limited primarily to the photoreceptor outer segments in the retina. Furthermore, the conversion of N-acetylserotonin to melatonin is

achieved by the enzyme of hydroxyindole-O-methyltransferase (HIOMT). This enzyme is present in high amount in the pineal gland, but it is nearly undetectable in the retina (Coon et al. 2002). Circadian clocks in the vertebrate retina optimize retinal function by driving rhythms in gene expression, photoreceptor outer segment membrane turnover, and visual sensitivity (Iuvone et al. 2005). Most of the regulatory functions of melatonin are mediated by two high-affinity G-protein-coupled receptors, named MT1and MT2 (Dubocovich et al. 2010), which are mainly expressed in the CNS but are also present in different peripheral organs (Slominski et al. 2012). The third melatonin-binding site MT3 is an enzyme named quinone reductase 2 (QR2). Protective effect of melatonin against oxidative stress is provided by the activation of MT3/QR2. All three melatonin receptors can be found in the gut (Chen et al. 2011). G-protein-coupled membrane receptors of melatonin modulate several intracellular messengers such as cyclic adenosine monophosphate (cAMP) and [Ca2+] which are highly effective in the production of melatonin (Klein 2007).

Chronic immune activation is encountered in different pathologies including granulomatous and functional bowel diseases (Prior et al. 1986; Clarke et al. 2009), atherosclerosis (Blasi 2008), cancer (Dalgleish and O'Byrne 2002), and obesity (Brandacher et al. 2007; Duncan and Schmidt 2001). Persistence of chronic inflammatory stimuli over time creates a biologic background for immunosenescence and favors the susceptibility to inflammatory age-related diseases (Franceschi et al. 2000; Candore et al. 2006). In this chapter, inflammatory bowel disease, irritable bowel syndrome, cancer, aging, atherosclerosis, and obesity are taken into consideration in terms of chronic immune activation and Trp metabolism.

6.2 Chronic Immune Activation in Inflammatory and Functional Bowel Diseases

Inflammatory bowel disease (IBD) results from an inappropriate immune response that occurs in genetically susceptible individuals. It represents a complex interaction between the intestinal immune system, environmental circumstances, and microbial factors (Danese and Fiocchi 2006). Actually, IBDs consist of two distinct pathologies: ulcerative colitis and Crohn's disease. The incidence and prevalence of IBD are increasing with time and in different regions around the world. In time-trend analyses, 75 % of Crohn's disease studies and 60 % of ulcerative colitis studies have an increased incidence of statistical significance. The highest reported prevalence values for IBD were in Europe, 505 per 100,000 persons and 322 per 100,000 persons, and in North America, 249 per 100,000 persons and 319 per 100,000 persons, for ulcerative colitis and for Crohn's disease, respectively (Molodecky et al. 2012).

The mechanisms of cell entry into the intestinal mucosa, bacterial and foreign antigen invasion, angiogenesis, and the control of gut inflammation through intestinal microvasculature are the most important issues considering the pathogenesis of IBD (Danese 2011).

Regardless of pathogenetic mechanisms, evaluation of urinary neopterin excretion in untreated ulcerative colitis patients shows a striking correlation between neopterin levels and the severity of disease. When the chronic cellular immune activation underlying ulcerative colitis is subsided, neopterin levels decrease and clinical remission is achieved (Niederwieser et al. 1985). Furthermore, fecal neopterin concentration is also increased in patients with clinically active or inactive Crohn's disease and in patients with clinically active ulcerative colitis when compared with controls. Therefore, neopterin represents a remarkable biomarker for the activity of IBD (Husain et al. 2013). On the other hand, expression of IDO mRNA is markedly induced in perifollicular regions of lymphoid follicles in colonic tissues of IBD patients. IDO is primarily expressed in CD123+ mononuclear cells. Upregulation of IDO is detected by the increase of Kyn and Kyn/Trp in supernatants from colonic tissues (Wolf et al. 2004). In a similar manner with neopterin, increase in IDO expression in the lesions of ulcerative colitis or Crohn's disease is positively related to the severity of inflammation. IDO-positive mononuclear cells also express CD11c, CD68, and toll-like receptor (TLR)4 (Zhou et al. 2012). Deficient TLR and nucleotide-binding-oligomerization domain function due to genetic variability is associated with an increased susceptibility to the development of inflammatory bowel disease (Mueller and Podolsky 2005). Actually, nucleotide-binding-oligomerization domain-containing-2 (NOD2) acts as a bacterial sensor in dendritic cells (DCs), and NOD2 variants are associated with Crohn's disease. DCs from individuals with Crohn's disease expressing Crohn's disease-associated NOD2 are defective in autophagy induction, bacterial trafficking, and antigen presentation (Cooney et al. 2010).

On the other hand, an induction of mRNA for TLR2, TLR4, and TLR5 expression in inflammation-associated human intestinal macrophages also contributes to the inflammatory process (Hausmann et al. 2002). TLRs play essential roles in innate immune responses by recognizing various pathogen-derived components. In this respect, they activate various transcription factors such as nuclear factor-kappa B (NF-kappaB), activating protein-1, and interferon regulatory factors, which are responsible for inflammatory responses. In addition, TLRs also mediate alternative pathways by utilizing TLR3, TLR4, TLR7/8, and TLR9. Specific combination of these adapter molecules induces type I interferon responses (Kawai and Akira 2006). Consequently, the classical proinflammatory TLR signaling pathway leads to the synthesis of inflammatory cytokines and chemokines, such as interleukin (IL)-1beta, IL-6, IL-8, IL-12, and tumor necrosis factor (TNF)-alpha, which are causally involved in the pathogenesis of IBD. Thus, treatment with the TNF-blocking antibody, "infliximab," indicates good clinical response to anti-TNF-alpha agents. This is accompanied with reduced IDO expression (Wolf et al. 2004; Frazão et al. 2013).

Chronic or recurrent abdominal pain or discomfort along with altered bowel function characterizes the irritable bowel syndrome (IBS) (Fukudo 2013). Gastrointestinal comorbidities, such as functional dyspepsia, gastroesophageal reflux disease, functional constipation, and anal incontinence, occur in almost 50 % of the patients. A broad variety of extraintestinal comorbidities, such as fibromyalgia,

chronic fatigue syndrome, and chronic pelvic pain, are best documented and appear in up to 65 % (Riedl et al. 2008). A web-based survey that was carried out shown that the subtypes of IBS were mixed IBS 36 %, IBS with diarrhea 33 %, IBS with constipation 18 %, and unsubtyped IBS 11 % (Krogsgaard et al. 2013). It is also thought that the disorder of the autonomic nervous system function, the neuroimmune axis, and the brain-gut-microbiota axis profiles are unique in IBS patients. Since 5-HT neurotransmission in IBS patients is regulated with the 5-HT3 antagonists, 5-HT4 agonists, and antidepressants, 5-HT appears to be strongly associated with brain-gut function (Fukudo 2013). Successive potentiation of 5-HT and desensitization of its receptor could account for the symptoms seen in diarrhea-predominant and constipation-predominant IBS, respectively (Gershon 2004). Hence, IBS is a complex disorder that is associated with altered gastrointestinal motility, secretion, and sensation. Actually, 5-HT modulates sensation and perception of visceral stimulation at peripheral and central sites. However, enteric 5-HT signaling may be defective and inactivated by the SERT in the enterocytes or neurons. Tegaserod, a 5-HT4 partial agonist, is used in constipation-predominant IBS, while alosetron, a 5-HT3 antagonist, is used in IBS with diarrhea (Sikander et al. 2009; Crowell and Wessinger 2007). Furthermore, mucosal 5-HT, TpH-1 mRNA, SERT mRNA, and SERT immunoreactivity are all significantly reduced in both IBS with constipation and IBS with diarrhea (Coates et al. 2004). These data suggested that reduced SERT in the IBS patients can be one of the factors contributing to the development of both diarrhea and constipation. Thus, SERT immunoreactivity intensity of all IBS, IBS with diarrhea and IBS with constipation, patients significantly differs from that of healthy controls (El-Salhy et al. 2013). There are conflicting data on the efficacy of selective 5-HT reuptake inhibitors in IBS, the association of the SERT gene promoter polymorphism serotonin transporter-linked polymorphic region (5-HTTLPR) with IBS, and the expression pattern of SERT in the intestinal mucosa of IBS patients (Colucci et al. 2008).

According to these evidences, IBS has been linked with abnormal serotonin functioning and immune activation. On the one hand, Trp is used as a substrate for serotonin biosynthesis, but it can alternatively be catabolized to Kyn by the enzyme IDO. While a positive correlation between IBS severity and Kyn to Trp ratio is evident in these patients, increase in IFN-gamma activity is significantly correlated with the rise of Kyn-Trp ratio (Fitzgerald et al. 2008). In this case two alternatives may be valid. Firstly, the increased Kyn-Trp originates from the increased activity of hepatic TDO; the alternative scenario of increased IDO activity is equally valid. However, the elevated neopterin levels in the IBS cohort strongly suggest that IDO is the main enzymatic player. Although, the majority of the neopterin measurements are below the cutoff value, 10 nM level, which are considered to be reliably indicative of a disease state (Schroecksnadel et al. 2005a). In some cases although both plasma Kyn levels and the Kyn-Trp ratio are significantly increased in the IBS cohort, no difference is found in plasma L-Trp levels between IBS patients and healthy subjects. These patients show significant increases in neopterin levels but below the cutoff value (Clarke et al. 2009). These evidences confirm that low-level chronic immune activation may be valid in IBS. Additionally, significant imbalances

in Trp concentrations and its metabolites may be frequently observed. This phenomenon might be associated either with a disturbance in albumin binding of Trp and an overcompensatory response to decreased Trp concentrations or a dysfunctional serotonergic system in IBS (Chen and Guillemin 2009; Shufflebotham et al. 2006).

As stated above, IBS patients exhibit a distinct Trp degradation profile through downstream of overall TLR activation that is different from that of healthy controls. However, TLR4 activation for Trp metabolism appears equivalent in both healthy controls and some subgroups of IBS patients (Clarke et al. 2012). Indeed, colonic gene and protein expression of TLR2 and TLR4 differs significantly between the subgroups of IBS patients, providing further support for the hypothesis of altered intestinal immune activation. A significant increase of TLR2 and TLR4 was shown only in diarrhea mixed bowel pattern (IBS-M) subgroup compared with healthy subjects. These results support the hypothesis, at least in constipation and IBS-M patients, that the innate immune system plays a key role in the pathophysiology of the disease. Thus the increased colonic expression of TLR2 and TLR4 in IBS-M patients are accompanied by the impaired expression of peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and enhanced production of mucosal proinflammatory cytokines, IL-8 and IL1-beta (Belmonte et al. 2012).

In fact IBS patients showed a significant amount, 72 % increase in number of mucosal immune cells, CD3+, CD4+, and CD8+ T cells, and mast cells compared to controls (Cremon et al. 2009). The increased level of T-cell activation is consistent with the hypothesis of low-grade immune activation in IBS (Ohman et al. 2009). Mild inflammation is involved in diarrhea-predominant IBS patients as proinflammatory cytokine TNF-alpha is significantly higher, although no difference in anti-inflammatory cytokine is observed (Rana et al. 2012). Although IBS is characterized by the increase of proinflammatory cytokines, IL-6 and IL-8, IBS patients with certain extraintestinal comorbid conditions are distinguished by additional elevations in IL-1beta and TNF-alpha (Scully et al. 2010).

6.3 Immune Escape Mechanism in Cancer

The dual host-protective and tumor-promoting actions of immunity are referred to as cancer immune editing which is comprised of elimination, equilibrium, and escape phases (Vesely and Schreiber 2013). IDO-reactive T cells are peptide-specific, cytotoxic effector cells. Hence, IDO-specific T cells effectively disrupt IDO+ cancer cell lines of different origin. IDO-specific cytotoxic T lymphocytes (CTLs) recognize and kill IDO+-matured CD19+ plasmacytoid DC, which mediates immune suppression. Indeed, IDO is upregulated in DC in tumor-draining lymph nodes and creates a tolerogenic microenvironment (Sørensen et al. 2009).

DNA molecules containing unmethylated CpG oligodeoxynucleotides (ODN) have potent immunostimulatory effects on plasmacytoid DCs through TLR9 recognition and signaling. Human plasmacytoid DCs are activated by

CpG-ODN-mediated TLR9 ligation. Later, they can induce the generation of CD4+ CD25+ forkhead boxp3(Foxp3)+ regulatory T cells (Tregs) from CD4+CD25 T cells (Moseman et al. 2004). In this process, human plasmacytoid DCs express high levels of IDO mRNA and protein in response to TLR9 ligation and use the IDO pathway to induce the differentiation of CD4+CD25+Foxp3+ Tregs from CD4+CD25- T cells. IDO inhibitor, 1-methyl-D-tryptophan, significantly impedes plasmacytoid DC-driven inducible Treg generation and suppressor cell function. However, Kyn supplementation suppresses the effect of 1-methyl-D-tryptophan and restores the differentiation of Treg cells (Chen et al. 2008).

IDO is spontaneously recognized by CTLs in patients with cancer (Sørensen et al. 2009). Thus, IDO-specific T cells are present in peripheral blood as well as in the tumor microenvironment. These IDO-reactive T cells are able to recognize and kill tumor cells as well as IDO-expressing DCs, that is, one of the main immune-suppressive cell populations (Sørensen et al. 2011). Inhibition of the expression and activity of IFN-gamma-induced IDO in bone marrow-derived dendritic cells (BMDCs) through the suppression of the activity of Janus kinase/signal transducers and activators of transcription (JAK/STAT) and protein kinase C causes antitumor activity by regulating CD8+ T-cell polarization and CTLs activity (Noh et al. 2013).

Spontaneous CTL reactivity against IDO exists not only in patients with cancer but also in healthy persons. IDO+ DCs inhibit T-cell proliferation because of Trp depletion and accumulation of toxic Trp metabolites (Platten et al. 2005; Munn and Mellor 2007). Actually, Trp metabolites of the Kyn pathway, such as 3-hydroxyanthranilic and QA, can induce the selective apoptosis of Th1 cells and can also effectively suppress T-cell proliferation (Fallarino et al. 2003). Furthermore, CTLs starved of Trp are unable to proliferate and go into G1 cell-cycle arrest (Munn et al. 2005).

Moreover, IDO-expressing plasmacytoid DCs activate the general control non-derepressible-2 (GCN2) kinase pathway in responding T cells. GCN2 kinase acts as a molecular sensor for T cells during IDO-induced Trp depletion and related immunosuppression (Munn et al. 2005). Endoplasmic reticulum (ER) transmembrane signaling protein, unfolded protein response (UPR)-mediated downregulation of protein synthesis, is accompanied by increased phosphorylation of eukaryotic translation initiation factor 2alpha (eIF2alpha). UPR initiates a rapid block in translation of cyclin D1 mRNA, and the cyclin D-dependent kinase activity is lost. During ER stress, one of the mammalian eIF2alpha kinases, protein kinase RNA-activated (PKR)-like ER kinase (PERK), contributes to cyclin D1 translation attenuation and provokes G1 arrest (Brewer et al. 1999). When considering all, both PERK and GCN2 contribute to the ER stress-mediated regulation of eIF2alpha phosphorylation and translation of cyclin D1 (Hamanaka et al. 2005). Consequently, the activation of GCN2 triggers a stress response program that can result in cell-cycle arrest, differentiation, adaptation, or apoptosis via eIF2alpha phosphorylation (De Haro et al. 1996).

Functionally, Trp-deprived DCs show a reduced capacity to stimulate T cells, which can be restored by blockade of specific immunoglobulin (Ig)-like transcripts (ILTs), ILT3. Trp deprivation generates human monocyte-derived DCs with a

marked upregulation of the inhibitory receptors ILT3 and ILT4 and increases the capacity to induce CD4+CD25+Foxp3+ Tregs in an ILT3-dependent manner. Moreover, ILT3high ILT4high DCs lead to the induction of CD4+CD25+Foxp3+ Tregs with suppressive activity from CD4+CD25- T cells. The generation of ILT3high ILT4high DCs with tolerogenic properties by Trp deprivation is linked to a stress response pathway mediated by the GCN2 kinase (Brenk et al. 2009).

IDO activation leads to many complex changes within the affected cells resulting in immunosuppression through breakdown of Trp in the tumor microenvironment and tumor-draining lymph nodes (Soliman et al. 2010). In human malignancies, overexpression of IDO can facilitate immune escape which is under control of tumor suppressor gene bridging integrator 1 (Bin1). Thus, Bin1 loss contributes to immune escape in cancer by increasing the STAT1 and NF-kappaB-dependent expression of IDO (Muller et al. 2005). Since IDO represents an antitumoral immune effector mechanism, IDO also can cause immune system failure by inhibiting T-cell responses. Therefore, tumor cells can escape from immune system through IDO activity. Kyn-Trp ratio correlates strongly with the concentrations of cytokine IL-6, soluble IL-2 receptor-alpha, TNF-alpha receptor, and the macrophage marker neopterin. In this respect accelerated Trp degradation represents an immune escape mechanism (Sperner-Unterweger et al. 2011). Within the tumor microenvironment, not only tumor cells but also other infiltrating cells such as DCs, monocytes, and others can be sources of IDO. In addition to the Trp depletion, accumulation of its metabolites into the tumor environment also propagates the suppression of antitumor immune responses (Zamanakou et al. 2007). On the other hand, Engin et al. have found that certain colon cancer subsets are different in their ability to express IDO, while significant correlation between IDO activity and immunostaining scores indicates an immunosuppressive activity in patients with high IDO expression in colorectal cancer. Thus, high total IDO immunostaining score is a strong predictor for immune tolerance, lymphatic invasion, and subsequent lymph node metastasis (Engin et al. 2010).

Treg cells have been defined as a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens (Sakaguchi 2005, 2006). CD4+ Treg cells are abundant in tumor tissues and prevent the induction of effective antitumor immunity. They express C-C chemokine receptor 4 (CCR4) in tumor tissues. CCR4+ Treg cells are predominant among tumor-infiltrating Foxp3+ T cells (Sugiyama et al. 2013). The chemokines which are specific ligands for CCR4 that are produced by tumor cells attract CCR4+ Treg cells to the tumor. These cells create a favorable environment for tumor escape from host immune responses. Thus, anti-CCR4 monoclonal antibodies eliminate the suppressive effect of CCR4+ Treg cells on the host immune response to tumor cells (Ishida and Ueda 2006). Actually, Foxp3+ Treg cells are associated with more advanced disease in cancers. As IDO promotes differentiation of Treg cells, it may become a suitable target to abolish the development of T-cell tolerance against the cancer development. Node-positive disease almost exclusively occurs in patients with Foxp3+/IDO+ tumors. Actually, the combined expression and immunosuppressive effects of IDO and Foxp3 on

metastatic lymph nodes support this assumption (Mansfield et al. 2009). Most Treg cells are defined based on expression of CD4, CD25, and the transcription factor, Foxp3. The combination of expression of CD4, CD25, and CD127 represents highly purified population of Treg cells and has an efficient suppressor function (Liu et al. 2006). Indeed, natural Treg cells have been observed to predominantly infiltrate tumor masses especially in the early phase of tumor progression (Yamaguchi and Sakaguchi 2006).

Activation of IDO in either tumor cells or nodal regulatory DCs appears to be sufficient to facilitate tumoral immune escape (Munn and Mellor 2007). Additionally, most human tumors can overexpress IDO (Uyttenhove et al. 2003). For instance, IDO is also expressed in human breast cancer cells. Estrogen receptor-negative breast cancer cells may evade the attention of the immune system through the expression of IDO together with its main substrate, L-Trp transport, into these cells (Travers et al. 2004).

In the tumor-draining lymph nodes (TDLNs), there are three strong regulatory mechanisms. IDO, functional activation of Tregs, and the inhibitory programmed cell death 1/programmed cell death 1 ligand (PD-1/PD-L) pathway are tightly linked and constitute an immunosuppressive milieu. When IDO+ plasmacytoid DCs present antigen to effector T cells in the presence of mature, resting Tregs, this initiates a GCN2-dependent activation of the Tregs by IDO. While GCN2 signaling is critical for allowing IDO-induced functional activation, Trp metabolites complete the full activation of the Tregs. Seventy-five to 90 % of this constitutive Treg activity in TDLNs is mediated via IDO-induced, PD-1/PD-L-dependent pathway. IDO-induced Treg activation is prevented by blockade of CTLs antigen 4, and IDO-Treg-PD-1/PD-L pathway is interrupted (Sharma et al. 2007). Eventually, the combination of these IDO-expressing plasmacytoid DCs and IDO-activated Treg cells renders the local milieu in the TDLNs profoundly inhibitory for T-cell activation (Munn and Mellor 2006).

On the other hand, Tregs exposed to certain inflammatory signals from activated DCs or TLR ligands can lose their suppressor activity (Pasare and Medzhitov 2003) and may alter their phenotype (be "reprogrammed") to resemble proinflammatory effector cells. The reprogrammed Treg cells downregulate Foxp3 expression and express proinflammatory cytokines, IFN-gamma, IL-17, and TNF-alpha. This phenotype conversion requires DC-Treg cell contact, which causes IL-6 secretion by the DC, and occurs in an antigen-specific manner (Radhakrishnan et al. 2008). That means IDO plus effector T cells activate Foxp3+ Tregs for suppression. In the absence of IDO, Tregs can lose their suppressor phenotype and undergo conversion to a Th17-like phenotype. Most of the reprogrammed Tregs coexpress IL-2 and TNF-alpha, in addition to IL-17 and IL-22. Only a small number of reprogrammed cells express interferon-gamma or IL-10. Thus, reprogrammed Treg is a source of multiple proinflammatory cytokines. Upregulation of IL-17 in Tregs is driven by IL-6. However, IL-6 expression occurs only when IDO is blocked (Sharma et al. 2009).

Trp degradation is also detectable in patients with gynecological cancer. The relationship between Kyn-Trp and neopterin concentrations indicates that cellular immune activation rather than tumor-mediated IDO activity is responsible for the

degradation of Trp (Schroecksnadel et al. 2005b). However, immunosuppressants are effective to inhibit IDO activity and neopterin production in a similar and dose-dependent manner (Schroecksnadel et al. 2011).

6.4 Aging and Chronic Immune Activation

Aging is associated with increased levels of circulating cytokines and proinflammatory markers. Age-related changes in the immune system, known as immunosenescence, and increased secretion of cytokines by adipose tissue represent the major causes of chronic inflammation (Michaud et al. 2013). Actually, impairment of immune defense with aging is a part of the age-associated neuroendocrine disorders which consist of hypertension, obesity, dyslipidemia, type 2 diabetes, menopause, late-onset depression, vascular cognitive impairment, and some forms of cancer (Oxenkrug 2010). On the other hand, progressive increase in Trp catabolism is also a part of the normal aging process (Frick et al. 2004). In this regard, a causal relationship is evident between the Trp metabolism and immune deficiency in elderly. Thus, neopterin, Kyn-Trp ratio, and all Kyn metabolites are 20–30 % higher in the older group, whereas Trp is 7 % lower (Theofylaktopoulou et al. 2013). Eventually, the reduced serum Trp concentrations and increased Kyn levels indicate increased chronic low-grade inflammation in elderly. In this case IDO-induced Trp degradation is associated with increase in neopterin and nitrite levels (Capuron et al. 2011). In addition to rising neopterin and Kyn levels, KA and homocysteine concentrations as well as the Kyn-Trp ratio also increase with older age. In this respect increasing neopterin concentrations and Kyn-Trp ratio in older age are associated with immune activation especially of the T-cell/macrophage system (Frick et al. 2004; Urbańska et al. 2006). As mentioned above, neopterin and Trp metabolites are strong predictive markers of the normal aging process and comorbidities of aging such as cardiovascular and neurodegenerative diseases or malignant tumors. Actually, aging and related pathological conditions critically involve an overwhelming production of reactive oxygen species (ROS) (Becker et al. 2014). During the exposure to oxidative stress, neopterin derivatives exhibit distinct biochemical effects, most likely via interactions with reactive oxygen or nitrogen intermediates (Hoffmann et al. 2003). The amounts of neopterin produced by activated monocytes/macrophages correlate with their capacity to release ROS. With this background, neopterin concentrations in body fluids can be taken into consideration as a degree of oxidative stress emerging during cell-mediated immune response (Murr et al. 1999). In this case the increased synthesis of BH4 in pteridine pathway is an adaptive response to inflammation; however, inflammation-induced oxidative stress could oxidize BH4 (Huang et al. 2005). In fact the enhanced production of neopterin occurs at the expense of BH4 formation (Fuchs et al. 2009). BH4 is the essential cofactor in the enzymatic hydroxylation of phenylalanine, tyrosine, and Trp. It is synthesized from GTP, and synthesis steps are catalyzed by GTPCH I, 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase (Shintaku 2002).

IFN-gamma-induced IDO promoter activity is enhanced synergistically by TNF-alpha. IFN-gamma-responsive elements, IFN regulatory factor-1, and two

IFN-gamma-stimulated response elements (ISRE-1 and ISRE-2) are critical for this synergistic activation (Robinson et al. 2005). The transcriptional regulation of GTPCH I is important in the control of BH4 metabolism during the coordinated induction of GTPCH I and inducible nitric oxide synthase (iNOS) gene expression. However, the combination of TNF-alpha and IFN-gamma induces a strong activation of GTPCH I mRNA, protein, and BH4 production (Peterson and Katusic 2005). Eventually, TNF-alpha acts synergistically with the Th1 type cytokine, IFN-gamma in age-related changes in both pteridine and Kyn pathways. Meanwhile, BH4 serves as an essential NOS cofactor, and Kyn catabolites, quinolinic acids, and picolinic acids transcriptionally activate iNOS. These evidences indicate that there is a connection between arginine and Trp metabolic pathways in the generation of reactive nitrogen intermediates in aging (Melillo et al. 1994). Consequently, demand for BH4 might be increased under the condition of Kyn-induced activation of iNOS triggered by IFN-gamma-induced upregulation of Kyn pathway (Oxenkrug 2007). The deficiency of BH4 results in uncoupling of NOS and shifting of arginine metabolism to the production of superoxide anion rather than nitric oxide (NO) (Pou et al. 1992).

Actually, IFN-gamma does not play a role in redox modulation of IDO activity in DCs. The cystine/glutamate antiporter controls intracellular and extracellular redox. Mattox et al. showed that the antiporter control of redox regulates IDO enzymatic activity and IDO protein levels in DCs. IDO-competent DCs arise under pathophysiologic conditions, which are characterized by imbalances in systemic redox as occurs in obesity and aging (Mattox et al. 2012). Blocking the antiporter activity exhausts intracellular glutathione and interferes with DC differentiation from monocyte precursors, thereafter significantly reducing DC presentation of exogenous antigen to T cells (D'Angelo et al. 2010).

Several intermediate products of the Kyn pathway are known to be neurotoxic. Among them, the NMDA receptor agonist and neurotoxin, QA, is likely to be most important in terms of biological activity (Stone 2001). Anthranilic acid, 3-hydroxyanthranilic acid (3-HAA), and 3-hydroxykynurenine (3-HK) have been shown to generate free radicals leading to neuronal damage similar to QA (Stone 2001). During the Trp supplementation, Trp can be used for the synthesis of serotonin, melatonin, and nicotinamide adenine dinucleotide (NAD^+) besides the Kyn production (Ruddick et al. 2006; Penberthy 2007). Moreover, under conditions of Trp depletion, supplementation with Trp downregulates enzymes directing Trp to non-NAD^+-dependent pathways. This suggests a shift of all available Trp catabolism to NAD^+ synthesis (Penberthy 2007). Kyn causes intracellular NAD^+ depletion and reduces cell viability at greater than physiological concentrations (Braidy et al. 2009). The third metabolic pathway of L-Trp degradation leads to synthesis of its major metabolite melatonin. Melatonin not only improves the antioxidant potential of the cell by stimulating the synthesis of antioxidant enzymes but also reduces free radical generation and keeps the adequate mitochondrial adenosine triphosphate (ATP) synthesis. The decline in melatonin production in aged individuals is one of the primary contributing factors for the development of age-associated neuronal damage (Pandi-Perumal et al. 2013).

6.5 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease initiated by the retention and accumulation of low-density lipoprotein (LDL) in the artery wall, leading to maladaptive responses of macrophages and T cells (Tabas et al. 2007). It could be caused by an immune reaction against autoantigens at the endothelial level, the most relevant of which are oxidized LDL and heat shock proteins (Blasi 2008). IDO suppresses T-cell activity and is upregulated by various inflammatory stimuli. IDO activity has a significant positive correlation in both sexes with carotid artery intima/media thickness as an early marker of atherosclerosis (Niinisalo et al. 2008; Pertovaara et al. 2007). Enhanced Trp degradation was reported in patients with coronary heart disease and was found to correlate with enhanced neopterin formation. In cardiovascular disease, IFN-gamma is the most important trigger for the formation and release of ROS. Chronic ROS production leads to the depletion of antioxidants. Furthermore, oxidative stress plays a major role in the atherogenesis and progression of cardiovascular disease (Schroecksnadel et al. 2006). In these patients, as traditional cardiovascular disease risk factors, IFN-gamma activity, plasma neopterin, and plasma Kyn-Trp ratio provide similar risk estimates for major coronary events and mortality (Pedersen et al. 2011). Neopterin and Kyn do not necessarily only serve as passive markers of IFN-gamma activity. Neopterin is released in parallel with its partially reduced derivative 7,8-dihydroneopterin (Fuchs et al. 2009). IFN-gamma-stimulated human macrophages generate ROS as well as neopterin and 7,8-dihydroneopterin. These pteridines may also have antioxidant effects depending on the circumstances (Herpfer et al. 2002).

The Kyn metabolite, 3-HAA, has immune regulatory properties and can inhibit Th1 and Th2 cells while increasing the amount of Tregs (Platten et al. 2005; Hayashi et al. 2007). Thus, 3-HAA modulates systemic adaptive immune responses and inhibits oxidized LDL (oxLDL) uptake in macrophages. Consequently, 3-HAA reduces local inflammation and atherosclerosis by impairing local antigen presentation and vascular infiltration of T cells (Zhang et al. 2012). However, 3-HAA, but not L-Kyn, markedly inhibits antigen-independent proliferation of CD8+ T cells induced by IL-2, IL-7, and IL-15 (Weber et al. 2006). A marked immunosuppressive effect of IDO expression is evident on human CD4+ and CD8+ T cells. Nevertheless, there is a significant difference in the suppressive effect of IDO on proliferation of CD8+ compared to that of CD4+ T cells (Forouzandeh et al. 2008). Actually, IFN-gamma is synthesized by CD4+ Th1 cells. This cytokine, a key regulator of immune function, is highly expressed in atherosclerotic lesions and has emerged as a significant factor in atherogenesis (McLaren and Ramji 2009). Neopterin is produced by human macrophages upon activation by proinflammatory stimuli like Th1-type cytokine IFN-gamma. Neopterin has prooxidative properties. Elevated neopterin concentrations are an independent marker for cardiovascular disease (Fuchs et al. 2009). Additionally, high neopterin levels also predict independently adverse prognosis in coronary artery disease patients (Grammer et al. 2009; Ray et al. 2007; Avanzas et al. 2005). Patients with hypertension and chest pain, but

without obstructive coronary artery disease and developed adverse events during follow-up period, have significantly higher neopterin levels compared with patients without events (Avanzas et al. 2004). Aging vasculature generates an excessive ROS and NO. Consequently, it facilitates the formation of the deleterious radical, peroxynitrite. Main sources of ROS are mitochondrial respiratory chain and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, although NOS uncoupling could also account for ROS generation. The redox-sensitive transcription factor, NF-kappaB, is upregulated in vascular cells and drives a proinflammatory shift (El Assar et al. 2013).

Actually, IFN-gamma regulates a number of steps during atherogenesis. Its cellular actions in human macrophages are mediated through the regulation of STAT1. IFN-gamma-induced expression of key genes implicated in atherosclerosis is extracellular signal-regulated kinase (ERK) 1/2 dependent. The ERK pathway is required for the IFN-gamma-induced activity of STAT1 and monocyte chemoattractant protein-1 promoter (Li et al. 2010). At the same time IFN-gamma is also a principal inducer of neopterin and Kyn formation. Positive correlation between circulating neopterin and Kyn-Trp ratio levels reflects IFN-gamma activity.

When macrophages are exposed to oxLDLs, increased nuclear factor erythroid 2-related factor 2 (Nrf2) expression protects the macrophages from oxLDL-mediated injury via expression of antioxidant enzymes, including catalase, glutathione peroxidase (GPx), glutathione reductase, glutathione S-transferase, and NADPH/quinone oxidoreductase 1 (Zhu et al. 2008). Circulating adipocyte fatty acid-binding protein (FABP4) levels are associated with long-term prognosis in patients with coronary heart disease and may represent an important pathophysiological mediator of atherosclerosis (Von Eynatten et al. 2012). In macrophages, FABP4 coordinates cholesterol trafficking and inflammatory responses. Nrf2 is a redox-sensitive transcription factor and provides a primary cellular defense against the oxidative stress. Akt and ERK/Nrf2-dependent FABP4 upregulation pathway in human macrophages responds to the oxidative effect of polyunsaturated fatty acids (Lázaro et al. 2013). The kelch-like ECH-associated protein (Keap1)-Nrf2-ARE (antioxidant response element) signaling pathway elicits an adaptive response for cell survival. During cell stress, Keap1 disrupts Nrf2, and Nrf2 translocates to the nucleus and upregulates genes containing an antioxidant response element in their promoter regions (Wakabayashi et al. 2010). The activation of Nrf2 suppresses IFN-gamma production while inducing the production of the Th2 cytokines IL-4, IL-5, and IL-13 (Rockwell et al. 2012). In fact the dual neuroprotective treatment with nicotinamide and an Nrf2 inducer indicates that redox environment is more important than ROS for neuron survival in aging (Ghosh et al. 2014).

Raising the bioavailability of NO in primary human endothelial cells by activating Nrf2 impairs the presence of superoxide and the subsequent formation of peroxynitrite. Eventually, active Nrf2 elicits an antioxidant response in endothelial cells and reduces endothelial NOS (eNOS) expression. BH4 levels are important to keep eNOS in the coupled and NO-producing state. Reduced BH4 levels lead to downregulated eNOS expression in an Nrf2-dependent manner. Activation of Nrf2 downregulates eNOS levels via elevation of heme oxygenase (HO-1) activity (Heiss

et al. 2009). HO-1 is important to prevent the endothelium from atherosclerosis. 3-HAA induces HO-1 expression and stimulates nuclear translocation of Nrf2 in human endothelial cells. Nrf2-dependent HO-1 expression induced by 3-HAA inhibits the monocyte chemoattractant protein (MCP)-1 secretion, vascular cell adhesion molecule (VCAM)-1 expression, and the activation of transcriptional NF-kappaB in endothelial cells. Subsequently, TNF-alpha stimulated vascular injury and inflammation is suppressed in atherosclerosis (Pae et al. 2006).

Oxidant compounds such as hydrogen peroxide (H_2O_2) have been shown to stimulate the release of arachidonic acid (AA) in a number of cell systems (Xu et al. 2003). Involvement of AA and its metabolites in the stimulation of both ERK and c-Jun-N-terminal kinase (JNK) following the oxidative stress evoked by H_2O_2 induces a cell-cycle arrest (Tournier et al. 1997). Fatty acid, AA, interferes with the transcriptional function of the IFN-gamma signaling pathway by reducing phosphorylation of STAT1. AA modulates the immunosuppressive activity of IDO by inhibiting the IFN-gamma/STAT1/IDO pathway (Bassal et al. 2012).

IDO expression is impaired in early prediabetic nonobese diabetic mouse strain. Virtually, IFN-gamma fails to induce IDO expression in cells with defective STAT1 phosphorylation in IFN-gamma-induced IDO signaling pathway of these animals (Hosseini-Tabatabaei et al. 2012).

The NF-kappaB subunits p65 and STAT1 cooperate to control iNOS gene transcription in response to proinflammatory cytokines (Burke et al. 2013). iNOS generates high concentrations of NO which is easily converted to peroxynitrite and superoxide in the prooxidant environment, a characteristic in essential hypertension. iNOS upregulates arginase activity, which limits NO production through eNOS and causes hypertension-associated endothelial dysfunction (Santhanam et al. 2007). Acute iNOS inhibition increases NO-dependent vasodilation likely through eNOS-mediated mechanisms (Smith et al. 2011).

The metabolism of arginine to NO is functionally in contrast with the metabolism of Trp to Kyn. Similar to iNOS, IDO is expressed in inflammatory conditions via IFN-gamma induction. IFN-gamma-induced endothelial IDO converts Trp to N-formylkynurenine, which decomposes spontaneously to Kyn. Kyn could directly modulate vascular tone and significantly attenuate the contractile response via activation of soluble guanylate cyclase (sGC). Eventual activation of adenylate cyclase by Kyn contributes to vessel relaxation via a cAMP-dependent pathway (Wang et al. 2010). Hence, Kyn formation within atherosclerotic arteries possibly represents a counter-regulatory protective mechanism (Niinisalo et al. 2010).

6.6 Human Hypertryptophanemia

Aging is characterized by a proinflammatory status which could contribute to the onset of major age-related diseases such as cardiovascular diseases, neurodegeneration, osteoarthritis and osteoporosis, and diabetes. In human hypertryptophanemia or in other neurodegenerative diseases, Trp accumulates in the body. Subsequent

events leading to the brain injury are involved in oxidative stress damage (Feksa et al. 2008). Actually, Trp significantly decreases the brain antioxidant defenses by reducing the values of total radical-trapping antioxidant potential, total antioxidant reactivity, and glutathione. Consequently, the overall content of antioxidant capacity of the brain is reduced by Trp. Furthermore, the Trp-induced increase of thiobarbituric acid-reactive substances is fully prevented by glutathione and by combination of catalase plus superoxide dismutase (Feksa et al. 2006). More recent studies of Trp loading have indicated that high doses of Trp cause an abnormal white blood cell accumulation in tissues (Gross et al. 1996, 1999; Ronen et al. 1999), suggesting that Trp or its metabolites are active in modulating immune system activity.

6.7 Obesity-Related Chronic Immune Activation

Obesity-related immune-mediated systemic inflammation is associated with the induction of the Trp-Kyn pathway which reflects the IDO activation. Although a markedly increased Kyn-Trp ratio is evident in adult obese subjects with metabolic syndrome, obese juveniles show contrary decrease in Kyn-Trp ratio (Mangge et al. 2014). In any case plasma Trp concentration of obese individual is reduced independent of the weight reduction and dietary intake. Because of the changes in Trp metabolism, serotonin production may decrease. Impaired satiety due to subsequent insufficient serotonin synthesis causes overfeeding and obesity (Brandacher et al. 2007). Kyn-Trp ratio and all kynurenines, except anthranilic acid, are 2–8 % higher in overweight and 3–17 % higher in obese, than in normal-weight individuals (Theofylaktopoulou et al. 2013). Bariatric surgery significantly diminishes immune mediators by substantial weight reduction. In addition to elevated levels of neopterin, Trp depletion still persists (Brandacher et al. 2006). Neopterin concentrations correlate with abdominal obesity and metabolic syndrome (MetS), which is the cause of increased mortality risk. Accordingly, neopterin concentrations also correlate with high-density lipoprotein (HDL) cholesterol, insulin resistance, and plasma pyridoxal-5′-phosphate (Oxenkrug et al. 2011). Dysregulation of Trp-Kyn and Kyn-NAD metabolic pathways plays an important role in the occurrence of insulin resistance. Thus, the key enzymes of Kyn-NAD pathway require pyridoxal-5-phosphate as a cofactor. Obesity, cardiovascular diseases, or aging associated by excessive Kyn and xanthurenic acid formation in combination with pyridoxal-5-phosphate deficiency impair the biological activity of insulin (Oxenkrug 2013). Inflammation is associated with a T-cell infiltration in obese adipose tissue, with predominance of Th17 in the omental compartment and of Treg in the subcutaneous depot. The Th17/Treg balance is decreased in subcutaneous fat and correlates with IDO1 activation. In contrast, in the omental compartment, despite IDO1 activation, the Th17/Treg balance control is impaired (Wolowczuk et al. 2012).

6.8 Conclusion

During the chronic immune activation, Trp-consuming pathways display extremely simple response to highly complex immune mechanisms. Trp depletion and Trp metabolites influence the immune response modulation and immune tolerance. The activation of IDO through IFN-gamma leads to many complex changes within the affected cells resulting in immunosuppression through breakdown of Trp. Despite the evidences, IDO is not a sole factor in chronic immune activation encountered diseases. In particular suppression of tumor-specific host immune response suggests that IDO might support the tumor progression by providing immune escape. Persistence of chronic inflammatory stimuli over time creates a biologic background for immunosenescence and favors the susceptibility to inflammatory age-related diseases. Thus, for better understanding of the mechanisms underlying the interaction between IDO and chronic immune activation-related disorders, further studies should be planned in more details.

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Chapter 7
Diabetes and Tryptophan Metabolism

Ugur Unluturk and Tomris Erbas

Abstract Tryptophan, an essential amino acid, can be metabolized to several kinds of physiologically active metabolites. Accumulating data indicate that an altered metabolism of tryptophan and its active metabolites have important roles for the pathogenesis and development of complications of diabetes mellitus. Changes in tryptophan–kynurenine and tryptophan–methoxyindole pathways are related to several pathophysiological mechanisms of type 1 or type 2 diabetes. Particularly, serotonin, melatonin, and their receptors would be novel targets not only to better understand the pathogenesis of diabetes but also to develop new antidiabetic agents.

Keywords Diabetes mellitus • Type 1 diabetes • Type 2 diabetes • Diabetic complications • Tryptophan • Tryptophan metabolism • Indoleamine 2,3-dioxygenase • Kynurenine • Serotonin • Melatonin

7.1 Introduction

In addition to the traditional knowledge, recent studies show that amino acids are also among the regulators of the phosphorylation cascade of proteins and expression of genes. Moreover, they are crucial precursors of hormones and nitrogenous substances with outstanding biological importance. Even though physiological concentrations of amino acids and their metabolites are necessary for physiological processes, their elevated levels might be pathogenic in several disorders. Several

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amino acids regulate key metabolic pathways that are necessary for homeostasis, growth, reproduction, and immunity. These amino acids are called "functional amino acids," and tryptophan is one of them (Wu 2009).

Diabetes mellitus defines a group of metabolic disorders characterized by hyperglycemia. Type 1 and type 2 diabetes are the most frequent ones. While type 1 diabetes is caused by absolute insulin deficiency, insulin resistance and relative impairment in insulin secretion lead to type 2 diabetes. In the last two decades, prevalence of type 2 diabetes mellitus has enormously increased worldwide in parallel with the increased prevalence of obesity. As a result, diabetic complications have become one of the leading causes of organ dysfunctions/losses and mortality as well as healthcare expenses. The pathogenesis of both type 1 and 2 diabetes still bears many secrets, and care of these frequent metabolic disorders needs to be improved with further treatment alternatives in order to decrease disease-related burden and mortality.

As a functional amino acid, tryptophan and altered tryptophan metabolism may have roles in diabetes mellitus pathogenesis. Additionally, the end products of tryptophan, i.e., serotonin and melatonin, as well as their receptors would be novel targets to develop new antidiabetic agents. In this chapter, we will discuss the association of diabetes with the tryptophan metabolism and its intermediary and end products under the scope of available literature.

7.2 Tryptophan Metabolism

Tryptophan is an essential amino acid and can be metabolized to several kinds of physiologically active metabolites such as L-kynurenine, L-kynurenic acid, quinolinic acid, and picolinic acid in addition to serotonin and nicotinic acid derivatives (Fig. 7.1). Tryptophan has two nonprotein metabolic pathways to produce these active substances: methoxyindole and kynurenine (Gal and Sherman 1980).

7.2.1 Tryptophan–Kynurenine Pathway

Approximately 95 % of tryptophan is metabolized by the tryptophan–kynurenine pathway. The rate-limiting enzyme of kynurenine formation from tryptophan is the indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) (Dang et al. 2000; Oxenkrug 2010a). Kynurenine is further metabolized in two different pathways, kynurenine–kynurenic acid pathway and kynurenine–nicotinamide adenine dinucleotide (NAD) pathway (Fig. 7.1). The end product of kynurenine–NAD pathway is NAD. More than 30 intermediary metabolites of this pathway, named as kynurenines, display free radical-generating properties (3-hydroxykynurenine and 3-hydroxyanthranilic acids) and may cause lipid peroxidation and activate an arachidonic acid cascade (3-hydroxykynurenine, 3-hydroxyanthranilic acids,

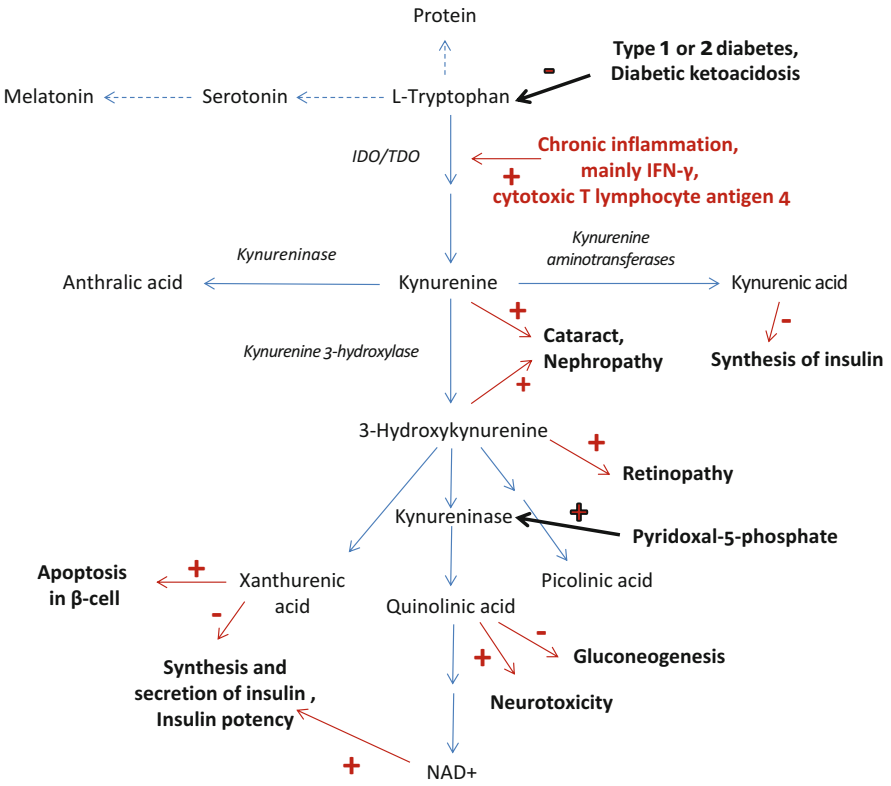

Fig. 7.1 The association of tryptophan–kynurenine pathway and its metabolites with several steps of the diabetes pathogenesis and diabetic complications. "+" sign represents stimulation and "–" sign represents inhibition

quinolinic and picolinic acids), which increases the production of inflammatory mediators (Gal and Sherman 1980; Oxenkrug 2007). Some of these mediators can induce apoptosis (kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acids) and have a neurotoxic potential (3-hydroxykynurenine) (Rongvaux et al. 2003; Schwarcz and Pellicciari 2002).

7.2.2 Tryptophan–Methoxyindole Pathway

The methoxyindole pathway leads to the generation of serotonin (5-hydroxytryptamine, 5-HT) and melatonin. The rate-limiting step of the serotonin biosynthesis is the hydroxylation of tryptophan, which is catalyzed by tryptophan hydroxylase. The availability of tryptophan as a substrate is an important rate-limiting factor for serotonin biosynthesis. This pathway metabolizes about 5 % of

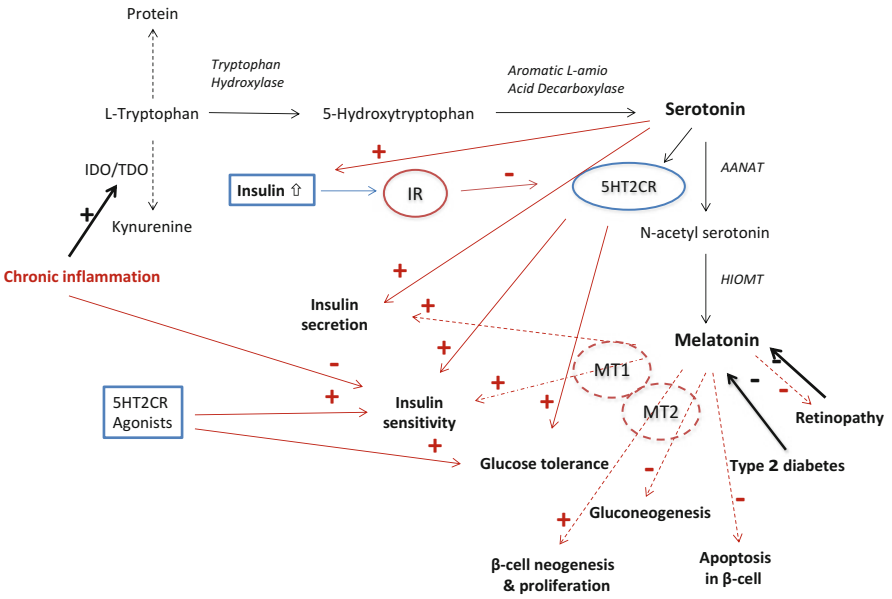

Fig. 7.2 The association of tryptophan–methoxyindole pathway and its metabolites with several steps of the diabetes pathogenesis and diabetic complications. "+" sign represents stimulation and "–" sign represents inhibition. *IR* insulin receptor, *MT1* and *MT2* melatonin receptor 1 and 2, *5-HT2CR* serotonin 5-HT2C receptor, *IDO/TDO* indoleamine 2,3-dioxygenase/tryptophan 2,3-dioxygenase, *AANAT* aralkylamine N-acetyltransferase, *HIOMT* hydroxyindole-O-methyl transferase

tryptophan (Gal and Sherman 1980). Serotonin is also a substrate for melatonin biosynthesis. After stimulation by night, activity of either aralkylamine N-acetyltransferase or serotonin N-acetyltransferase, which are the rate-limiting enzymes in melatonin synthesis, is enhanced, and they convert serotonin into N-acetyl serotonin (NAS), which is then converted to melatonin, with the additional help of hydroxyindole-O-methyl transferase, also known as acetyl serotonin N-methyltransferase (Fig. 7.2) (Claustrat et al. 2005).

7.3 Diabetes Mellitus Definition and Classification

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and/or insulin action. The vast majority of diabetic cases are classified within two etiopathogenetic categories: type 1 and type 2 diabetes mellitus. Type 1 diabetes is characterized by the absolute deficiency of insulin secretion and results from a cell-mediated autoimmune destruction of the β cells of the pancreas. Type 1 diabetes accounts for only 5–10 % of all diabetic cases. Type 2 diabetes is characterized by the combination of insulin resistance and

Table 7.1 Other rare types of diabetes mellitus

1. Genetic defects of β-cell function (MODYs, mitochondrial DNA, and others)
2. Genetic defects in insulin action (type A insulin resistance, lipoatrophic diabetes, and others)
3. Endocrinopathies (Cushing's syndrome, acromegaly, and others)
4. Diseases of the exocrine pancreas
5. Drug or chemical induced (glucocorticoids, diazoxide, and others)
6. Infections
7. Uncommon forms of immune-mediated diabetes
8. Other genetic syndromes associated with diabetes

Adapted from the Diagnosis and Classification of Diabetes Mellitus of the American Diabetes Association (2011)

an inadequate compensatory insulin secretory response (relative insulin deficiency). Type 2 diabetes accounts for 90–95 % of all diabetic cases (2011, Genuth et al. 2003). More than 2 % of pregnant women develop gestational diabetes mellitus. Pregnancy is associated with insulin resistance, caused mainly by the diabetogenic hormones secreted from the placenta (e.g., estrogen, prolactin, human chorionic somatomammotropin, cortisol, and progesterone), and gestational diabetes occurs when the insulin resistance surpasses a pregnant woman's pancreatic function. The other causes of diabetes are very rare (Table 7.1).

7.4 Diabetes Mellitus and Tryptophan Metabolism

7.4.1 Type 1 Diabetes

Type 1 diabetes is characterized by the absolute deficiency of insulin secretion and usually results from autoimmune destruction of the β cells of the pancreas. Several antibodies are detected in the course of type 1 diabetes such as islet cell antibodies (ICA); antibodies to glutamic acid decarboxylase; to insulin; to the tyrosine phosphatases, IA-2 and IA-2β; and zinc transporter ZnT8. Detecting such antibodies in serum of the patients can help us make the differential diagnosis of diabetes. Especially, a positive result for the antibody against glutamic acid decarboxylase is indicative for the immune-mediated or type 1A diabetes. In some cases of absolute insulin deficiency, there might be no evidence of autoimmunity and we may not detect any other known causes for the β-cell destruction. Such cases are called as idiopathic or type 1B diabetes mellitus.

The lifelong risk for type 1 diabetes is about 6 % in an offspring and 5 % in the siblings (Redondo et al. 1999). The risk of type 1A diabetes is associated with polymorphisms of multiple genes and is more closely associated with certain human

leukocyte antigen (HLA) alleles; however, this genetic locus accounts for less than 50 % of genetic contributions to disease susceptibility (Concannon et al. 2009). The HLA region includes the genes that encode major histocompatibility complex (MHC) class II molecules, which is expressed on the membrane of antigen-presenting cells such as macrophages (Tisch and McDevitt 1996). The antigens implicated in the pathogenesis of type 1 diabetes are bound to the MHC class II molecules and are presented to the antigen receptors of T cells, which have pivotal roles in autoimmune diseases. Amino-acid sequences of these class II molecules are important for antigen binding, and any substitutions at critical positions could increase or decrease the susceptibility to type 1 diabetes (Rowe et al. 1994). In addition to MHC genes, polymorphisms in non-MHC genes such as a promoter of the insulin gene, the protein tyrosine phosphatase gene, and the cytotoxic T-lymphocyte-associated antigen-4 are also implicated in the pathogenesis of type 1 diabetes (Davies et al. 1994; Polychronakos and Li 2011; Santin and Eizirik 2013).

The nonobese diabetic (NOD) strain of mice has become the prototypic model for autoimmune diseases and is used widely as an animal model of type 1A diabetes (Kolb 1987). This strain of mice has an autoimmune infiltration at the β-cell microenvironment (insulitis) and develops clinical diabetes around 120 days of age, mimicking the type 1 diabetes in humans. The progression to the state of clinical type 1 diabetes begins with the infiltration of the perivascular ducts and peri-islet regions of the pancreas, initially by macrophages and dendritic cells and subsequently by T and B lymphocytes (Delovitch and Singh 1997). It is accepted that Th_1 cells, which produce IFN-γ, are the central mediators of insulitis seen in NOD mice. Furthermore, anti-interferon gamma antibodies can suppress the damage in the islet cells and the initiation of insulitis seen after increases in levels of IL-12 and IL-18 (potent inducers of interferon gamma) (Rothe et al. 1997). Th_2 cells also have an ability to induce the destruction in islet cells. It is proposed that the process of inducing and sustaining type 1 diabetes is dependent on both Th_1 and Th_2 lymphocytes (Almawi et al. 1999).

Several autoantigens were also proposed to be implicated in the onset and progression of type 1 diabetes. About 85 % of type 1 diabetic patients have islet antibodies at the time of diagnosis (Atkinson and Maclaren 1994). According to the data obtained from NOD mice model, insulin/proinsulin itself is one of the primary target autoantigens, and changing a specific amino acid of insulin can prevent diabetes in them (Nakayama et al. 2005). Glutamic acid decarboxylase enzyme (GAD), which is present in islet cells, is another important autoantigen, and 70 % of newly diagnosed type 1 diabetes patients have antibodies to GAD (Baekkeskov et al. 1990). A number of additional type 1 diabetes-related islet autoantigens have also been identified (Verge et al. 1996). The data obtained from the NOD mice (Yang et al. 1997) and the patients who have X-linked agammaglobulinemia (Martin et al. 2001) suggest that the autoimmunity seen in type 1 diabetes is principally mediated by T cells, so B cells are not required for the initiation of the disease.

Triggering factors of the autoimmunity seen in type 1 diabetes have not been fully identified yet. Environmental factors such as prenatal factors, viral infections, and cow's milk are important factors in the development of type 1 diabetes.

Especially enteroviruses can cause β-cell damage. One theory is molecular mimicry, such as the homology between GAD and Coxsackie B virus (Ko et al. 1994). Self-antigens are expressed in the thymus (Pugliese et al. 1997), and tolerance to self-molecules is most likely to start at the thymus (Nitta et al. 2008). The thymocytes that express T cell receptor with an affinity toward self-molecules are exposed to negative selection during the development of immune system. The insulin gene has also thymic expression (Nitta et al. 2008) and studies show that the length of variable number of tandem repeat polymorphisms of the promoter of insulin gene controls the expression of insulin mRNA in the thymus and thus leads to the susceptibility to type 1 diabetes (Vafiadis et al. 2001).

7.4.2 Type 1 Diabetes and Tryptophan Metabolism

Accumulating data indicate an altered metabolism of tryptophan and increased levels of kynurenine pathway metabolites in diabetes (Rosen et al. 1955; Hundley et al. 1956; Auricchio et al. 1960; Hattori and Kotake 1984; Oxenkrug 2013). Tryptophan plasma levels were found to be lower in both an animal model (Masiello et al. 1987) and type 1 diabetic patients (Herrera et al. 2003; Fierabracci et al. 1996; Koenig et al. 2010).

Protein deficiency could impair immune functions, thus increasing the susceptibility to diseases. There has been a growing interest recently in the role of tryptophan on immune responses. Notably, progressive decline in plasma levels of tryptophan was observed in animals with chronic lung inflammation (Melchior et al. 2003). IDO is expressed in various tissues and the highest levels of its expression are found in immune cells. Catabolism of tryptophan via IDO appears to be pivotal for functions of both macrophages and lymphocytes (Macchiarulo et al. 2009). Especially, IDO plays a significant role in modulation of T cell-mediated immune responses (Munn et al. 1998; Baban et al. 2005; Jalili et al. 2010). Both IDO-induced tryptophan depletion and the resulting accumulation of kynurenine can contribute to the suppression of T cell-mediated immune responses. IDO is a cytokine-inducible enzyme and IFN-γ is known to be the main inducer of IDO (Yasui et al. 1986). Tumor necrosis factor (TNF)-alpha stimulates IDO activity and enhances IFN-γ-induced IDO expression (Robinson et al. 2005). IFN-γ fails to induce tolerating properties in dendritic cells from highly susceptible NOD female mice that are in early stages of diabetes. This defect is associated with defective tryptophan catabolism and is related to transient blockade of the STAT1 pathway of intracellular signaling by IFN-γ. This condition results in impaired transcriptional activation of the IDO gene and is caused by peroxynitrite production. Furthermore, the use of a peroxynitrite inhibitor may rescue tryptophan catabolism and provide tolerance in those mice (Grohmann et al. 2003). Similarly, dermal fibroblasts of diabetic NOD mice, regardless of their gender, fail to express IDO in response to IFN-γ treatment, and a defect in STAT1 phosphorylation is also shown as the potential underlying mechanism (Hosseini-Tabatabaei et al. 2012). Furthermore, local

overexpression of IDO by fibroblasts, co-transplanted with pancreatic islets, can delay immune rejection and prolongs islet allograft survival, even in the absence of any antirejection treatment (Jalili et al. 2010).

These findings showing a defect in tryptophan catabolism in dendritic cells and in diabetic fibroblasts may give hints about impaired tolerance in autoreactive T cells that affect selective destruction of the β cells in type 1 diabetes.

The activities of the enzymes in the metabolic pathway of tryptophan affect the production of tryptophan metabolites. Hepatic activity of a-amino-b-carboxymuconate-semialdehyde decarboxylase was demonstrated to be much higher in rats with type 1 diabetes (Tanabe et al. 2002). Furthermore, the amounts of urinary excreted niacin metabolites per tryptophan intake in streptozotocin-induced diabetic rats (one way to generate type 1 diabetic animal model is to use streptozotocin to destroy β cells of pancreas) were significantly less than those in the normal rats (Egashira et al. 1995). Kynurenine or quinolinic acid was found to be three times more in the hepatocytes prepared in vitro from streptozotocin-induced diabetic rats, compared with that of normal hepatocytes (Sasaki et al. 2009). Decreased kynureninase activity was also reported in diabetic rabbits with type 1 diabetes (Allegri et al. 2003).

Quinolinic acid results in neurotoxicity and this molecule was considered to be involved in the pathogenesis of neurodegenerative disorders (Heyes et al. 1991). Kynurenine and L-kynurenic acid demonstrate neuroprotective effects (Robotka et al. 2008; Rozsa et al. 2008). These metabolites also affect the immune system (Belladonna et al. 2007). Quinolinic acid, by inhibiting phosphoenolpyruvate carboxykinase, was proposed to suppress gluconeogenesis (Lelli et al. 2008). It has been suggested that type 1 diabetes mellitus augmented both kynurenine and quinolinic acid generation in the liver (Sasaki et al. 2009).

There exists experimental evidence that high levels of glucose and/or the purified Amadori albumin lead to specific oxidative modifications in tryptophan residues in lysozymes, thus inhibiting their activity (Chetyrkin et al. 2008). Chetyrkin et al. also demonstrated that pyridoxamine reduced the oxidation of tryptophan residues. Oxidized tryptophan residues are also elevated in cardiac proteins of streptozotocin-induced diabetic rats (Hamblin et al. 2007). Therefore, these results suggest that oxidative stress and tryptophan oxidation could be among the reasons responsible from the reduced serum levels of tryptophan both in animal models of type 1 diabetes and also in diabetic patients (Jain 2008).

7.4.3 Type 2 Diabetes Mellitus

Type 2 diabetes is characterized by the combination of varying degrees of insulin resistance and an inadequate compensatory insulin secretory response (relative insulin deficiency) (1997, Genuth et al. 2003). Its prevalence has increased dramatically in the last decade. Sedentary lifestyle and the resulting obesity are the main causes of this increase (Sullivan et al. 2005). The importance of insulin resistance and impaired insulin secretion in the pathogenesis of type 2 diabetes has been well

documented. Insulin resistance is the best predictor of developing type 2 diabetes. Five years before the onset of diabetes, insulin resistance is generally evident, and insulin secretion usually increases 3–4 years before the development of overt diabetes and then decreases until diagnosis (Tabak et al. 2009). Insulin resistance in type 2 diabetes is probably due to a post-receptor defect, affecting one of the intracellular enzymes involved in glucose metabolism. Genetic background of insulin resistance has been observed in a study conducted with lean normoglycemic offsprings of parents with type 2 diabetes (Rothman et al. 1995). These offsprings represented to have reduced non-oxidative glucose metabolism (indicative of insulin resistance) and muscle glycogen synthesis secondary to a defect in muscle glucose transport/hexokinase activity prior to the onset of overt diabetes. Increased muscle cell lipid content has also been observed in these subjects, which indicates a relation between the dysregulation of fatty acid metabolism and insulin resistance. Subsequent studies also showed that this dysregulation was due to an inherited defect in mitochondrial functions of skeletal muscle (Petersen et al. 2004). Studies indicate that insulin resistance alone is insufficient to cause diabetes (Lauro et al. 1998; Moller and Flier 1991). There is a close link between high-fat diet and development of diabetes. Glucose is transported into β cells to induce insulin secretion, and the glucose transporter-2 (GLUT-2) mediates this transportation. A mouse model of genetically defective GLUT-2 represented glucose intolerance, and a similar phenotype was also reported in high-fat diet-fed (lipotoxicity) wild-type mice (Ohtsubo et al. 2005). Hyperglycemia itself contributes to decrease in insulin secretion (glucotoxicity) by β cells as well (Moran et al. 1997).

Increasing knowledge about the genetic background of diabetes indicates that monogenic causes of type 2 diabetes constitute only a small fraction of cases, and most of type 2 diabetic cases have complex polygenic risk factors. Type 2 diabetes is essentially considered to represent a multipart interaction between complex polygenic inheritance and environmental factors. Observational studies clearly demonstrated a genetic influence on the development of type 2 diabetes, i.e., about 39 % of type 2 diabetics have at least one parent with the disease (Klein et al. 1996), and ethnic groups living in the same environment may have different diabetes prevalence (Carter et al. 1996). On the other hand, environmental factors also play a major role in the development of diabetes. For example, the prevalence of type 2 diabetes is much higher among Pima Indians in the United States than those in Mexico (Schulz et al. 2006). Genome-wide association analysis has also identified several diabetes susceptibility loci, such as the genes involved in pancreatic development and insulin synthesis, β-cell function, and insulin action (Sladek et al. 2007; Zeggini et al. 2008; Voight et al. 2010). Requirements for multiple abnormalities in the genes controlling insulin secretion and action may explain the non-Mendelian inheritance and the variable penetrance of type 2 diabetes. Genome-wide association studies indicate that type 1 and type 2 diabetes' genetic loci do not overlap.

Type 2 diabetic patients usually present with hypertension, dyslipidemia, and visceral obesity, which are named together as metabolic syndrome that indicates an increased cardiovascular risk (DeFronzo and Ferrannini 1991). Insulin resistance is considered to be the underlying factor for all these pathologies, and hyperinsulinemia that occurs as a result of insulin resistance leads to increases in free fatty acids in the

circulation and inflammatory cytokines from adipose tissue. Obesity causes insulin resistance and probably decreases the sensitivity of the β cells to glucose and losing weight can reverse these effects (Henry et al. 1985). An exercise regimen also improves the glucose tolerance and delays the development of overt diabetes. The exact mechanism of how obesity induces diabetes is not clearly understood. Obese patients have high plasma free fatty acid levels. Such high levels suppress insulin secretion and insulin-stimulated glucose uptake and thus constitute a risk factor for type 2 diabetes (Paolisso et al. 1995; Boden and Chen 1995; Ohtsubo et al. 2005). Low-grade inflammation is also considered as a common mediator linking obesity and type 2 diabetes (Hotamisligil 2006). Increased levels of inflammatory markers such as TNF-alpha, IL-6, and plasminogen activator inhibitor-1 are correlated with incidence of type 2 diabetes (de Rekeneire et al. 2006). Adipose tissue secretes numerous adipokines that can induce low-grade inflammatory activity as well. Leptin, expressed primarily in adipocytes, is a major adipokine, and its levels are in proportion to the adipocyte mass (Zhang et al. 1994; Weigle et al. 1997; Gautron and Elmquist 2011). Leptin is a hormone made and released by the adipose tissue and regulates metabolism, energy intake/expenditure, and behavior through leptin receptors located in the central nervous system (Friedman and Halaas 1998). Leptin gives a signal to the hypothalamus about fat storage status. It is well demonstrated that leptin deficiency and/or resistance is associated with obesity and type 2 diabetes. The adipoinsular axis has long been proposed as the link between the adipose tissue and the β cells through the insulin and leptin interaction (Kieffer and Habener 2000). Effects of leptin on β-cell mass and functions according to the presence of insulin resistance were also reported in an animal study (Morioka et al. 2007). Another important adipokine in the pathogenesis of obesity and diabetes is adiponectin. Numerous studies revealed that low adiponectin levels were associated with obesity, the development of insulin resistance, and subsequent type 2 diabetes (Kadowaki et al. 2006). Studies with animal models of obesity revealed that increased release of TNF-alpha from adipose tissue leads to insulin resistance (Hotamisligil et al. 1993; Uysal et al. 1997).

7.4.4 Type 2 Diabetes and Tryptophan Metabolism

7.4.4.1 Type 2 Diabetes and Kynurenine Pathway

A common feature of insulin resistance-related disorders, e.g., metabolic syndrome, type 2 diabetes, and obesity, is the low-grade chronic inflammation. Chronic inflammation may be one of the mechanisms that trigger IDO (Oxenkrug 2013). Tryptophan metabolism shifts from serotonin synthesis to formation of kynurenine metabolites when IDO is activated (Fig. 7.2).

Plasma tryptophan levels are decreased in obese rats (Finkelstein et al. 1982) and in obese patients (Brandacher et al. 2007). It was demonstrated that after bariatric surgery in morbidly obese patients, preoperative high kynurenine/tryptophan ratio

(as an index of IDO activity) did not decrease (Brandacher et al. 2006). Recently, serum kynurenine/tryptophan ratio and IDO expression in the omental and subcutaneous adipose tissues and also in the liver were shown to be high in obese women (Wolowczuk et al. 2012).

It was reported that xanthurenic acid induced experimental diabetes in rats (Kotake et al. 1975). Several mechanisms have been proposed for the effects of xanthurenic acid (reviewed in (Oxenkrug 2013)), such as decreasing the effects of insulin (Kotake et al. 1975) or insulin release (Rogers and Evangelista 1985), direct toxic effects (Meyramov et al. 1998), or induction of apoptosis in pancreatic islets (Malina et al. 2001). Xanthurenic acid, kynurenic acid, and their derivatives, quinaldic acid and 8-hydroxyquinaldic acid, inhibit proinsulin synthesis in isolated rat pancreatic islets (Noto and Okamoto 1978). In a study with a small sample size, an increased urine excretion of xanthurenic acid was reported in type 2 diabetic patients (Hattori and Kotake 1984). A recent metabolomics study showed an increased level of kynurenic acid in the urine of nonhuman primates with type 2 diabetes mellitus (Patterson et al. 2011).

Kynurenine pathway needs pyridoxal-5-phosphate as a cofactor, which is an active form of vitamin B6, for NAD^+ synthesis (van de Kamp and Smolen 1995). Pyridoxal-5-phosphate deficiency causes downregulation of kynureninase and leads to a shift in tryptophan metabolism from the formation of NAD to excessive production of xanthurenic acid (Bender et al. 1990). Decreased formation of NAD leads to the inhibition of synthesis and secretion of insulin as well as the death of pancreatic β cells (Okamoto 2003). It was shown that vitamin B6 decreased insulin levels and improved insulin sensitivity in a dose-dependent manner, in an animal model of type 2 diabetes (Murakoshi et al. 2009).

Most of the chronic hepatitis C virus (HCV) infections are associated with insulin resistance and the molecular mechanisms concerning this condition are still under debate (Romero-Gomez 2006). An activated tryptophan–kynurenine metabolism (IDO activation) was previously reported in patients with HCV infection (Larrea et al. 2007). In a recent study, significant correlations were reported between insulin resistance/insulin secretion and tryptophan as well as kynurenine concentrations (Oxenkrug et al. 2013).

7.4.4.2 Type 2 Diabetes and Methoxyindole Pathway

7.4.4.2.1 Type 2 Diabetes and Serotonin

Two different tryptophan hydroxylase enzymes catalyze serotonin synthesis according to central or peripheral serotonin needs. While tryptophan hydroxylase-1 is used at peripheral tissues, tryptophan hydroxylase-2 acts at the central nervous system (CNS) (Sakowski et al. 2006). The monoamine-signaling molecule, serotonin, cannot cross the blood–brain barrier, and the synthesis of serotonin depends on the presence of circulatory tryptophan (Fernstrom 2013). Tryptophan-free diet leads to a rapid decrease in brain serotonin (Reilly et al. 1997). Serotonin is involved in the

maintenance of energy balance through regulating many behavioral and physiological processes (Tecott 2007). The suppression of food intake is the predominant global effect of CNS serotonin signaling. Serotonin in CNS is synthesized at the raphe nuclei. Administration of serotonin or its precursor 5-hydroxytryptophan into CNS causes a decrease in foot intake and increases the metabolic rate (Yamada et al. 2006). At least 18 receptors of serotonin have been identified (Marston et al. 2011). In hypothalamus, the 5-HT2CR receptor of serotonin is the predominantly expressed type (Yadav et al. 2009), and serotonin exerts its anorectic action through this receptor (Nonogaki et al. 1998).

Insulin has also an anorexigenic effect in CNS and its receptors are co-localized with serotonin receptors in the hypothalamus (Bruning et al. 2000). There is an interaction between the insulin and serotonin systems. The function of 5-HT2CR may be inhibited by insulin receptor activation in cells expressing both of these receptors (Hurley et al. 2003). Systemic administration of 5-HT2CR agonists reduces elevated serum insulin levels and improves glucose tolerance and insulin sensitivity in mice with insulin resistance (Zhou et al. 2007). These effects were achieved at doses that did not show any impact on food intake or body weight. These data were further supported by the finding that genetic inactivation of the 5-HT2CR in mice impaired glucose homeostasis (Wade et al. 2008). Administering fenfluramine, a drug that increases synaptic serotonin concentrations by inducing vesicular release and inhibiting reuptake, into the hypothalamus leads to increases in hypothalamic extracellular insulin levels (Orosco et al. 2000). Systemic dexfenfluramine treatment was also reported to increase serum insulin levels (Papazoglou et al. 2012). Furthermore, increased serum insulin levels and pancreatic islet cell density (indicating increased insulin production) were reported in the serotonin reuptake transporter-deficient mice (Chen et al. 2012). These findings suggest that the 5-HT2CR may be a novel target for the treatment of type 2 diabetes.

Serotonin in circulation has complex effects on peripheral glucose regulation. While serotonin induces hyperglycemia, likely by inhibiting glucose uptake, it also leads to hyperinsulinemia, presumably by stimulating pancreatic β cells (Hajduch et al. 1999; Moore et al. 2005). Serotonin is also synthesized and stored in secretory β granules within β cells (Ekholm et al. 1971; Richmond et al. 1996). The concept of "microserotonergic systems" in peripheral tissues has emerged with the recent advances in intracellular serotonin functions (Paulmann et al. 2009), which at least partially underlie the regulation of insulin release by serotonin. Serotonin is concomitantly released with insulin during the stimulation of β cells by glucose (Smith et al. 1999). Studies on tryptophan hydroxylase-1$^{-/-}$ mice (lack of peripheral serotonin) identified the "serotonylation" of small GTPases as a 5-HT receptor-independent, intracellular signaling mechanism of monoamines (Walther et al. 2003). Intracellular Ca^{+2} mobilization and monoamine accumulation in the cytoplasm together trigger vesicular exocytosis through activating covalent binding of serotonin to GTPases, in a reaction that is conferred by the Ca^{+2}-dependent transglutaminases (TGases) (Walther et al. 2003). TGase2$^{-/-}$ mice are glucose intolerant (Bernassola et al. 2002). It was shown that intracellular serotonin regulates insulin secretion in peripheral tryptophan hydroxylase-1$^{-/-}$ mice. Due to the lack of

serotonin in the pancreas, these mice are diabetic and have an impaired insulin secretion. The damaged insulin secretion was also reversed by pharmacological restoration of the peripheral serotonin levels in vivo (Paulmann et al. 2009). In summary, serotonin primarily acts not only as an intercellular signaling molecule but also as an intracellular agent by regulating the activity of target proteins through covalent coupling.

7.4.4.2.2 Type 2 Diabetes and Melatonin

The methoxyindole pathway of tryptophan results in the generation of serotonin and then the synthesis of melatonin. In mammals, melatonin is mainly derived from pineal body and peaks at night in response to light information received through retinohypothalamic pathway and from the suprachiasmatic nucleus in the hypothalamus where the circadian clock is located. This enables the synchronization of the phases of the circadian clock with the light–dark cycle. This pathway is stimulated during the night and inhibited by light stimulation. Along with clock and calendar functions, melatonin has an antioxidant action and is a biological modulator of mood, sleep, sexual behavior, and circadian rhythm (Singh and Jadhav 2014). The suprachiasmatic nucleus is the master oscillator of the light–dark cycle. It controls the release of melatonin and adrenal glucocorticoids and transmits information to other systems to control the sleep–wake cycle, cardiovascular system activity, endocrine behavior, and dietary activity. Any deficiency in melatonin or in melatonin precursors leads to impaired circadian rhythms, depressed mood, and sleep disorders (Oxenkrug 2010b; Oxenkrug and Requintina 2003). Accumulating data indicate that disrupted circadian system is associated with metabolic syndrome, including type 2 diabetes and obesity (Pulimeno et al. 2013).

Melatonin has especially an important role in the regulation of glucose metabolism. Experimentally induced desynchronization of sleep–wake cycles has been reported to significantly contribute to higher fasting plasma glucose levels (Scheer et al. 2009). The removal of the pineal gland, under basal conditions, leads to higher glucose and glucagon levels and lower insulin levels in rats (Diaz and Blazquez 1986; Rodriguez et al. 1989). In type 2 diabetic rats, pinealectomy resulted in increased insulin resistance and accelerated disease progression (Scheer et al. 2009; Nishida et al. 2003). These findings indicate a potential direct role of melatonin on pancreatic functions in rats. Pinealectomized rats also displayed hepatic insulin resistance, increased gluconeogenesis, and phosphoenolpyruvate carboxykinase (PEPCK) expression at the end of the nocturnal feeding period (Kosa et al. 2001). In the early hours of the morning, in parallel to decreased levels of circulating melatonin, increased gluconeogenesis and hyperglycemia have also been observed in type 2 diabetic patients (Radziuk and Pye 2006). A disrupted rhythm of insulin secretion was also reported in the isolated islets of pinealectomized rats (Picinato et al. 2002). These data indicate that melatonin is involved in the control of hepatic gluconeogenesis and acts as a synchronizer of biological rhythms associated with the development of type 2 diabetes.

Melatonin synthesis has been reported to be decreased in a type 2 diabetic rat model (Peschke et al. 2006). An improvement in glucose metabolism after treatment with melatonin was also demonstrated in an insulin-resistant mouse model (Sartori et al. 2009). Whereas administering melatonin or insulin alone provided a limited protection against hyperglycemia-induced oxidative damage in diabetic rats, combined treatment prevented the oxidative damage and ameliorated hyperglycemia (Sartori et al. 2009). Several studies on diabetic rats have demonstrated a protective role of melatonin on the preservation of the pancreatic β cell, such as inducing β-cell neogenesis, proliferation, and also prevention from apoptosis (Karamitri et al. 2013). Melatonin receptors have two subtypes (MT1 and MT2) (Pandi-Perumal et al. 2008), which also exist in pancreatic islet cells. Removing the MT1 receptor of melatonin leads to impaired glucose metabolism and increased insulin resistance (Bazwinsky-Wutschke et al. 2014).

Human studies concentrating on the association of melatonin with diabetes are very limited in number. Nocturnal levels of melatonin were observed to be decreased in type 2 diabetic patients (Tutuncu et al. 2005; Peschke et al. 2006). Recently, lower melatonin secretion was reported to be independently associated with a higher risk of developing type 2 diabetes (McMullan et al. 2013). In addition, several polymorphisms linked to the MTNR1B gene, which encodes the melatonin MT2 receptor, have been identified as a risk factor for developing type 2 diabetes (Bonnefond et al. 2012).

Nevertheless, it is not yet known whether melatonin has positive effects on metabolic parameters in type 2 diabetic patients and diabetic complications, as observed in animal studies.

7.4.5 Diabetic Complications and Tryptophan Metabolism

Diabetic complications are mainly categorized as acute and chronic. Acute complications of diabetes include diabetic ketoacidosis, hyperosmolar hyperglycemic nonketotic coma, and hypoglycemia. Chronic complications are specifically classified as either microvascular (i.e., neuropathy, retinopathy, and nephropathy) or macrovascular (i.e., cerebrovascular, cardiovascular, and peripheral vascular disease). Chronic hyperglycemia is central to the pathophysiology of chronic complications and leads to long-term damage, dysfunction, and failure in different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. An altered metabolism of tryptophan and its active metabolites have also been implicated in most of diabetic complications (Carl et al. 2002; Raju et al. 2007; Zarnowski et al. 2007; Kanth et al. 2009; Pawlak et al. 2009; Munipally et al. 2011; Hirayama et al. 2012).

Kynurenine pathway metabolites are present in the human eye (Van Heyningen 1971). Kynurenine and 3-hydroxykynurenine derivatives are found in the lens and they absorb UV radiation; therefore, they may have roles in protecting the retina and lens from UV light (Chiarugi et al. 1999). Accumulation of kynurenine was reported in cataractous lenses of animals with diabetes (Raju et al. 2007; Zarnowski et al.

2007) and also in senile nuclear human cataracts (Zarnowski et al. 2007). On the other hand, nuclear cataract contains extensive oxidation of proteins and 3-hydroxykynurenine which oxidize lens crystalline and cause cataract formation (Aquilina et al. 1997). Glycated lens proteins can produce reactive oxygen radicals. By this way, they can oxidize tryptophan into kynurenine. IDO is expressed in the human lens and it was reported that IDO was induced in type 1 diabetic cataractous lenses (Kanth et al. 2009). IDO-mediated oxidation of tryptophan may also lead to increases in kynurenine and kynurenine metabolites in the lenses of diabetics.

A recent study showed increased levels of xanthurenic acid precursors, kynurenine, and 3-hydroxykynurenine as well as elevated expression of IDO in serum samples of patients with diabetic retinopathy (Munipally et al. 2011). These results indicate that IDO enzyme and tryptophan–kynurenine pathway metabolites might be involved in the diabetic retinopathy pathogenesis by the mechanism of oxidative stress. 3-Hydroxykynurenine has a role in producing H_2O_2 (Eastman and Guilarte 1990), which could be a cause of oxidative damage seen in diabetic retinopathy (Munipally et al. 2011). In addition, it was reported that nocturnal melatonin levels in type 2 diabetic patients with proliferative diabetic retinopathy were significantly lower than those in type 2 diabetic patients without proliferative retinopathy (Hikichi et al. 2011). Upon this result, the authors proposed that decreased retinal light perception might be linked to low levels of melatonin, which could also accelerate the diabetic complications. Furthermore, the protective role of melatonin on retina has been recently shown both in diabetic rats (Li et al. 2013) and in an experimental animal model of retinopathy associated with type 2 diabetes (Salido et al. 2013). Decreased levels of melatonin were also reported in type 2 diabetic patients with cardiac autonomic neuropathy (Tutuncu et al. 2005).

The kynurenine pathway tryptophan metabolites are also associated with inflammation and oxidative stress markers, as well as cardiovascular diseases in patients with end-stage renal disease, including diabetics (Pawlak et al. 2009). Activation of the kynurenine pathway and its enzymatic activity could be related to increased inflammatory reactions seen in uremic patients. Higher levels of kynurenine were reported in hemodialysis patients with type 1 or 2 diabetes (Koenig et al. 2010).

As an index of IDO activity, the ratio of kynurenine to tryptophan is frequently used in clinical studies (Oxenkrug 2010a). A significant proportion of coronary heart disease patients had a higher kynurenine/tryptophan ratio (Wirleitner et al. 2003). Furthermore, it was reported that IDO activity was correlated with atherosclerosis risk factors, such as age, carotid artery intima/media thickness, low-density lipoprotein, body mass index, and C-reactive protein (Pertovaara et al. 2007; Niinisalo et al. 2008). A recent metabolomics study reported higher kynurenine levels in diabetic patients with nephropathy compared with those without nephropathy (Hirayama et al. 2012).

Diabetic ketoacidosis is an acute and life-threatening complication of diabetes and is associated with insulin deficiency. Low levels of tryptophan were reported in patients with diabetic ketoacidosis and this depletion was proposed to be a possible predisposing factor for affective disorders secondary to the neurotransmitter

imbalances seen in diabetic patients (Carl et al. 2002). Several studies in animal models evaluating the serotonergic activity of brain in type 1 diabetes have been published (Crandall et al. 1981; Trulson et al. 1986; Lackovic and Salkovic 1990; Yang and Lin 1995; Herrera et al. 2003, 2005). The level of tryptophan, the rate of serotonin synthesis, and the activity of tryptophan–hydroxylase have been reported to be decreased in the brains of animals with type 1 diabetes (Crandall et al. 1981; Trulson et al. 1986; Herrera et al. 2005). These results indicate a diminished activity of the serotonergic system. Tryptophan–hydroxylase demonstrates an unsaturated enzyme activity with respect to tryptophan. Therefore, a decrease in available tryptophan could be one of the causes of the reduced activity of the metabolic pathway of tryptophan in the brain, as seen in animal models of type 1 diabetes. There could be other intrinsic factors that lead to the sustained tryptophan–hydroxylase activity. In relation to this, a study reported a significantly decreased affinity of tryptophan–hydroxylase for tryptophan and lower rates of enzyme activity in both the cortex and the brainstem of rats with type 1 diabetes, compared to the healthy controls (Herrera et al. 2005). These changes could be related to the epigenetic modifications of functional protein systems after developing a diabetic condition and might affect the pathophysiology of the psychoneurological complications of diabetes.

7.5 Conclusions

There is growing evidence about the role of tryptophan metabolism in the pathogenesis of diabetes and its complications (summarized in Figs. 7.1 and 7.2). While most of the studies were conducted with animal models of type 1 diabetes, there exist a limited number of human studies about tryptophan metabolism. Accumulating literature suggests that tryptophan metabolism is upregulated during diabetes and affects several systems, such as β-cell secretory capacity; insulin sensitivity; development of microvascular, macrovascular, and psychoneurological complications of diabetes; as well as immune responses. Recent data suggest that the altered tryptophan metabolism, serotonin, melatonin, and their receptors in diabetes would be novel targets to understand the secrets of the pathogenesis of diabetes and related complications as well as to develop new antidiabetic agents. However, there is a huge need for further studies in humans in order to support the existing data obtained from animal models. Encouraging results of the animal studies may accelerate the conduct of human trials.

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Chapter 8
3-Hydroxykynurenic Acid and Type 2 Diabetes: Implications for Aging, Obesity, Depression, Parkinson's Disease, and Schizophrenia

Gregory Oxenkrug

Abstract Aging, obesity, depression, Parkinson's and other neurodegenerative diseases, schizophrenia, and treatment with antipsychotic drugs are highly associated with insulin resistance (IR) and type 2 diabetes mellitus (T2D). Molecular mechanisms of increased associations remain uncertain. Current review of literature and our data suggest that one such mechanism is the overproduction of diabetogenic factors resulting from dysregulation of upstream and downstream pathways of tryptophan (TRP)–kynurenine (KYN) metabolism. Proinflammatory factors and stress hormones activate two upstream steps of TRP–KYN pathway: TRP conversion into KYN, a substrate for formation of kynurenic acid (KYNA), and KYN conversion into 3-hydroxyKYN (3-HK). The first step of downstream pathway of 3-HK metabolism, formation of 3-hydroxyanthranilic acid (3-HAA), is catalyzed by pyridoxal-5-phosphate (P5P)-dependent kynureninase (KYNase). P5P deficiency, associated with inflammation, stress, and treatment with antipsychotic drugs, diverts metabolism of overproduced 3-HK from formation of 3-HAA to the excessive formation of 3-hydroxykynurenic acid (3H-KYNA). Human and experimental studies suggested diabetogenic (e.g., impairment of production, release, and biological activity of insulin) effect of KYN, 3H-KYNA, KYNA, and their metabolites. We propose that one of the mechanisms of increased association of IR (and T2D) with aging, obesity, depression, neurodegenerative diseases, schizophrenia, and treatment with antipsychotic drugs is overproduction of 3H-KYNA resulting from upregulated formation of 3-HK augmented by P5P deficiency. Pharmacological regulation of up- and downstream TRP–KYN metabolic pathways might be a new approach for prevention and treatment of IR (and IR progression to T2D) associated with aging, obesity, depression, neurodegenerative diseases, schizophrenia, and treatment with antipsychotic drugs.

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8.1 Introduction

Aging, obesity, depression (including induced by antiviral treatment of HCV), Parkinson's disease, schizophrenia and treatment with antipsychotic drugs, and neurodegenerative disorders (e.g., vascular, Alzheimer's, and HIV-1-associated dementias and Huntington disease) are chronic inflammation-/stress-related conditions that are highly associated with insulin resistance (IR) and type 2 diabetes (T2D). While IR and T2D might increase the risk of inflammation/stress-related conditions, the reverse causality is possible, i.e., that these conditions increase the risk of developing IR and T2D. Alternatively, these conditions might facilitate progression from prediabetes to T2D.

We suggest that one of the mechanisms of high association between chronic inflammation/stress-related conditions and IR (T2D) is dysregulation of tryptophan (TRP)–kynurenine (KYN)–NAD+ metabolism (Oxenkrug 2013).

8.1.1 Upstream KYN Metabolic Pathways

TRP is an essential (for humans) amino acid. About 5 % of nonprotein route of TRP metabolism is utilized for the formation of methoxyindoles: serotonin, N-acetylserotonin, and melatonin (Oxenkrug 2007). The major nonprotein route of TRP metabolism is formation of KYN (via production of N-formyl-KYN), catalyzed by rate-limiting enzymes: either inflammation-inducible indoleamine 2,3-dioxygenase (IDO) (Murray 2001) or stress-inducible TRP 2,3-dioxygenase (TDO) (Fig. 8.1) (Schwarcz et al. 2012).

8.1.2 Downstream KYN Metabolic Pathways

KYN is metabolized into kynurenic acid (KYNA), by pyridoxal 5′-phosphate (P5P)-dependent KYN-aminotransferases I, II, and III (KATs), or oxidated into 3-hydroxyKYN (3-HK), by flavin adenine dinucleotide-dependent KYN 3-monooxygenase (KMO) (Amori et al. 2009). 3-HK is a substrate for two competitive pyridoxal-5-phosphate (P5P)-dependent pathways: formation of 3-hydroxyanthranilic acid (3-HAA) (along the NAD+ biosynthetic pathway) catalyzed by kynureninase (KYNase) and formation of 3-hydroxy-KYNA (3H-KYNA) (also known as xanthurenic acid) catalyzed by HK-transaminase (HKT) (Fig. 8.1). It is noteworthy that insects' HKT has sequence identity with mammalian alanine aminotransferases (Han et al. 2002) known to be elevated in T2D (Deboer et al. 2013).

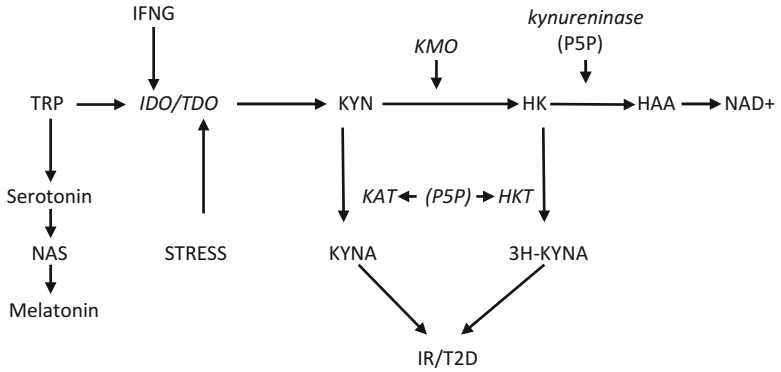

Fig. 8.1 KYN metabolic pathways and type 2 diabetes. Abbreviations: *TRP* tryptophan, *IFNG* interferon-gamma, *IDO* indoleamine 2,3-dioxygenase, *TDO* TRP 2,3-dioxygenase, *KYN* kynurenine, *KAT* KYN-aminotransferase, *KYNA* kynurenic acid, *KMO* KYN-3-monooxygenase, *HK* 3-hydroxyKYN, *HKT* HK-transaminase, *3H-KYNA* 3-hydroxyKYNA (xanthurenic acid), *P5P* pyridoxal 5′-phosphate, *HAA* 3-hydroxyanthranilic acid, *NAD+* nicotinamide adenine dinucleotide, *IR* insulin resistance, *T2D* type 2 diabetes, *NAS* N-acetylserotonin

8.2 Regulation of Upstream KYN Metabolic Pathways

8.2.1 Inflammation

Proinflammatory factors, in particular, Th1-type cytokine interferon-gamma (IFNG), activate IDO, catalyzing conversion of TRP into KYN (precursor of KYNA and 3-HK) (Taylor and Feng 1991), and KMO, catalyzing conversion of KYN into 3-HK (precursor of 3-HAA and 3H-KYNA) (Alberati-Giani et al. 1996). The effect of IFNG on IDO activity is affected by polymorphic gene that encodes IDO production (Smith et al. 2012).

8.2.2 Stress

Stress upregulates TDO (Oxenkrug 2007; Schwarcz et al. 2012; Su et al. 2011) and KMO activities. Thus, electric foot shock elevates rat brain levels of KYN, 3-HK, and KYNA (Pawlak et al. 2000).

It is noteworthy that overproduction of 3H-KYNA metabolic precursors, KYN and 3-HK, may not be sufficient to increase formation of 3H-KYNA. Thus, mouse lung infection, resulted in a 100-fold induction of IDO, was accompanied by a 16- and threefold increase of KYN and 3-HK levels, respectively, without increase of 3H-KYNA (Christen et al. 1990). In the same vein, chronic administration of IFNG increased the release of KYN and KYNA but not of 3H-KYNA from cultured rat astrocytes (Fukuyama et al. 2014).

However, upregulation of 3-HK production may be a prerequisite to P5P-induced shift of 3-HK metabolism from formation of HAA toward overproduction of 3H-KYNA.

8.3 Regulation of Downstream KYN Metabolic Pathways

Recent reviews indicated that low levels of plasma P5P are associated with a variety of inflammatory disease conditions independent of dietary intake of vitamin B6, excessive catabolism of the vitamin B6, or congenital defects in its metabolism (Paul et al. 2013; Ueland and Selhub 2012). Authors suggested that the inverse association between plasma P5P and inflammation may be the result of mobilization of P5P for use in downstream metabolism of KYN overproduced in response to inflammation-induced upregulation of IDO, i.e., a passive consequence of P5P pivotal role as a coenzyme for the key enzymes of KYN metabolism: KATs, HKT, and KYNase. However, continuous decline of plasma P5P might, in its turn, affect downstream metabolism of KYN. Because KYNase is more sensitive to P5P deficiency than other P5P-dependent enzymes of KYN metabolism (Kamp and Smollen 1995), P5P deficiency results in inhibition of KYNase and downregulation of conversion of overproduced 3-HK into HAA. Overproduced 3-HK, therefore, became available as a substrate for formation of an excessive amount of 3H-KYNA (Allegri et al. 2003; Ogasawara et al. 1962).

Recent study found that impact of P5P deficiency on stress/substrate-inducible TDO-driven KYN metabolism is dose dependent: moderate deficiency yielded increased 3-HK and decreased KYNA formation, while more severe P5P deficiency yielded an additional increase in 3H-KYNA and KYN (Rios-Avila et al. 2013).

The other consequence of P5P deficiency-induced downregulation of *KYNase* is the decreased formation of NAD+ that leads to inhibition of synthesis and secretion of insulin and the death of pancreatic beta cells (Okamoto 2003). Considering that NAD inhibits TDO (Cho-Chung and Pitot 1967), decreased formation of NAD+ caused by P5P deficiency might lead to further activation of TDO and increased production of KYN.

Besides P5P deficiency, *KYNase* might be inhibited by 3H-KYNA, thus sustaining the accumulation of HK, KYNA, KYN, and 3H-KYNA at the expense of NAD+ production (Shibata et al. 1996). Additionally, 3H-KYNA might perpetuate P5P deficiency by inhibiting pyridoxal kinase, the enzyme catalyzing the formation of P5P from vitamin B 6 (Takeuchi et al. 1985)

Clinical and experimental studies indicate that P5P deficiency combined with upregulated TRP conversion to KYN leads to increased availability of 3-HK as substrate for formation of 3KYNA and 8-HQ and increased availability of KYN as substrate for KYNA and QA in the cerebellum, corpus striatum, frontal cortex, and pons/medulla (Guilarte and Wagner 1987), blood (Midttun et al. 2011; Ciorba 2013) and pancreatic islets (Rogers and Evangelista 1985).

Vitamin B6 depletion drastically increased while vitamin B6 supplementation normalized urinary HK and 3-HKYNA excretion after TRP load in cardiac (Rudzite et al. 2003) and obese patients (Yess et al. 1964) and rats (Okamoto 2003). In addition, vitamin B6 dose-dependently decreased insulin levels and improved insulin sensitivity in the KK-A(y) mice, animal model of type 2 diabetes (Murakoshi et al. 2009; Unoki-Kubota et al. 2010).

Therefore, inflammation or stress augmented by P5P deficiency might result in overproduction of KYN, KYNA precursor, and 3-HK, precursor of 3H-KYNA.

8.4 Diabetogenic Effects of KYN Derivatives

8.4.1 KYNA

KYNA is an endogenous broad-spectrum antagonist at all subtypes of ionotropic glutamate receptors that preferentially activate at the strychnine-insensitive glycine allosteric site of the N-methyl-D-aspartate (NMDA) receptor and a noncompetitive antagonist at the alpha7 nicotinic receptor (Schwarcz et al. 2012; Turski et al. 2013). Increased urine excretion of KYNA was found in nonhuman primate and mouse models of T2D in a recent metabolomic study (Patterson et al. 2011). KYNA (in micromolar concentrations) was detected in pancreatic juice of pigs (Kuc et al. 2008). The possible mechanisms of diabetogenic effect of KYNA might be related to the KYNA's ability to block NMDA receptors. Thus, NMDA antagonist and pharmacological precursor of KYNA, 7-KYNA, and NMDA antagonist, MK-801, negated the inhibition of glucose production induced by NMDA agonists injected into dorsal vagal complex in rodents (Lam et al. 2010).

8.4.2 3H-KYNA

3H-KYNA was discovered (and designated as xanthurenic acid) in 1943 in urine of vitamin B6 deficient rats after administration of TRP (Lepkovsky et al. 1943). Apart from a specific role for 3H-KYNA in the signaling cascade resulting in gamete maturation in mosquitoes (Billker et al. 1998), nothing was known about its functions in other species including mammals, except it pro-convulsive effect (Lapin 1978). 3H-KYNA was reported to induce experimental diabetes in rats (Kotake et al. 1975), and its elevated urinary excretion was found in alloxan- and streptozotocin-induced diabetic rats (Okamoto 2003; Ikeda and Kotake 1986; Hattori et al. 1984; Connick and Stone 1985), while decreased activity of P5P-dependent KYNase was observed in alloxan-induced diabetic rabbits (Allegri et al. 2003). Increased urine excretion of 3H-KYNA was found in diabetic patients (Kotake et al. 1975) and in subjects with prediabetes (Manusadzhian et al. 1974). It

is noteworthy that insulin dose dependently decreases urine excretion of 3H-KYNA (and increases excretion of KYNA) in rats after TRP load (Kotake et al. 1975). Metabolomic study revealed high association between alloxan and 3-HK in rats fed with high caloric diet (Gu et al. 2007). The possible mechanisms mediating 3H-KYNA contribution to development of diabetes might be (a) induction of pathological apoptosis of pancreatic beta cells through 3H-KYNA-induced activation of caspase 3 (Malina et al. 2001), (b) formation of 3H-KYNA-Zn++ complexes in beta cells that exert a toxic effect in isolated pancreatic islets (Meyramov et al. 1984), (c) inhibition of proinsulin synthesis in isolated rat pancreatic islets (Noto and Okamoto 1978), (d) inhibition of insulin release from pancreas observed in the rat (Rogers and Evangelista 1985), and (e) formation of chelate complexes with insulin that are indistinguishable from insulin as antigens but have 49 % lower activity than pure insulin (Ikeda and Kotake 1986). In addition, 3H-KYNA, KYNA, and their derivatives, quinaldic acid (QA) (Takahashi et al. 1956) and 8-hydroxyquinaldic acid (8-HQ) (Takahashi and Price 1956) (in millimolar concentrations, i.e., much higher than their micromolar concentrations in pig's pancreatic juice), inhibited proinsulin synthesis in isolated rat pancreatic islets (Noto and Okamoto 1978).

8.5 Clinical Markers of Upregulation of TRP–KYN Pathway

Rate of TRP conversion into KYN might be assessed by evaluation of ratio between substrate (TRP) and end product (KYN) of enzymatic reaction catalyzed by IDO or TDO. Serum (or plasma) KYN to TRP ratio (KTR) is a generally accepted clinical marker of IDO activity (Midttun et al. 2011). However, considering that both IDO and TDO regulate the rate of TRP conversion into KYN, serum concentrations of TRP and KYN might be affected by the activity of stress hormone-inducible TDO as well. Since IDO and TDO do not activate concurrently (Takikawa et al. 1986), assessment of inflammation factors might help to decide whether increased KTR is triggered by activation of IDO or TDO. IFNG, the most powerful inducer of IDO, is produced by microglia and macrophages and, after release into the circulation, is rapidly neutralized by soluble receptors or binds to target structures. Therefore, the half-life of circulating IFNG is short, and its activity cannot be reliably evaluated by systemic measurements, e.g., IFNG concentrations in blood (Fuchs et al. 2009). The more reliable method to differentiate between IFNG and stress-induced activation of TRP conversion into KYN is evaluation of IFNG-induced marker of inflammation, neopterin. Concurrently with IDO, IFNG transcriptionally induces the rate-limiting enzyme of pteridine biosynthesis, guanosine triphosphate cyclohydrolase 1 (GTPCH) (Schoedon et al. 1986; Fuchs 2002; Neurauter et al. 2008). In humans, IFNG-induced stimulation of GTPCH results in accumulation of 7,8-dihydroneopterin and its stable water-soluble derivative, neopterin. Therefore, elevated neopterin concentration might be considered not only as a clinical marker of inflammation (as e.g., C-reactive protein and

fibrinogen) but an indirect marker of increased of KYN production during inflammation (Sucher et al. 2010). Blood neopterin levels correlate with KTR in healthy humans (Spenser et al. 2010; Capuron et al. 2014) and cardiovascular patients (Midttun et al. 2011; Murr et al. 2011).

8.6 Dysregulation of TRP–KYN Metabolism in T2D

Impaired accumulation of TRP in the brain concomitantly with a much faster disappearance of the administered TRP from the bloodstream was observed in streptozotocin-diabetic rats after TRP load (Masiello et al. 1987). Surplus of dietary TRP, the initial substrate for the formation of KYN, KYNA, 3-HK, and 3H-KYNA, induces insulin resistance (IR), a precursor of T2D, in pigs (Koopmans et al. 2009). Decreased KYNase activity was observed in liver and kidney of alloxan-diabetic rabbits (Allegri et al. 2003). Increased expression of IDO and serum levels of 3H-KYNA precursors, KYN and 3-HK, was reported in patients with diabetic retinopathy (Munipally et al. 2011). As it was mentioned earlier, 3H-KYNA was identified in prediabetes subjects (Manusadzhian et al. 1974). Recent studies revealed decreased plasma TRP concentrations and increased KYN and KTR in 21 hemodialysis patients with diabetes in comparison with 40 healthy controls patients (Koenig et al. 2010). Neopterin, an inflammatory marker of IFNG-induced upregulation of IDO (Fuchs et al. 2009; Oxenkrug 2011), was increased in these patients and correlated with KYN concentrations ($rs = 0.393$, $p < 0.01$), indicating that increased TRP degradation was a result of IDO activation.

Neopterin negatively correlated with IR in Caucasian population (Fuchs et al. 1982; Spencer et al. 2010). We found correlation between plasma neopterin concentrations and IR (HOMA-IR, $r = 0.08$, $P < 0.03$) and P5P ($r = -0.13$, $P = 0.002$) in 592 adult (45–75 years of age) participants of community dwellers of Boston Puerto Rican Health Study. The strongest ($r = 0.15$) and most significant ($P < 0.0002$) correlation was observed between HOMA–IR and neopterin/P5P ratio (index of combination of increased inflammation and P5P deficiency) (Oxenkrug et al. 2011a).

8.6.1 Dysregulation of TRP–KYN Metabolism in Aging

Animal and human studies suggested that aging is associated with upregulation of TRP–KYN metabolism (Oxenkrug 2007). The increased dioxygenation of mitochondrial TRP to N-formyl-KYN was consistently present among conserved biomarkers across ageing models in five species (Groebe et al. 2007). KTR, a marker of the rate of KYN formation from TRP, increased with aging in humans when comparing three age groups (34–60, 61–71, and 72–93 years) (Frick et al. 2004) and nonagenarians with 45-year-old subjects (Pertovaara et al. 2006). Increased

formation of KYNA was observed in aged rat brain (Moroni et al. 1988; Gramsbergen et al. 1992) and in human serum (Urbanska et al. 2006; Theofylaktopoulou et al. 2013). The higher rate of TRP conversion into KYN at the entry into the study was predictive of higher mortality in 10-year prospective study of nonagenarians (Pertovaara et al. 2007). 3H-KYNA accumulates in organs with aging and activates caspases 9 and 3, leading to apoptosis of pancreatic beta cells (Malina et al. 2001).

Aging-associated upregulation of TRP–KYN metabolism might be triggered by activation of IDO due to age-associated chronic inflammation or TDO due to age-associated elevation of cortisol production.

8.6.2 Aging and TDO (Stress)

Aging is characterized by elevated production of TDO inducer, cortisol, due to disinhibition of the brain–pituitary–adrenal axis (Dilman et al. 1979; Oxenkrug and Gershon 1987; Oxenkrug et al. 1983, 1984a, b). TRP–KYN pathway and related genes were described in *Drosophila melanogaster* (Savvateeva-Popova et al. 2003). TDO is the rate-limiting enzyme of KYN formation from TRP in Drosophila, as in the other species. However, the end product of TRP–KYN pathway in Drosophila is not NAD but brown eye pigment (Tearle 1991). Besides TDO, TRP–KYN metabolism is affected by ATP-binding cassette (ABC) transporter regulating TRP access to intracellular TDO (Sullivan and Sullivan 1975). *Drosophila melanogaster* mutants with impaired KYN production and TDO-deficient (vermilion) (Kamyshev 1980) and ABC transport-impaired (white) eye mutants had longer life span than wild-type flies (Oxenkrug 2010c). Furthermore, TDO inhibitor, alpha-methyl tryptophan (aMT), and ABC transported inhibitor, 5-methyl tryptophan (5MT), prolonged mean and maximum life span (by 27 % and 43 % and 21 % and 23 %, respectively) (Oxenkrug et al. 2011b).

8.6.3 Inflammation and Aging: IDO

Aging is associated with a chronic low-grade inflammation triggered by a shift from the homeostatic balance of pro- and anti-inflammatory mediators to a proinflammatory Th1 (cellular)-type state (Vasto et al. 2007) and by increased reactivity upon immune stimulation due to priming of brain microglial cells and peripheral macrophages (Henry et al. 2009). Activation of macrophages and microglia requires both a "priming" stimulus (i.e., IFNG) and a secondary "triggering" stimulus such as stress (Sparkman and Johnson 2008) or infection (e.g., gram-negative bacterial endotoxin, lipopolysaccharide (Henry et al. 2009). Microglia-derived IFNG was

shown to stimulate astrocytes via IFNG receptor in the injured hippocampus of SAMR1 mice (Hasegawa-Ishii et al. 2011).

Involvement of IDO inducer, IFNG, in mechanisms of aging is supported by identification of interferon-related genes among six pathways regulating senescence/immortalization: the cell cycle pRB/p53, cytoskeletal, interferon related, insulin growth factor related, MAP kinase, and oxidative stress pathway (Fridman and Tainsky 2008). Prolonged treatment with IFNG induces cellular senescence in human vascular endothelial cells via upregulation of senescence-associated genes (Kim et al. 2009). Age-dependent increases in IFNG production have been reported in in vitro and in vivo studies, with minor changes in the remaining evaluated cytokines in senescence-accelerated mice (Rodrigez et al. 2007). The frequency of A (low-producer) alleles of IFNG(+874) gene that encodes the production of IFNG protein increased with aging in line with the other evidences that centenarians are characterized by a higher frequency of genetic markers associated with better control of inflammation (Lio et al. 2002). Down's syndrome, a condition representing an accelerated aging, was associated with higher percentages of IFNG-producing cell in comparison with mentally retarded and healthy controls (Baran et al. 1996).

8.6.4 Inflammation and Aging: Neopterin

Increased plasma levels of neopterin (but not other 33 independent immune parameters) separated the aged and a healthy younger group (Fahey et al. 2000). Neopterin levels increased with age (Fuchs et al 2009; Pertovaara et al. 2006; Theofylaktopoulou et al. 2013; Spencer et al. 2010) with no gender differences (Schennach et al. 2002), while age-associated increase of IDO was more prominent in women than in men (Raitala et al. 2005). Elevation of neopterin, as a consequence of upregulation of IFNG production, has been shown to correlate with several components of inflammation-associated metabolic syndrome, including IR in populations of European ancestry (Grammer et al. 2009). We assessed neopterin correlations with IR and other clinical markers of metabolic syndrome and mortality risk in population with a different genetic background, i.e., Puerto Ricans residents of Boston (592 subjects (45–75 years of age). Neopterin concentrations correlated with insulin resistance (HOMA-IR, $r=0.08$, $P<0.03$), abdominal obesity (waist circumference, $r=0.085$, $p<0.038$), and HDL cholesterol ($r=-0.15$, $p<0.0001$). Neopterin concentrations of >16 nmol/L at baseline were associated with the dramatically increased risk of mortality in 113 subjects followed for 6 years (Oxenkrug et al. 2011a).

Since inflammation is associated with P5P deficiency (Morris et al. 2010; Shen et al. 2010), we assessed correlations of neopterin with P5P. Neopterin concentrations correlated with plasma (PLP ($r=-0.13$, $P=0.002$) (Oxenkrug et al. 2011a).

8.6.5 Aging and P5P

Aging is associated with vitamin B6 (P5P) deficiency (Schennach et al. 2002; Gori et al. 2006; Selhub et al. 2010; Middtun et al. 2011). Since P5P deficiency is associated with the increased production of diabetogenic kynurenine derivative, 3H-KYNA (Middtun et al. 2011), it was suggested that aging-associated combination of increased formation of KYN from TRP with P5P deficiency contributes to the development of IR in aging (Oxenkrug 2013b).

8.7 Dysregulation of TRP–KYN Metabolism in Obesity

Human obesity is characterized by chronic low-grade inflammation in white adipose tissue that releases many inflammatory mediators, including KYN (Watts et al. 2011; Scarpellini and Tack 2012). Activation of IDO1, a rate-limiting enzyme that converts TRP to KYN and is induced by IFNG, has been suggested to trigger a metabolic syndrome (including obesity) in response to chronic inflammation (Oxenkrug 2010b). Overexpression of IDO1 in the liver and white adipose tissues of obese patients and increased serum KTR in obese in comparison with lean women were discovered by Wolowczuk et al. (2012). Free TRP was decreased in the plasma of obese rats (Finkelstein 1982), while obese mice produced more IFNG than controls and had deficient IFNG receptor (Rocha et al. 2008). Secretion of IFNG was significantly higher in the obese than in the control subjects that might be partly depend on action of leptin, an adipocyte-secreted hormone, that shifts Th cells toward a Th1 phenotype (Mouzaki et al. 2012; You et al. 2008). In obese children, a shift to Th1-cytokine profile is dominated by the production of IFNG and is related to IR (Pacifico et al. 2006). Proinflammatory cytokines exacerbate IR, impair insulin action, and, thus contribute to the development of T2DM (Lann and LeRoith 2007). Significantly higher serum neopterin levels were reported in subjects with increased waist-to-hip ratio (Bozdemir et al. 2006) and increased BMI (Ledochowski et al. 1999; Ursavas et al. 2008). Low plasma TRP and high plasma KYN levels (elevated KTR) were observed in obese subjects, independently of weight reduction or dietary intake, and likely result from the inflammatory response of the adipose tissue (Ashley et al. 1985; Breum et al. 2003). Serum levels of KTR and neopterin and inflammatory markers, including C-reactive protein, in morbidly obese patients were significantly increased compared to the control group, but only KTR and neopterin remained below normal after weight reduction induced either by caloric restriction (Gatti et al. 1994) or by bariatric surgery (Brandacher et al. 2006). These studies suggest that IFNG-induced IDO and GTPCH activities have unique role as the trait (vs state) inflammatory markers in obesity. Nevertheless, it has been demonstrated that weight loss improves the inflammatory profile of obese subjects

through a decrease of proinflammatory factors and an increase of anti-inflammatory molecules (Forsythe et al. 2008). In addition, these TRP metabolic changes may subsequently reduce 5-HT production and cause mood disturbances, depression, and impaired satiety ultimately leading to increased caloric uptake and obesity (Brandacher 2007).

Obesity is one of the major risk factor for the development of IR (Stanworth and Johns 2009) and is considered as an independent cause of IR (Park et al. 2005). In most cases, IR exists because of the obesity and will disappear with weight loss (Gomez-Ambrosi et al. 2008).

8.7.1 P5P Deficiency

P5P deficiency was noted in 11 % of morbid obese individuals before laparoscopic sleeve gastrectomy (Damms-Machado et al. 2012). Significantly lower P5P concentrations were reported in the morbidly obese Norwegian women and men (Aasheim et al. 2008).

8.7.2 Dysregulation of TRP–KYN Metabolism in Depression

The stress-induced TDO activation shunting TRP metabolism from formation of serotonin toward production of KYN in depression was originally suggested in 1969 (Lapin and Oxenkrug 1969; Oxenkrug 2010a, 2013a). Association of depression with the increased production of cortisol (Leonard 2005) is described elsewhere and might be further supported by the results of recent animal experiments (Gibney et al. 2014). Discovery of inflammation-inducible IDO added another mechanism of upregulation of KYN formation from TRP in depression (Hayaishi 1976). Increased production of IDO inducers, proinflammatory factors, was reported in depression (Leonard and Myint 2009). Gene set analysis reported upregulation of IFNG, the most powerful IDO inducer, in postmortem brain tissue samples from Brodmann area 10 in the prefrontal cortex from psychotropic drug-free persons, with the history of depression (Shelton et al. 2010). Both IDO and TDO activations lead to the same major consequences described in depression: (1) deficiency of serotonin (and its metabolites, melatonin and N-acetylserotonin) (Oxenkrug and Ratner 2012) contributing to insomnia, dysregulation of biological rhythms (Oxenkrug and Requintina 2003), and impairment of neurogenesis (Duman and Aghajanian 2014); (2) upregulated formation of KYN and its neuroactive derivatives exerting anxiogenic, pro-oxidative, and cognitive impairment effects (Lapin 1973, 2003).

Depression is associated with low plasma concentrations of P5P (Merete et al. 2008; Moorthy et al. 2012) and increased urine secretion of 3H-KYNA acid independent from P5P status (Cazzulo et al. 1974).

Therefore, upregulated formation of KYN and 3-HK in depression in combination with P5P deficiency might contribute to the increased association between depression and T2DM (Rustad et al. 2011; Demakakos et al. 2010) and increased (by 65 %) risk of development of T2DM (Campayo et al. 2010) supporting the hypothesis that depression leads to diabetes (Eaton et al. 1996).

8.7.3 Dysregulation of TRP–KYN Metabolism in Depression Associated with Hepatitis C Virus

Depression is an often (30–50 %) side effect of IFN-alpha treatment (Loftis et al. 2013). There is a strong correlation between increased serum KYN levels and severity of depression, associated with IFN-alpha treatment of HCV or melanoma patients (Larrea et al. 2007). We found that the presence of high-producer (T) allele of IFNG (+874) T/A gene that encodes the production of proinflammatory cytokine, IFNG, increases the risk of development of depression during IFN-alpha treatment (Oxenkrug et al. 2011c). There was strong ($r=0.7$) and highly significant ($p<0.0001$) correlation between serum KYN and neopterin levels in our cohort of HCV patients. High serum TRP was the risk factor for the development of depression (Oxenkrug et al. 2014, in press), while high neopterin levels predicted poor response to IFN-alpha treatment of HCV patients (Oxenkrug et al. 2012).

The incidence of IR among HCV patients is 50 %, which is fourfold higher than in non-HCV population (Negro and Alaei 2009). HCV infection significantly lowered vitamin B6 (Lin and Yin 2009). IFN-alpha treatment was associated with the increased risk of developing IR and higher incidence of T2DM in comparison with the group of nonviral chronic liver disease (Brischetto et al. 2003) or patients with chronic hepatitis B virus (Imazeki et al. 2008). Moreover, antecedent HCV infection markedly increases the risk of developing diabetes in susceptible subjects, while even nondiabetic HCV patients have IR and specific defects in the insulin-signaling pathway (Knobler and Schattner 2005). Serum KYN and neopterin concentrations are higher in HCV patients than in non-HCV population (Fuchs et al. 1982). We found correlations between plasma levels of KYN and homeostasis model of insulin resistance (IR) assessment (HOMA2-IR) scores ($p=0.32$, $p=0.01$) and between KYN and scores of HOMA-beta (pancreatic beta cell function) ($r=0.30$, $p=0.02$) in 60 hepatitis C virus (HCV) patients (Oxenkrug et al. 2013). There was no correlation between KYNA and IR, probably because of downregulation of P5P-dependent KATs despite increased availability of KYN as a substrate for KYNA formation. The absence of KYNA correlations with HOMA–IR in our

study of HCV subjects does not preclude possible KYNA involvement in the development of T2DM in non-HCV subjects because of additional impact of HCV-inducible apoptosis-like death of pancreatic beta cells through a caspase 3-dependent pathway (Wang et al. 2012).

Therefore, exposure to HCV and treatment with IFN-alpha might predispose to the development of T2DM in HCV patients because of upregulation of TRP–KYN metabolism and inflammation-associated P5P deficiency.

8.7.4 Dysregulation of TRP–KYN Metabolism in Parkinson's Disease

Over half of Parkinson's disease (PD) patients have abnormal glucose tolerance (prediabetes) (Lipman et al. 1974; Sandyk 1993). Development of T2D in PD patients is associated with decreased efficacy of dopamine replacement therapy (Sandyk 1993), worsening of rigidity and gait (Papapetropoulos et al. 2004), and increased cost of medical care (Pressley et al. 2003).

While type 2 diabetes (T2D) was suggested as a risk factor for PD (Hu et al. 2007), there is a possibility for a reverse causality, i.e., that PD increased a risk for T2D. Alternatively, PD might facilitate progression from prediabetes to T2D. One might suggest that one of the mechanisms of high association between IR and PD is dysregulation of TRP–KYN metabolism. Thus, upregulated conversion of TRP into KYN (Widner et al. 2002) and increased concentrations of 3H-KYNA precursor, 3-HK, were reported in brain tissues (Ogawa et al. 1992) and spinal fluid (Lewitt et al. 2013) of PD patients.

8.7.5 Dysregulation of TRP–KYN Metabolism in Neurodegenerative Disorders

Besides Parkinson's disorder, peripheral IR and T2D are associated with other neurodegenerative conditions such as Huntington's disease (Russo et al. 2013) and vascular disease (Umegaki 2014), Alzheimer's disease (de la Monte 2009), and HIV-1-associated dementias (Calvo and Martinez 2014).

Dysregulation of TRP–KYN pathway might contribute to the association of these conditions with IR considering the findings of drastically elevated levels of 3-HK in spinal fluid and serum of patients (Sardar et al. 1995; Schwarz et al. 2013) and brains of animal models of neurodegenerative disorders (Guidetti et al. 2006; Campesan et al. 2011).

8.7.6 Dysregulation of TRP–KYN Metabolism in Schizophrenia

Atypical antipsychotic drugs (AAD) are widely prescribed to treat various disorders, most notably schizophrenia and bipolar disorder. Treatment with AAD is associated with the increased incidence of IR and T2DM that are considered as damaging side effects of AAD (Citrome et al. 2013). On the other hand, recent review suggested that schizophrenia might predispose patients to diabetes (Leonard et al. 2012). In schizophrenia, metabolic syndrome incidence is double that of the general population, with women having a higher incidence (Ellingrod et al. 2012). Increased formation of KYNA in schizophrenia is well documented (Carlborg et al. 2013; Sathyasaikumar et al. 2011). Although we could not find studies of 3H-KYNA in patients with schizophrenia, plasma levels of its immediate precursor, 3-HK, were higher (by 50 %) in 35 medication-naïve and 18 medication-free patients with schizophrenia compared with 48 healthy controls and decreased after 6 weeks of treatment (Myint et al. 2011). Both baseline and proinflammatory factor-stimulated levels of 3-HK were higher in ex vivo study of skin fibroblast from patients with schizophrenia than in control subjects (Johansson et al. 2013).

Elevation of 3-HK might result from increased conversion of KYN into HK, catalyzed by KMO and/or decreased metabolism of 3-HK, catalyzed by P5P-dependent KYNase. We could not find studies of the effect of AAD on KMO and P5P. However, typical antipsychotic drug, chlorpromazine, was reported to activate KMO in mouse liver in both in vivo and in vitro studies (Mostafa et al. 1982) and to cause P5P deficiency in rat brains (Gey and Georgi 1974). The state of P5P deficiency might be further perpetuated by 3-HK and 3H-KYNA-induced inhibition of P5P (Karawya et al. 1981).

Therefore, it is possible that upregulated formation of KYNA and 3H-KYNA contributes to predisposition of patients with schizophrenia to T2D. AAD might further increase 3H-KYNA formation by activation of KMO, an enzyme catalyzing KYN conversion into 3-HK, a precursor of 3H-KYNA, and by triggering deficiency of P5P, cofactor of KYNase, catalyzing degradation of 3-HK.

8.8 Conclusion

Review of literature and our data suggest that inflammation- and/or stress-induced upregulation of TRP–KYN metabolism, resulting in the excessive production of KYN, KYNA, and 3-HK, is one of the factors predisposing to T2D. Deficiency of P5P, a cofactor of the key enzyme of 3-HK–NAD pathway, diverts the excessive amount of 3-HK from formation of NAD toward production of 3H-KYNA. Overproduction of diabetogenic KYN, KYNA, 3-HK, and 3H-KYNA might contribute to increased association of T2D with aging, obesity, depression (including triggered by IFN-alpha treatment), Parkinson's disease, schizophrenia

and use of antipsychotic drugs, and neurodegenerative conditions, e.g., vascular, Alzheimer's, and HIV-1-associated dementias and Huntington disease.

Pharmacological regulation of TRP–KYN and KYN–NAD pathways and maintenance of adequate vitamin B6 status might contribute to the prevention and treatment of T2D in the abovementioned conditions.

One of the possible interventions is the inhibition of enzymes of TRP–KYN pathways. The standard IDO inhibitor, 1-methyl-L-TRY (Cady and Sono 1991), is not available for human use. The family of IDO inhibitors were identified among alkaloids isolated from *Berberis aristata*, a herb widely used in Indian and Chinese systems of medicine (Yu et al. 2010). One of them, berberine, improves IR in diabetic hamsters (Li et al. 2011) and diabetic patients (Zhao et al. 2012; Di Pierro et al. 2012) and prolonged life span and improved health span in Drosophila model (Navrotskaya et al. 2012, 2014).

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Chapter 9
Therapeutical Implications of Melatonin in Alzheimer's and Parkinson's Diseases

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Abstract Neurodegenerative diseases like Alzheimer's disease (AD) and Parkinson's disease (PD) are major health problems, and a growing recognition exists that efforts to prevent them must be undertaken by both governmental and nongovernmental organizations. In this context, the pineal product melatonin has a promising significance because of its chronobiotic/cytoprotective properties. One of the features of advancing age is the gradual decrease in endogenous melatonin synthesis. A limited number of therapeutic trials have indicated that melatonin has a potential therapeutic value as a neuroprotective drug in the treatment of AD, minimal cognitive impairment (which may evolve to AD), and PD. Both in vitro and in vivo, melatonin prevented the neurodegeneration seen in experimental models of AD and PD. For these effects to occur, doses of melatonin about two orders of magnitude higher than those required to affect sleep and circadian rhythmicity are needed. More recently, attention has been focused on the development of potent melatonin analogs with prolonged effects which were employed in clinical trials in sleep-disturbed or depressed patients in doses considerably higher than those employed for melatonin. In view that the relative potencies of the analogs are higher than that of the natural compound, clinical trials employing melatonin in the range of 50–100 mg/day are needed to assess its therapeutic validity in neurodegenerative disorders.

Keywords Melatonin • Neurodegeneration • Free radicals • Oxidative stress • Aging • Parkinson's disease • Alzheimer's disease • Mild cognitive impairment • Melatonin analogs

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Abbreviations

6-OHDA	6-hydroxydopamine
Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AFMK	N^1-Acetyl-N^2-formyl-5-methoxykynuramine
AMK	N^1-Acetyl-5-methoxykynuramine
APP	Amyloid precursor protein
Aβ	Aggregated β-amyloid
Bcl-2	B cell lymphoma proto-oncogene protein
ChAT	Choline acetyltransferase
Cox	Cyclooxygenase
DA	Dopamine
GABA	γ-Aminobutyric acid
GPR50	G-protein receptor 50 ortholog
GPx	Glutathione peroxidase
GRd	Glutathione reductase
GSH	Reduced glutathione
GSK–3	Glycogen synthase kinase-3
iNOS	Inducible nitric oxide synthase
L-DOPA	L-Dihydroxyphenylalanine
MAO	Monoamine oxidase
MAP	Microtubule-associated protein
MCI	Mild cognitive impairment
MPP^+	1-Methyl-4-phenylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid
MT_1	Melatonin receptor 1
MT_2	Melatonin receptor 2
MT_3	Melatonin receptor 3
NF κB	Nuclear factor κB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
PD	Parkinson's disease
PK	Protein kinase
RBD	REM-associated sleep behavior disorder
REM	Rapid eye movement
RNS	Reactive nitrogen species
ROR	Retinoic acid receptor-related orphan receptor
ROS	Reactive oxygen species
RZR	Retinoid Z receptor
SCN	Suprachiasmatic nuclei
SNpc	Substantia nigra pars compacta
SOD	Superoxide dismutase

9.1 Introduction

Neurodegenerative disorders are a group of chronic and progressive diseases characterized by selective and symmetric losses of neurons in cognitive, motor, or sensory systems. Alzheimer's disease (AD) and Parkinson's disease (PD) are the most clinically relevant examples of neurodegenerative disorders. Although the origin of specific neurodegeneration in these disorders remains mostly undefined, three major and frequently interrelated processes, namely, free radical-mediated damage, mitochondrial dysfunction, and excitotoxicity, have been identified as common pathophysiological mechanisms for neuronal death (Reiter et al. 1998).

Neurodegenerative diseases have become a major health problem, and a growing recognition exists that efforts to prevent these diseases at an early stage of development must be undertaken by both governmental and nongovernmental organizations. Regular intake of antioxidants by the elderly has been recommended for prevention of age-associated, free radical-mediated, and neurodegenerative diseases, although the efficacy of this treatment is discussed (Johnson et al. 2013). In this context, the use of melatonin as a cytoprotective agent becomes of interest.

Melatonin is a well-preserved methoxyindole found in most phyla having remarkable cytoprotective actions in addition to chronobiotic properties. The source of circulating melatonin is the pineal gland, and a substantial amount of data support that plasma melatonin decrease is one of the features of advancing age (Bubenik and Konturek 2011). In this chapter we will first summarize the efficacy of melatonin to decrease basic processes of brain degeneration in animal models of AD and PD. We will then assess the clinical data that support the possible therapeutic efficacy of melatonin in AD and PD.

9.2 Basic Biology of Melatonin Relevant to Neurodegeneration

Tryptophan serves as the precursor for melatonin biosynthesis. It is hydroxylated at C5 position and then decarboxylated to form serotonin. Serotonin is N-acetylated by the enzyme serotonin-N-acetyl transferase and the produced N-acetylserotonin is finally O-methylated by the enzyme hydroxyindole-O-methyl transferase to form melatonin.

In all mammals, circulating melatonin derives primarily from the pineal gland (Claustrat et al. 2005). In addition, melatonin is locally synthesized in many cells, tissues, and organs including lymphocytes, bone marrow, thymus, gastrointestinal tract, skin, and eyes, where it may play either an autocrine or paracrine role (see for (Hardeland et al. 2011)). Both in animals and in humans, melatonin participates in diverse physiological functions signaling not only the length of the night but also enhancing free radical scavenging and the immune response, showing relevant cytoprotective properties (Hardeland et al. 2011).

Circulating melatonin binds to albumin (Cardinali et al. 1972) and is metabolized mainly in the liver where it is hydroxylated in the C6 position by the cytochrome P_{450} monooxygenases A2 and 1A (Facciola et al. 2001; Hartter et al. 2001). Melatonin is then conjugated with sulfate to form 6-sulfatoxymelatonin, the main melatonin metabolite found in urine. Melatonin is also metabolized in tissues by oxidative pyrrole ring cleavage into kynuramine derivatives. The primary cleavage product is N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), which is deformylated, either by arylamine formamidase or by hemoperoxidase, to N^1-acetyl-5-methoxykynuramine (AMK) (Hardeland et al. 2009). It has been proposed that AFMK is the primitive and primary active metabolite of melatonin to mediate cytoprotection (Tan et al. 2007). Melatonin is also converted into cyclic 3-hydroxymelatonin in a process that directly scavenges two hydroxyl radicals (Tan et al. 2007).

Melatonin exerts many physiological actions by acting on membrane and nuclear receptors while other actions are receptor independent (e.g., scavenging of free radicals or interaction with cytoplasmic proteins) (Reiter et al. 2009). The two membrane melatonin receptors cloned so far (MT_1 and MT_2) have seven membrane domains and belong to the superfamily of G-protein-coupled receptors (Dubocovich et al. 2010). MT_1 and MT_2 receptors are found in the cell membrane as dimers and heterodimers. GPR50, a G-protein-coupled melatonin receptor ortholog that does not bind melatonin itself, dimerizes with MT_1 receptors and can block melatonin binding (Levoye et al. 2006). The human MT_2 receptor exhibits a lower affinity than the human MT_1 receptor and becomes desensitized after exposure to melatonin, presumably by an internalization mechanism.

As representatives of the G-protein-coupled receptor family, MT_1 and MT_2 receptors act through a number of signal transduction mechanisms (Dubocovich et al. 2010). The MT_1 receptor is coupled to G proteins that mediate adenylyl cyclase inhibition and phospholipase C activation. The MT_2 receptor is also coupled to the inhibition of adenylyl cyclase, and it additionally inhibits the soluble guanylyl cyclase pathway.

By using receptor autoradiography with the nonselective 2-[^{125}I]iodomelatonin ligand and real-time quantitative reverse transcription–polymerase chain reaction to label melatonin receptor mRNA, MT_1 and MT_2 receptors have been identified in the retina, suprachiasmatic nuclei (SCN), thalamus, hippocampus, vestibular nuclei, and cerebral and cerebellar cortex. At the level of the hippocampus, MT_2 receptors were detected in CA3 and CA4 pyramidal neurons, which receive glutamatergic excitatory inputs from the entorhinal cortex, whereas MT_1 receptors were predominantly expressed in CA1.

In addition to binding to MT_1 and MT_2 receptors, melatonin has been shown to display affinity for another binding site, originally considered to represent a membrane-bound receptor (MT_3), but then confirmed to be an enzyme, quinone reductase 2 (QR2) (Nosjean et al. 2000). Polymorphisms in the promoter of the human *QR2* gene are associated with PD and a decline in cognitive ability over time (Harada et al. 2001).

Melatonin also binds to transcription factors belonging to the retinoic acid receptor superfamily, in particular, splice variants of RORα (RORα1, RORα2, and RORα isoform d) and RZRβ (Wiesenberg et al. 1995; Lardone et al. 2011). Retinoic acid receptor subforms are ubiquitously expressed in mammalian tissues, and relatively high levels were detected especially in T- and B-lymphocytes, neutrophils, and monocytes (Lardone et al. 2011).

Melatonin is a powerful antioxidant that scavenges •OH radicals as well as other radical oxygen species (ROS) and radical nitrogen species (RNS) and that gives rise to a cascade of metabolites that share antioxidant properties (Galano et al. 2011). Melatonin also acts indirectly to promote gene expression of antioxidant enzymes and to inhibit gene expression of prooxidant enzymes (Antolin et al. 1996; Pablos et al. 1998; Rodriguez et al. 2004; Jimenez-Ortega et al. 2009). In particular, this holds for glutathione peroxidase (GPx) and for glutathione reductase (GRd), presumably in response to GPx-dependent increases in GSSG, the oxidized form of glutathione (GSH). Melatonin contributes to maintain normal brain GSH levels (Subramanian et al. 2007) by stimulating GSH biosynthesis via γ-glutamylcysteine synthase and glucose-6-phosphate dehydrogenase (Rodriguez et al. 2004; Kilanczyk and Bryszewska 2003).

As abovementioned, the antioxidative efficiency of melatonin is high because the metabolites formed after free radical scavenging also act as free radical scavengers with an activity even higher than the native compound. Melatonin has a demonstrated superiority to vitamin C and E in protection against oxidative damage and in scavenging free radicals (Galano et al. 2011). Additionally, melatonin potentiates effects by other antioxidants, such as vitamin C, Trolox (a water-soluble vitamin E analog), and NADH.

Melatonin has significant anti-inflammatory properties presumably by inhibiting nuclear factor κ B (NF κB) binding to DNA thus decreasing the synthesis of proinflammatory cytokines, by inhibiting cyclooxygenase (Cox) (Cardinali et al. 1980) particularly Cox-2 (Deng et al. 2006) and by suppressing inducible nitric oxide (NO) synthase (iNOS) gene expression (Costantino et al. 1998). Melatonin was shown to protect from oxidotoxicity already at physiological concentrations (Galano et al. 2011; Tan et al. 1994). Although melatonin's direct action as an antioxidant agent is mostly independent on receptor interaction (Leon-Blanco et al. 2004), the upregulation of antioxidant enzymes involves nuclear transcription and in some cases RZR/RORα receptors (Urata et al. 1999).

The efficacy of melatonin in inhibiting oxidative damage has been tested in a variety of neurological disease models where free radicals have been implicated as being at least partial causal agents of the condition. Besides the animal models of AD and PD discussed below, melatonin has been shown to lower neural damage due to cadmium toxicity (Poliandri et al. 2006; Jimenez-Ortega et al. 2011), hyperbaric hyperoxia (Shaikh et al. 1997; Pablos et al. 1997), δ-aminolevulinic acid toxicity (Princ et al. 1997; Carneiro and Reiter 1998; Onuki et al. 2005), γ radiation (Erol et al. 2004; Shirazi et al. 2011; Taysi et al. 2008), focal ischemia (Lee et al. 2004; Tai et al. 2011), brain trauma (Beni et al. 2004; Tsai et al. 2011; Kabadi and Maher 2010), and a number of neurotoxins (Reiter et al. 2010).

Melatonin's neuroprotective properties, as well as its regulatory effects on circadian disturbances, validate melatonin's benefits as a therapeutic substance in the preventive treatment of neurodegenerative diseases discussed below. Moreover, melatonin exerts anti-excitatory, and at sufficient dosage, sedating effects (Golombek et al. 1996; Caumo et al. 2009) so that a second neuroprotective mode of action may exist involving the γ-aminobutyric acid (GABA)-ergic system as a mediator. This view is supported by studies indicating that melatonin protects neurons from the toxicity of the amyloid-β (Aβ) peptide (a main neurotoxin involved in AD) via activation of GABA receptors (Louzada et al. 2004).

Melatonin has also anti-excitotoxic actions. Early studies in this regard employed kainate, an agonist of ionotropic glutamate receptors, and gave support to the hypothesis that melatonin prevents neuronal death induced by excitatory amino acids (Giusti et al. 1996; Manev et al. 1996). It has also been reported that administration of melatonin reduces the injury of hippocampal CA1 neurons caused by transient forebrain ischemia (Cho et al. 1997; Kilic et al. 1999) or high glucocorticoid doses (Furio et al. 2008).

The various types of toxicities listed above can result in cell death by necrosis or apoptosis. Apoptotic neuronal death requires RNA and protein synthesis and depletion of trophic factors. Apoptosis also involves single-strand breaks of DNA and neurotrophic factors have been found to rescue neurons from this type of death (Dodd et al. 2013). They may act via cellular antiapoptotic components, such as the B cell lymphoma proto-oncogene protein (Bcl-2). Bcl-2 is capable of blocking the apoptotic pathway in the mitochondria by preventing the formation of a functional mitochondrial permeability transition pore (mtPTP) and, thus, the release of the mitochondrial enzyme cytochrome c, which represents the final and no-return signal of the apoptotic program (Khandelwal et al. 2011). Studies in vitro indicate that melatonin enhances expression of Bcl-2 and prevents apoptosis (Jiao et al. 2004; Koh 2011; Radogna et al. 2010). In addition, melatonin directly inhibits the opening of the mtPTP, thereby rescuing cells (Peng et al. 2012; Jou 2011; Andrabi et al. 2004).

9.3 Basic Aspects of Melatonin Activity in Animal Models of AD

The pathological signature of AD includes extracellular senile plaques, formed mainly by Aß deposits, and intracellular neurofibrillary tangles, resulting mainly from abnormally hyperphosphorylated microtubule-associated protein (MAP) tau. Aß is generally believed to play an important role in promoting neuronal degeneration in AD turning neurons vulnerable to age-related increases in the levels of oxidative stress and an altered cellular energy metabolism. Concerning the microtubule-associated protein tau, it promotes microtubule assembly and is a major factor to stabilize microtubules.

Aß is composed by 39–43 amino acid residues derived from its precursor, the amyloid precursor protein (APP) (Selkoe 2004). APP is proteolytically processed by α- or β-secretases in different pathways. The α-non-amyloidogenic pathway

involves cleavage of APP by α-secretase to release a fragment of APP N – terminal, which after cleavage by γ-secretase precludes the formation of Aß (Selkoe 2004). The β-amyloidogenic pathway includes β-secretase which results in the formation of intact Aß peptide and is mediated by the sequential cleavage of β-secretase and γ-secretase at the N- and C-terminal of Aß sequence (Selkoe 2004). Melatonin inhibited the normal levels of soluble APP secretion in different cell lines interfering with APP maturation (Lahiri and Ghosh 1999). Additionally, the administration of melatonin efficiently reduces Aß generation and deposition in vivo (Matsubara et al. 2003; Lahiri et al. 2004) and in vitro (Lahiri and Ghosh 1999; Song and Lahiri 1997; Zhang et al. 2004; Olivieri et al. 2001).

Generally, the results in transgenic mice support the view that melatonin regulates APP and Aß metabolism mainly by preventing the pathology, with little anti-amyloid and antioxidant effects occurring after the deposition of Aß. Thus, melatonin therapy in old Tg2576 mice starting at 14 months of age could not prevent additional Aß deposition (Quinn et al. 2005) while a similar treatment starting at the 4th month of age was effective to reduce Aß deposition (Matsubara et al. 2003). Since amyloid plaque pathology is typically seen in 10–12-month-old Tg2576 mice (Hsiao et al. 1996), the data point out to the effectiveness of melatonin in preventing amyloid plaque formation rather than afterwards.

How melatonin exerts its inhibitory effect on the generation of Aß remains undefined. The proteolytic cleavage of APP by α-secretase pathway is regulated by many physiological and pathological stimuli particularly through protein kinase (PK) C activation and secretase-mediated cleavage of APP. The inhibition of glycogen synthase kinase-3 (GSK-3) and upregulation of c-Jun N-terminal kinase result in high activity of matrix metalloproteinases with increasing degradation of Aß (Donnelly et al. 2008). GSK-3 interacts with presenilin-1, a cofactor of γ-secretase, the phosphorylation of GSK-3, by PKC leading to γ-secretase inactivation. Indeed, GSK-3 can be one of the common signaling pathways increasing Aß generation and tau hyperphosphorylation, and melatonin could regulate APP processing through PKC and GSK-3 pathways.

Melatonin interacts with $Aß_{40}$ and $Aß_{42}$ and inhibits progressive β-sheet and/or amyloid fibrils (Poeggeler et al. 2001; Pappolla et al. 1998). This interaction between melatonin and Aß appears to depend on structural melatonin characteristics rather than on its antioxidant properties, since it could not be mimicked by melatonin analogs or other free radical scavengers (Poeggeler et al. 2001). By blocking the formation of secondary sheets, melatonin not only reduces neurotoxicity but also facilitates peptide clearance by increasing its proteolytic degradation.

Oxidative stress plays a central role in Aß-induced neurotoxicity and cell death. Accumulating data support that melatonin effectively protects cells against Aß-induced oxidative damage and cell death in vitro (Feng et al. 2004a; Zatta et al. 2003) and in vivo (Matsubara et al. 2003; Feng et al. 2004a; Furio et al. 2002; Shen et al. 2002; Rosales-Corral et al. 2003). In cells and animals treated with Aß, melatonin could exert its protective activity through an antioxidant effect, whereas in APP transfected cells and transgenic animal models, the underlying mechanism may involve primarily the inhibition of generation of β-leaves and/or amyloid fibrils. Aggregated Aβ generates ROS that produce neuronal death by damage of neuronal

membrane lipids, proteins, and nucleic acids. Protection from Aβ toxicity by melatonin was observed, especially at the mitochondrial level (Olcese et al. 2009; Dragicevic et al. 2011).

As far as the hyperphosphorylation of tau, it reduces tau capacity to prevent microtubule changes and the disruption of the cytoskeleton arrangement ensues (Brion et al. 2001; Billingsley and Kincaid 1997). Indeed, the extent of neurofibrillary pathology correlates with the severity of dementia in AD patients. The level of hyperphosphorylated tau is three to four times higher in the brain of AD patients than in normal adult brains (Khatoon et al. 1992; Iqbal et al. 2005). More than 30 serine or threonine phosphorylation sites have been identified in the brains of AD patients (Nelson et al. 2012).

Melatonin efficiently attenuates tau hyperphosphorylation by affecting protein kinases and phosphatases in a number of experimental models including exposure of N2a and SH-SY5Y neuroblastoma cells to wortmannin (Deng et al. 2005), calyculin A (Li et al. 2004, 2005; Xiong et al. 2011), and okadaic acid (Benitez-King et al. 2003; Montilla-Lopez et al. 2002; Montilla et al. 2003; Wang et al. 2004). Melatonin also antagonizes the oxidative stress that arises by the action of these agents (Liu and Wang 2002; Wang et al. 2005).

The inhibition of melatonin biosynthesis in rats not only resulted in impairment of spatial memory but also induced an increase in tau phosphorylation, an effect prevented by melatonin supplementation (Zhu et al. 2004). Melatonin also prevented the oxidative damage and organelles injury found in animal models. The results point out to the involvement of decreased melatonin levels as a causative factor in the pathology of AD.

The oxidative stress is known to influence tau phosphorylation state (Gomez-Ramos et al. 2003; Lovell et al. 2004). The accumulation of misfolded and aggregated proteins in brain neurons of AD is considered a consequence of oxidative stress, in addition to the molecular structural changes due to age (Kenyon 2010). Since melatonin prevents, as an antioxidant and free radical scavenger, overproduction of free radicals, it seems feasible that the prevention of tau phosphorylation by melatonin is partly due to its antioxidant activity. In addition several studies indicated that melatonin may act as a modulator of enzymes in a way that is unrelated to its antioxidant properties. These include the regulation by melatonin of PKA (Schuster et al. 2005; Peschke et al. 2002), PKC (Witt-Enderby et al. 2000; Rivera-Bermudez et al. 2003), Ca^{2+}/calmodulin-dependent kinase II (Benitez-King et al. 1996), and mitogen-activated protein kinase (Chan et al. 2002).

A major and early event in the pathogenesis of AD is the deficit in cholinergic function (Struble et al. 1982). Neurons in the nucleus basalis of Meynert, the major source of cholinergic innervation to the cerebral cortex and the hippocampus, undergo a profound and selective degeneration in AD brains (Samuel et al. 1994). The levels of acetylcholine (ACh) are reduced at the early stage of AD, whereas the activities of the synthesizing enzyme choline acetyltransferase (ChAT) and of the degradating enzyme acetylcholinesterase (AChE) do not change until a late phase of AD (Terry and Buccafusco 2003; Rinne et al. 2003). Since a profound decrease in ChAT activity in the neocortex of AD patients correlated with the severity of

dementia, the use of AChE inhibitors as a standard treatment of mild to moderate AD is now widely employed (Spencer et al. 2010).

Melatonin has a protective effect on the cholinergic system. It prevents the peroxynitrite-induced inhibition of choline transport and ChAT activity in synaptosomes and synaptic vesicles (Guermonprez et al. 2001). Melatonin treatment of 8-month-old APP695 transgenic mice significantly improved the profound reduction in ChAT activity in the frontal cortex and the hippocampus (Feng et al. 2004a). Melatonin also antagonizes the spatial memory deficit and the decreased ChAT activity found in adult ovariectomized rats (Feng et al. 2004b). However, in rats perfused intracerebroventricularly with Aß for 14 days, melatonin was unable to restore the activity of ChAT (Tang et al. 2002). Melatonin inhibited lipopolysaccharide- and streptozotocin-induced increase in AChE activity (Agrawal et al. 2009). Recently hybrids of the AChE inhibitor tacrine and melatonin were synthesized as new drug candidates for treating AD (Fernandez-Bachiller et al. 2009; Spuch et al. 2010). These hybrids showed better antioxidant- and cholinergic-preserving activity tacrine or melatonin alone. The direct intracerebral administration of one of these hybrids decreased induced cell death and Aß load in the APP/PS1 mouse brain parenchyma accompanied by a recovery of cognitive function (Spuch et al. 2010).

Another common factor in the pathogenesis of AD is the activation of microglia with consequent more expression of proinflammatory cytokines (Arends et al. 2000; Combadiere et al. 2007; Streit et al. 2004; Shen et al. 2007). Epidemiological studies have shown that the use of anti-inflammatory drugs decreases the incidence of AD (Stuchbury and Munch 2005). Aß-induced microglial activation is a major source of inflammatory response (Park et al. 2012). Melatonin attenuated the production of proinflammatory cytokines induced by Aß, NF kB, and nitric oxide in the rat brain (Rosales-Corral et al. 2003; Lau et al. 2012). Moreover, the DNA-binding activity of NF kB was inhibited by melatonin (Mohan et al. 1995; Chuang et al. 1996).

9.4 Clinical Aspects of Melatonin Application in AD

Normal aging is characterized by a decline of cognitive capacities including reasoning, memory, and semantic fluency, which is detectable as early as the fifth decade of life (Singh-Manoux et al. 2014). Although there is a high variability across cognitive domains measured and among individuals in the degree and timing of age-related cognitive losses, there is evidence for a preclinical stage in dementia in which cognitive performance is borderline as compared to normal aging (Silveri et al. 2007). In community-based studies, up to 28 % of a sample of healthy community-dwelling elder shows deficits in performance that were not explained by age-related changes, education levels, mood, or health status. This strongly suggests the existence of early pathological changes which is a transitional state taking place between normal aging and early AD (Grundman et al. 2004).

Cross-sectional studies reveal that sleep disturbances are associated with memory and cognitive impairment (Fotuhi et al. 2009; Beaulieu-Bonneau and Hudon 2009; Cochen et al. 2009; Vecchierini 2010). A severe disruption of the circadian timing system occurs in AD as indicated by alterations in numerous overt rhythms like body temperature, glucocorticoids, and/or plasma melatonin (Weldemichael and Grossberg 2010; Harper et al. 2001; Mishima et al. 1999). The internal desynchronization of rhythms is significant in AD patients (Van Someren 2000). One emerging symptom is "sundowning," a chronobiological phenomenon observed in AD patients in conjunction with sleep–wake disturbances. Sundowning includes symptoms like disorganized thinking, reduced ability to maintain attention to external stimuli, agitation, wandering, and perceptual and emotional disturbances, all appearing in late afternoon or early evening (Weldemichael and Grossberg 2010; Klaffke and Staedt 2006; Pandi-Perumal et al. 2002). Chronotherapeutic interventions such as exposure to bright light and/or timed administration of melatonin in selected circadian phases alleviated sundowning symptoms and improved sleep–wake patterns of AD patients (der Lek et al. 2008).

A number of studies have revealed that melatonin levels are lower in AD patients as compared to age-matched control subjects (Mishima et al. 1999; Skene et al. 1990; Ohashi et al. 1999; Liu et al. 1999). The decreased CSF melatonin levels of AD patients were attributed to a decreased melatonin production. CSF melatonin levels decreased even in preclinical stages (Braak stages-1) when patients did not manifest cognitive impairment (Zhou et al. 2003) suggesting thereby that reduction in CSF melatonin may be an early marker (and cause) for incoming AD. The decrease of melatonin levels in AD was attributed to a defective retinohypothalamic tract or SCN-pineal connections (Skene and Swaab 2003). Decreased MT_2 immunoreactivity and increased MT_1 immunoreactivity have been reported in the hippocampus of AD patients (Savaskan et al. 2002, 2005). Additionally β_1-adrenoceptor mRNA levels decreased and the expression and activity of monoamine oxidase gene augmented in the pineal gland of AD patients (Wu et al. 2003).

The impaired melatonin production at night correlates significantly with the severity of mental impairment in demented patients (Magri et al. 1997). As AD patients have profound deficiency of endogenous melatonin, replacement of levels of melatonin in the brain could be a therapeutic strategy for arresting the progress of the disease. Melatonin's neuroprotective and vasoprotective properties would help in improving the clinical condition of AD patients (Srinivasan et al. 2006).

There is published information indicating that melatonin, as a chronobiotic agent, is effective in treating irregular sleep–wake cycles and sundowning symptoms in AD patients (Fainstein et al. 1997; Jean-Louis et al. 1998a; Mishima et al. 2000; Cohen-Mansfield et al. 2000; Mahlberg et al. 2004; Brusco et al. 1998a; Cardinali et al. 2002; Asayama et al. 2003; Singer et al. 2003; Pappolla et al. 2000) (Table 9.1). In an initial study on 14 AD patients with 6–9 mg of melatonin given for a 2–3-year period, it was noted that melatonin improved sleep quality (Brusco et al. 1998a). Sundowning, diagnosed clinically, was no longer detectable in 12 out of 14 patients. Reduction in cognitive impairment and amnesia was also noted. This should be contrasted with the significant deterioration of the clinical conditions expected from patients after 1–3 year of evolution of AD.

Table 9.1 Studies including treatment of AD patients with melatonin

Subjects	Design	Study's duration	Treatment	Measured	Results	Reference(s)
10 demented patients	Open-label study	3 weeks	3 mg melatonin p.o./daily at bedtime	Daily logs of sleep and wake quality completed by caretakers	7 out of 10 dementia patients having sleep disorders treated with melatonin showed a significant decrease in sundowning and reduced variability of sleep onset time	Fainstein et al. (1997)
14 AD patients	Open-label study	22–35 months	9 mg melatonin p.o./daily at bedtime	Daily logs of sleep and wake quality completed by caretakers. Neuropsychological assessment	Sundowning was not longer detectable in 12 patients and persisted, although attenuated in 2 patients. A significant improvement of sleep quality was found. Lack of progression of the cognitive and behavioral signs of the disease during the time they received melatonin	Brusco et al. (1998a)
Monozygotic twins with AD of 8 years duration	Case report	36 months	One of the patients was treated with melatonin 9 mg p.o./daily at bedtime	Neuropsychological assessment Neuroimaging	Sleep and cognitive function severely impaired in the twin not receiving melatonin as compared to the melatonin-treated twin	Brusco et al. (1998b)
11 AD patients	Open-label study	3 weeks	3 mg melatonin p.o./daily at bedtime	Daily logs of sleep and wake quality completed by the nurses	Significant decrease in agitated behaviors in all three shifts; significant decrease in daytime sleepiness	Cohen-Mansfield et al. (2000)
14 AD patients	Open-label, placebo-controlled trial	4 weeks	6 mg melatonin p.o./daily at bedtime or placebo	Daily logs of sleep and wake quality completed by caretakers. Actigraphy	AD patients receiving melatonin showed a significantly reduced percentage of nighttime activity compared to a placebo group	Mishima et al. (2000)

(continued)

Table 9.1 (continued)

Subjects	Design	Study's duration	Treatment	Measured	Results	Reference(s)
25 AD patients	Randomized double-blind placebo-controlled crossover study	7 weeks	6 mg of slow-release melatonin p.o. or placebo at bedtime	Actigraphy	Melatonin had no effect on median total time asleep, number of awakenings, or sleep efficiency	Serfaty et al. (2002)
45 AD patients	Open-label study	4 months	6–9 mg melatonin p.o./ daily at bedtime	Daily logs of sleep and wake quality completed by caretakers. Neuropsychological assessment	Melatonin improved sleep and suppressed sundowning, an effect seen regardless of the concomitant medication employed	Cardinali et al. (2002)
157 AD patients	Randomized, placebo-controlled clinical trial	2 months	2.5-mg slow-release melatonin, or 10-mg melatonin, or placebo at bedtime	Actigraphy. Caregiver ratings of sleep quality	Nonsignificant trends for increased nocturnal total sleep time and decreased wake after sleep onset were observed in the melatonin groups relative to placebo. On subjective measures, caregiver ratings of sleep quality showed a significant improvement in the 2.5-mg sustained-release melatonin group relative to placebo	Singer et al. (2003)
20 AD patients	Double-blind, placebo-controlled study	4 weeks	Placebo or 3 mg melatonin p.o./ daily at bedtime	Actigraphy. Neuropsychological assessment	Melatonin significantly prolonged the sleep time and decreased activity in the night. Cognitive function was improved by melatonin	Asayama et al. (2003)
7 AD patients	Open-label study	3 weeks	3 mg melatonin p.o./daily at bedtime	Actigraphy. Neuropsychological assessment	Complete remission of day-night rhythm disturbances or sundowning was seen in 4 patients, with partial remission in other 2	Mahlberg et al. (2004)

17 AD patients	Randomized, placebo-controlled study	2 weeks	3 mg melatonin p.o./daily at bedtime (7 patients). Placebo (10 patients)	Actigraphy. Neuropsychological assessment	In melatonin-treated group, actigraphic nocturnal activity and agitation showed significant reductions compared to baseline	Mahlberg and Walther (2007)
68-year-old man with AD who developed rapid eye movement (REM) sleep behavior disorder	Case report	20 months	5–10 mg melatonin p.o./ daily at bedtime	Polysomnography	Melatonin was effective to suppress REM sleep behavior disorder	Anderson et al. (2008)
50 AD patients	Randomized, placebo-controlled study	10 weeks	Morning light exposure (2,500 lx, 1 h) and 5 mg melatonin ($n=16$) or placebo ($n=17$) in the evening. Controls ($n=17$) received usual indoor light	Nighttime sleep variables, day sleep time, day activity, day/night sleep ratio, and rest–activity parameters were determined using actigraphy	Light treatment alone did not improve nighttime sleep, daytime wake, or rest-activity rhythm. Light treatment plus melatonin increased daytime wake time and activity levels and strengthened the rest-activity rhythm	Dowling et al. (2008)
41 AD patients	Randomized, placebo-controlled study	10 days	Melatonin (8.5 mg immediate release and 1.5 mg sustained release) ($N=24$) or placebo ($N=17$) administered at 10:00 P.M	Actigraphy	There were no significant effects of melatonin, compared with placebo, on sleep, circadian rhythms, or agitation	Gehrman et al. (2009)

The administration of melatonin (6 mg/day) for 4 weeks to AD patients reduced nighttime activity as compared to placebo (Mishima et al. 2000). An improvement of sleep and alleviation of sundowning were reported in 11 AD patients treated with melatonin (3 mg/day at bedtime) and evaluated by using actigraphy (Mahlberg et al. 2004). Improvement in behavioral signs was reported with the use of 6–9 mg/day of melatonin for 4 months in AD patients with sleep disturbances (Cardinali et al. 2002).

In a double-blind study conducted on AD patients, it was noted that 3 mg/day of melatonin significantly prolonged actigraphically evaluated sleep time, decreased activity in night, and improved cognitive functions (Asayama et al. 2003). In a multicenter, randomized, placebo-controlled clinical trial of a sample of 157 AD patients with sleep disturbances, melatonin or placebo was administered for a period of 2 months (Singer et al. 2003). In actigraphic studies a trend to increased nocturnal total sleep time and decreased wake after sleep onset was noted in the melatonin-treated group. On subjective measures by caregiver ratings, significant improvement in sleep quality was noted with 2.5 mg sustained-release melatonin relative to placebo (Singer et al. 2003).

Negative results with the use of melatonin in fully developed AD were also published. For example, in a study in which melatonin (8.5 mg fast release and 1.5 mg sustained release) was administered at 10:00 PM for ten consecutive nights to patients with AD, no significant difference was noticed with placebo on sleep, circadian rhythms, and agitation (Gehrman et al. 2009). Although the lack of beneficial effect of melatonin in this study on sleep could be attributed to the short period of time examined, it must be noted that large interindividual differences among patients suffering from a neurodegenerative diseases are not uncommon. It should be also taken into account that melatonin, though having some sedating and sleep latency-reducing properties, does not primarily act as a sleeping pill, but mainly as a chronobiotic.

A review of the published results concerning melatonin use in AD (Cardinali et al. 2010) yielded eight reports (five open-label studies, two case reports) ($N=89$ patients) supporting a possible efficacy of melatonin: sleep quality improved and in patients with AD sundowning was reduced and cognitive decay slowed progression. In six double-blind, randomized placebo-controlled trials, a total number of 210 AD patients were examined. Sleep quality increased, sundowning decreased significantly, and cognitive performance improved in four studies ($N=143$), whereas there was absence of effects in two studies ($N=67$) (Cardinali et al. 2010).

Another systematic search of studies published between 1985 and April 2009 on melatonin and sundowning in AD patients was published (de Jonghe et al. 2010). All papers on melatonin treatment in dementia were retrieved, and the effects of melatonin on circadian rhythm disturbances were scored by means of scoring sundowning/agitated behavior, sleep quality, and daytime functioning. A total of nine papers, including four randomized controlled trials ($n=243$) and five case series ($n=87$), were reviewed. Two of the randomized controlled trials found a significant improvement in sundowning/agitated behavior. All five case series found an improvement. The results on sleep quality and daytime functioning were inconclusive (de Jonghe et al. 2010).

Therefore, whether melatonin has any value in preventing or treating AD remains uncertain. It must be noted that one of the problems with AD patients with fully developed pathology is the heterogeneity of the group examined. Moreover, the reduced hippocampal expression of MT_2 melatonin receptors in AD patients (Savaskan et al. 2005) and of MT_1 receptors in the circadian apparatus at later stages of the disease may explain why melatonin treatment is less effective or erratic at this stage (Wu et al. 2007).

Mild cognitive impairment (MCI) is diagnosed in those who have an objective and measurable deficit in cognitive functions, but with a preservation of daily activities. The estimates of annual conversion rates to dementia vary across studies but may be as high 10–15 % (Farias et al. 2009), MCI representing a clinically important stage for identifying and treating individuals at risk. Indeed, the degenerative process in AD brain starts 20–30 years before the clinical onset of the disease (Davies et al. 1988; Price and Morris 1999). During this phase, plaques and tangle loads increase and at a certain threshold the first symptom appears (Braak and Braak 1995, 1998).

CSF melatonin levels decrease even in preclinical stages of AD when the patients do not manifest any cognitive impairment, suggesting that the reduction in CSF melatonin may be an early trigger and marker for AD (Zhou et al. 2003; Wu et al. 2003). Although it is not known whether the relative melatonin deficiency is either a consequence or a cause of neurodegeneration, it seems clear that the loss in melatonin aggravates the disease and that early circadian disruption can be an important deficit to be considered.

We previously reported a retrospective analysis in which daily 3–9 mg of a fast-release melatonin preparation p.o. at bedtime for up to 3 years significantly improved cognitive and emotional performance and daily sleep–wake cycle in 25 MCI patients (Furio et al. 2007). Recently we reported data from another series of 96 MCI outpatients, 61 of whom had received daily 3–24 mg of a fast-release melatonin preparation p.o. at bedtime for 15–60 months in comparison to a similar group of 35 MCI patients who did not receive it (Cardinali et al. 2012a). In addition, all patients received the individual standard medication considered appropriate by the attending psychiatrist.

Patients treated with melatonin exhibited significantly better performance in mini–mental state examination and the cognitive subscale of the AD Assessment Scale. After application of a neuropsychological battery comprising a Mattis' test, digit–symbol test, Trail A and B tasks, and the Rey's verbal test, better performance was found in melatonin-treated patients for every parameter tested (Cardinali et al. 2012a). Abnormally high Beck Depression Inventory scores decreased in melatonin-treated patients, concomitantly with the improvement in the quality of sleep and wakefulness. These results further support that melatonin is a useful add-on drug for treating MCI in a clinic environment.

Thus, an early initiation of treatment can be decisive for therapeutic success (Quinn et al. 2005). In Table 9.2, published data concerning melatonin treatment in MCI are summarized. Six double-blind, randomized placebo-controlled trials and two open-label retrospective studies ($N=782$) consistently showed that the

Table 9.2 Studies including treatment of MCI patients with melatonin

Subjects	Design	Study's duration	Treatment	Measured	Results	Reference(s)
10 patients with MCI	Double-blind, placebo-controlled, crossover study	10 days	6 mg melatonin p.o./ daily at bedtime	Actigraphy. Neuropsychological assessment	Melatonin enhanced the rest-activity rhythm and improved sleep quality. Total sleep time unaffected. The ability to remember previously learned items improved along with a significant reduction in depressed mood	Jean-Louis et al. (1998b)
26 individuals with age-related MCI	Double-blind, placebo-controlled pilot study	4 weeks	1 mg melatonin p.o. or placebo at bedtime	Sleep questionnaire and a battery of cognitive tests at baseline and at 4 weeks	Melatonin administration improved reported morning "restedness" and sleep latency after nocturnal awakening. It also improved scores on the California Verbal Learning Test-interference subtest	Peck et al. (2004)
354 individuals with age-related MCI	Randomized, double-blind, placebo-controlled study	3 weeks	Prolonged-release melatonin (Circadin, 2 mg) or placebo, 2 h before bedtime	Leeds Sleep Evaluation and Pittsburgh Sleep Questionnaires, and Clinical Global Improvement scale score and quality of life	PR-melatonin resulted in significant and clinically meaningful improvements in sleep quality, morning alertness, sleep onset latency, and quality of life	Wade et al. (2007)
60 MCI outpatients	Open-label, retrospective study	9–24 months	35 patients received daily 3–9 mg of a fast-release melatonin preparation p.o. at bedtime. Melatonin was given in addition to the standard medication	Daily logs of sleep and wake quality. Initial and final neuropsychological assessment	Abnormally high Beck Depression Inventory scores decreased in melatonin-treated patients, concomitantly with an improvement in wakefulness and sleep quality. Patients treated with melatonin showed significantly better performance in neuropsychological assessment	Cardinali et al. (2010) and Furio et al. (2007)

189 individuals with age-related cognitive decay	Long-term, double-blind, placebo-controlled, 2×2 factorial randomized study	1–3.5 years	Long-term daily treatment with whole-day bright (1,000 lx) or dim (300 lx) light. Evening melatonin (2.5 mg) or placebo administration	Standardized scales for cognitive and noncognitive symptoms, limitations of activities of daily living, and adverse effects assessed every 6 months	Light-attenuated cognitive deterioration and ameliorated depressive symptoms. Melatonin-shortened sleep onset latency and increased sleep duration but adversely affected scores for depression. The combined treatment of bright light plus melatonin showed the best effects	der Lek et al. (2008)
22 individuals with age-related cognitive decay	Prospective, randomized, double-blind, placebo-controlled, study	2 months	Participants received 2 months of melatonin (5 mg p.o. /day) and 2 months of placebo	Sleep disorders were evaluated with the Northside Hospital Sleep Medicine Institute (NHSMI) test. Behavioral disorders were evaluated with the Yesavage Geriatric Depression Scale and Goldberg Anxiety Scale	Melatonin treatment significantly improved sleep quality scores. Depression also improved significantly after melatonin administration	Garzon et al. (2009)
25 MCI outpatients	Randomized, double-blind, placebo-controlled study	12 weeks	11 patients received an oily emulsion of docosahexaenoic acid phospholipids containing melatonin (10 mg) and tryptophan (190 mg)	Neuropsychological assessment of orientation and cognitive functions, short-term and long-term memory, attentional abilities, executive functions, visuo-constructional and visuospatial abilities, language, and mood	Older adults with MCI had significant improvements in several measures of cognitive function when supplemented with an oily emulsion of DHA-phospholipids containing melatonin and tryptophan for 12 weeks, compared with the placebo. The antioxidant capacity of erythrocytes and membrane lipid composition improved after treatment	Cazzola et al. (2012) and Rondanelli et al. (2012)

(continued)

Table 9.2 (continued)

Subjects	Design	Study's duration	Treatment	Measured	Results	Reference(s)
96 MCI outpatients	Open-label, retrospective study	15–60 months	61 patients received daily 3–24 mg of a fast-release melatonin preparation p.o. at bedtime. Melatonin was given in addition to the standard medication	Daily logs of sleep and wake quality. Initial and final neuropsychological assessment	Abnormally high Beck Depression Inventory scores decreased in melatonin-treated patients, concomitantly with an improvement in wakefulness and sleep quality. Patients treated with melatonin showed significantly better performance in neuropsychological assessment. Only 6 out of 61 patients treated with melatonin needed concomitant benzodiazepine treatment vs. 22 out of 35 MCI patients not receiving melatonin	Cardinali et al. (2012a)

administration of daily evening melatonin improves sleep quality and cognitive performance in MCI patients. Therefore, melatonin treatment could be effective at early stages of the neurodegenerative disease.

There are two reasons why the use of melatonin is convenient in MCI patients. In the course of the neurodegenerative process, the age-related deterioration in circadian organization becomes significantly exacerbated and is responsible of behavioral problems like sundowning (Wu and Swaab 2007). Age-related cognitive decline in healthy older adults can be predicted by the fragmentation of the circadian rhythm in locomotor behavior. Hence, replacement of the low melatonin levels occurring in the brain (Zhou et al. 2003; Wu et al. 2003) can be highly convenient in MCI patients. On the other hand, the bulk of information on the neuroprotective properties of melatonin derived from experimental studies (see for ref. (Pandi-Perumal et al. 2013; Rosales-Corral et al. 2012)) turns highly desirable to employ pharmacological doses in MCI patients with the aim of arresting or slowing disease's progression.

The sleep-promoting activity of melatonin in humans has been known for years (Vollrath et al. 1981; Waldhauser et al. 1990), and a number of studies pointed to a beneficial effect of melatonin in a wide variety of sleep disorders (see for ref. (Cardinali et al. 2012b)). However, controversy continues to surround claims of melatonin's therapeutic potential. A meta-analysis on the effects of melatonin in sleep disturbances at all age groups (including young adults with presumably normal melatonin levels) failed to document significant and clinically meaningful effects of exogenous melatonin on sleep quality, efficiency, and latency (Buscemi et al. 2006). However, another meta-analysis involving 17 controlled studies in old subjects has shown that melatonin was effective in increasing sleep efficiency and in reducing sleep onset latency (Brzezinski et al. 2005). After the approval by the European Medicines Agency of a prolonged-release form of 2 mg melatonin (Circadin®, Neurim, Tel Aviv, Israel) for treatment of insomnia in patients ≥55 years of age, a recent consensus of the British Association for Psychopharmacology on evidence-based treatment of insomnia, parasomnia, and circadian rhythm sleep disorders concluded that prolonged-release melatonin is the first-choice treatment when a hypnotic is indicated in old patients (Wilson et al. 2010).

In addition to sleep promotion, melatonin has a mild sedating effect. This may be the cause for the decrease in Beck's score seen in MCI studies. Melatonin has a facilitatory effect on GABAergic transmission (Cardinali et al. 2008) which may be responsible for the anticonvulsant, anxiolytic, antihyperalgesic, and antinociceptive effects of the methoxyindole.

The mechanisms accounting for the therapeutic effect of melatonin in MCI patients remain to be defined. Melatonin treatment mainly promotes slow-wave sleep in the elderly (Monti et al. 1999) and can be beneficial in MCI by augmenting the restorative phases of sleep, including the augmented secretion of GH and neurotrophins. As outlined above, melatonin acts at different levels relevant to the development and manifestation of AD. The antioxidant, mitochondrial, and anti-amyloidogenic effects can be seen as a possibility of interfering with the onset of the disease. Therefore, to start melatonin treatment as soon as possible can be decisive for the final response (Quinn et al. 2005).

One important aspect to be considered is the melatonin dose employed, which may be unnecessarily low when one takes into consideration the binding affinities, half-life, and relative potencies of the different melatonin agonists on the market. In addition to being generally more potent than the native molecule, melatonin analogs are employed in considerably higher amounts (Cardinali et al. 2011a). Licensed doses of the melatonin receptor agonist ramelteon vary from 8 to 32 mg/day while agomelatine has been licensed for treatment of major depressive disorder at doses of 25–50 mg/day. In clinical studies involving healthy human subjects, tasimelteon, another melatonin receptor agonist (Vanda Pharmaceuticals, Washington, DC, USA), was administered at doses of 10–100 mg/day (Rajaratnam et al. 2009), while pharmacokinetics, pharmacodynamics, and safety of the melatonin receptor agonist TIK-301 (Tikvah Pharmaceuticals, Atlanta, GA, USA) have been examined in a placebo-controlled study using 20–100 mg/day (Mulchahey et al. 2004). Therefore, studies in MCI with melatonin doses in the range of 75–100 mg/day are further warranted.

Indeed, melatonin has a high safety profile; it is usually remarkably well tolerated and, in some studies, it has been administered to patients at very large doses. Melatonin (300 mg/day) for up to 3 years decreased oxidative stress in patients with amyotrophic lateral sclerosis (Weishaupt et al. 2006). In children with muscular dystrophy, 70 mg/day of melatonin reduced cytokines and lipid peroxidation (Chahbouni et al. 2010). Doses of 80 mg melatonin hourly for 4 h were given to healthy men with no undesirable effects other than drowsiness (Waldhauser et al. 1984). In healthy women given 300 mg melatonin/day for 4 months, there were no side effects (Voordouw et al. 1992). A recent randomized controlled double-blind clinical trial on 50 patients referred for liver surgery indicated that a single preoperative enteral dose of 50 mg/kg melatonin (i.e., an equivalent to 3 g for a 60-kg adult) was safe and well tolerated (Nickkholgh et al. 2011).

Another outcome of the study reported in (Cardinali et al. 2012a) was that when melatonin is employed much less benzodiazepines are needed to treat sleep disturbances in MCI. Since, as abovementioned, melatonin and benzodiazepines shared some neurochemical (i.e., interaction with GABA-mediated mechanisms in the brain (Cardinali et al. 2008)) and behavioral properties (e.g., a similar day-dependent anxiolytic activity (Golombek et al. 1996)), melatonin therapy was postulated to be an effective tool to decrease the dose of benzodiazepines needed in patients (Fainstein et al. 1997; Dagan et al. 1997; Garfinkel et al. 1999; Siegrist et al. 2001). A recent retrospective analysis of a German prescription database identified 512 patients who had initiated treatment with prolonged-release melatonin (2 mg) over a 10-month period (Kunz et al. 2012). From 112 patients in this group who had previously used benzodiazepines, 31 % discontinued treatment with benzodiazepines 3 months after beginning prolonged-release melatonin treatment. The discontinuation rate was higher in patients receiving two or three melatonin prescription (Kunz et al. 2012). The prolonged use of benzodiazepines and benzodiazepine receptor agonists (Z-drugs) is related to severe withdrawal symptoms and potential dependency which has become a public health issue leading to multiple campaigns to decreases consumption of these drugs. A recent pharmacoepidemiological study concluded that these campaigns generally failed when they were not associated with the availability and market of melatonin (Clay et al. 2013).

In conclusion, the question as to whether melatonin has a therapeutic value in preventing or treating MCI, affecting disease initiation or progression of the neuropathology and the driving mechanisms, deserved further analysis in future studies. Double-blind multicenter studies are needed to further explore and investigate the potential and usefulness of melatonin as an antidementia drug at the early stage of disease.

9.5 Basic Aspects of Melatonin Activity in Animal Models of PD

PD is a major neurodegenerative disease characterized, in its clinically relevant stages, by the progressive degeneration of dopamine (DA)-containing neurons in the substantia nigra (Rothman and Mattson 2012; Seppi et al. 2011). Typical of PD are cellular inclusions called Lewy bodies. They are single or multiple intraneuronal inclusions selectively distributed in the cytoplasm and having various sizes and shapes depending on the brain area that is affected. Lewy bodies have a relatively restricted distribution and are usually associated with DA neurons of the substantia nigra pars compacta (SNpc) and ventral tegmental region, noradrenergic neurons of the locus coeruleus, catecholamine cells of the medulla oblongata, serotoninergic neurons of the raphe nuclei, and specific cholinergic neurons (Rothman and Mattson 2012; Seppi et al. 2011).

Several studies indicate that accumulation of fibrillar α-synuclein aggregates is associated with PD and other Lewy body diseases (Fornai et al. 2005). Mitochondrial dysfunction plays a role in this process. Protein misfolding and aggregation in vivo can be suppressed or promoted by several factors, among them free radicals. It has thus been postulated that aggregation of α-synuclein might be one of many possible links that connect mitochondrial dysfunction to neurodegeneration (Fornai et al. 2005).

Animal models of altered brain DA function have been developed by injecting 6-hydroxydopamine (6-OHDA) into the nigrostriatal pathway of the rat or by injecting the neurotoxin 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP). MPTP-induced parkinsonism in animals is preferred over the other neurotoxin-induced models due to its potential to cause the disease in humans and in subhuman primates. MPTP is selective to the neurons in SNpc region and causes severe loss of striatal spines in nonhuman primates (Herraiz and Guillen 2011), a consistent neuropathologic phenomenon observed in postmortem PD brains.

MPTP administered to rats is selectively taken up by astrocytes and is metabolized into methyl 1-4 phenyl pyridinium (MPP^+). This cation is selectively taken up by dopaminergic neurons and causes increased production of free radicals, depletion of ATP, and apoptosis. In the case of 6-OHDA, the neurotoxin selectively destroys nigrostriatal neurons by causing enhanced release of free radicals. It should be stressed, however, that these animal models do not reflect the prodromal early changes in upper spinal cord and brain stem seen in PD and therefore are presumably meaningless in terms of etiology.

With some exceptions the role of melatonin in prevention and treatment of experimental PD is now supported by experimental data. Acuña-Castroviejo et al.

used an MPTP model to show that melatonin could counteract MPTP-induced lipid peroxidation in striatum, hippocampal, and midbrain regions (Acuña-Castroviejo et al. 1997). Using the 6-OHDA model, Mayo et al. showed that when added to incubation medium containing 6-OHDA, melatonin significantly prevented the increased lipid peroxidation which normally would have occurred in cultured PC 12 cells (Mayo et al. 1998). Melatonin also increased the levels of antioxidant enzymes (Mayo et al. 1998). Additionally, melatonin reduced pyramidal cell loss in the hippocampus, a cellular area which undergoes degeneration in the brains of PD patients and which presumably causes memory deficits in affected patients. Thomas and Mohanakumar similarly demonstrated in vitro and ex vivo models, as well as in an in vivo MPTP rodent model, that melatonin had potent hydroxyl radical scavenger activity in the mouse striatum and in isolated mitochondria (Thomas and Mohanakumar 2004). In addition to these primary effects, the investigators also found secondary increases in SOD activity.

The attenuation of MPTP-induced superoxide formation indicates an additional neuroprotective mechanism by melatonin. Intra-median forebrain bundle infusion of a ferrous-ascorbate-DA hydroxyl radical (•OH) generating system, which causes significant depletion of striatal DA, could be significantly attenuated by melatonin administration (Borah and Mohanakumar 2009). In another study, Antolín et al. used the MPTP model and found that melatonin was effective in preventing neuronal cell death in the nigrostriatal pathway as indicated by the number of preserved DA cells, of tyrosine hydroxylase levels, and other ultrastructural features (Antolin et al. 2002). The findings thus demonstrated that melatonin clearly prevents nigral dopaminergic cell death induced by chronic treatment with MPTP.

α-Synuclein assembly is a critical step in the development of Lewy body diseases such as PD and dementia with Lewy bodies. Melatonin attenuated kainic acid-induced neurotoxicity (Chang et al. 2012) and arsenite-induced apoptosis (Lin et al. 2007) via inhibition of α-synuclein aggregation. Melatonin also decreased the expression of α-synuclein in dopamine-containing neuronal regions after amphetamine both in vivo (Sae-Ung et al. 2012) and in vitro (Klongpanichapak et al. 2008). In another study melatonin effectively blocked α-synuclein fibril formation and destabilized preformed fibrils. It also inhibited protofibril formation, oligomerization, and secondary structure transitions of α-synuclein as well as reduced α-synuclein cytotoxicity (Chang et al. 2012; Brito-Armas et al. 2013).

MPTP elicits its neurotoxic effects by increasing the amount of •NO derived from iNOS. This action mainly affects DA neurons while •NO derived from neuronal NOS (nNOS) has a damaging effect on dopaminergic fibers and terminals in the striatum. A future therapy for PD may require agents that inhibit the degenerative effects of iNOS in the substantia nigra pars compacta (Zhang et al. 2000). Since melatonin can effectively downregulate iNOS and prevent •NO formation in the brain (Cuzzocrea et al. 1997; Escames et al. 2004), it should be regarded as a drug of choice for arresting the neuronal degeneration associated with PD.

MPTP, through its metabolite MPP^+, causes direct inhibition of Complex I of the mitochondrial electron transport chain. Such an inhibition of Complex I has been reported in the substantia nigra of patients suffering from PD. By increasing Complex I and IV activities of the mitochondrial electron transport chain, melatonin

exerts one of its antioxidant effects (Acuña-Castroviejo et al. 2011). Melatonin also stimulates the gene expression of three antioxidant enzymes Cu/Zn-SOD, Mn-SOD, and GPx in cultured dopaminergic cells (Mayo et al. 1998).

Symptomatically effective treatment for PD in modern medicine is by supplementation of DA in its precursor form that crosses the blood–brain barrier. However, long-term administration DA precursor typically leads to motor complications, such as L-dihydroxyphenylalanine (L-DOPA)-induced dyskinesias (Carta et al. 2004; Werneke et al. 2006). It is also shown that administration of this drug in high doses leads to generation of neurotoxic molecules such as 6-OHDA. Therefore, efforts are in the vogue to reduce the intake or to compensate for the side effects of this drug. In a recent study undertaken to examine whether melatonin could potentiate the effect of a low dose of L-DOPA in MPTP-induced experimental parkinsonism in mice, melatonin, but not L-DOPA, restored spine density and spine morphology of medium spiny neurons in the striatum suggesting that melatonin could be an ideal adjuvant to L-DOPA therapy in PD, making it possible to bring down the therapeutic doses of L-DOPA (Naskar et al. 2013).

It has been proposed that an abnormal assembly of the cytoskeleton is involved in the pathogenesis of neurodegenerative diseases. Lewy bodies, which are considered to be cytopathologic markers of parkinsonism, comprise abnormal arrangements of tubulin, MAP 1 and MAP 2 (Beach et al. 2009). Melatonin is very effective in promoting cytoskeletal rearrangements and thus may have a potential therapeutic value in the treatment of neurodegenerative diseases including parkinsonism (Benitez-King et al. 2004).

It must be noted that other studies do not support the hypothesis that melatonin is of therapeutic benefit in parkinsonism. For instance, reduction of melatonin by pinealectomy, or by exposure of rats to bright light to inhibit melatonin synthesis, has been found to enhance recovery from parkinsonism, i.e., spontaneous remission of symptoms following 6-OHDA or MPTP have been observed, whereas melatonin administration aggravated them (Willis and Armstrong 1999; Tapias et al. 2010), using a rotenone model of PD in rats, found that melatonin administration led to striatal catecholamine depletion, striatal terminal loss, and nigral DA cell loss and thus was not neuroprotective. Indeed, the use of melatonin as an adjunct therapy to either halt progressive degeneration or for providing symptomatic relief in PD patients has been questioned (Willis and Robertson 2004).

9.6 Clinical Aspects of Melatonin Application in PD

Key symptoms of PD such as tremor, rigidity, bradykinesia, and postural instability develop when about three-fourth of dopaminergic cells are lost in the SNpc, and consequently the smooth, coordinated regulation of striatal motor circuits is hampered (Maguire-Zeiss and Federoff 2010; Tansey et al. 2007). However, PD does not start in the nigrostriatum, but rather in the brainstem or even the spinal cord of subjects who remain asymptomatic for a long period of time (Braak et al. 2003).

Other, non-motor symptoms are seen in PD, and some of them, such as hyposmia, depression, or rapid eye movement (REM)-associated sleep behavior disorder (RBD), can precede the onset of disease. Non-motor symptoms are often misdiagnosed and untreated, although their appearance is an index of a worse prognosis and lower quality of life. Indeed up to 65 % of patients diagnosed with RBD, which is characterized by the occurrence of vivid, intense, and violent movements during REM sleep, subsequently developed PD within an average lag time of 12–13 years.

Administration of melatonin 3–12 mg at bedtime has been shown to be effective in the treatment of RBD (Kunz and Bes 1997, 1999; Takeuchi et al. 2001; Boeve et al. 2003; Anderson and Shneerson 2009). A total of 119 patients have been reported (Table 9.3). For example, in a study reporting the records of 45 consecutive RBD patients seen at Mayo Clinic between 2008 and 2010, 25 patients receiving melatonin (6 mg daily) reported significantly reduced injuries and fewer adverse effects (McCarter et al. 2013).

Polysomnography showed statistically significant decreases in the number of R epochs without atonia and in the movement time in R. This contrasted with the persistence of tonic muscle tone in R sleep seen with patients treated with clonazepam. Because of these data a clinical consensus recommended melatonin use in RBD at Level B, i.e., "assessment supported by sparse high grade data or a substantial amount of low-grade data and/or clinical consensus by the task force" (Aurora et al. 2010). In another consensus statement generated in 2011, a claim for eventual trials with disease-modifying and neuroprotective agents in RBD was urged based on the high conversion rate from idiopathic RBD to parkinsonian disorders (Schenck et al. 2013). Six inclusion criteria and 24 exclusion criteria were identified for symptomatic therapy and neuroprotective trials (Schenck et al. 2013).

At this time, there is no treatment that will delay or stop the progression of PD, and medications currently available are mostly symptomatic. The increasing incidence of age-associated neurodegenerative diseases has been attributed to the augmented generation of free radicals and the associated oxidative stress, which is enhanced in certain regions of the aging brain (Gibson et al. 2010; Olanow 1992; Fahn and Cohen 1992). Increased lipid peroxidation, decreased levels of GSH, and increased iron levels occur in the brains of patients suffering from parkinsonism (Dexter et al. 1989). As the increased iron levels can promote the Fenton reaction, it seems feasible that an increased hydroxyl radical formation induces free radical damage. Free radical damage of lipids, proteins, and nucleic acids has all been reported in the substantia nigra of parkinsonian patients (Alam et al. 1997). Oxidative stress has been suggested to be the major cause of dopaminergic neuronal cell death. Exposure to high concentrations of H_2O_2 that are formed during oxidation of DA by monoamine oxidase (MAO) may also be a major cause for destruction of dopaminergic neurons in parkinsonism (Fahn and Cohen 1992). Therefore, within this context the cytoprotective properties exhibited by melatonin are promising as a tool in PD prevention.

The study of melatonin secretion in PD has revealed some interesting findings. In related studies a phase advance in nocturnal melatonin levels in L-DOPA-treated parkinsonian patients was noted, but this was not observed in untreated patients when

Table 9.3 Studies including treatment of PD and RBD patients with melatonin

Subjects	Design	Study's duration	Treatment	Measured	Results	Reference(s)
40 PD patients	Open-label, placebo-controlled trial	2 weeks	5–50 mg melatonin p.o./daily at bedtime. All subjects were taking stable doses of antiparkinsonian medications	Actigraphy	Relative to placebo, treatment with 50 mg of melatonin significantly increased nighttime sleep, as revealed by actigraphy. As compared to 50 mg or placebo, administration of 5 mg of melatonin was associated with significant improvement of sleep in the subjective reports	Dowling et al. (2005)
18 PD patients	Open-label, placebo-controlled trial	4 weeks	3 mg melatonin p.o./ daily at bedtime	Polysomnography (PSG). Subjective evaluation by the Pittsburgh Sleep Quality Index and Epworth Sleepiness Scale	On initial assessment, 14 patients showed poor-quality sleep EDS. Increased sleep latency (50 %), REM sleep without atonia (66 %), and reduced sleep efficiency (72 %) were found in PSG. Melatonin significantly improved subjective quality of sleep. Motor dysfunction was not improved by the use of melatonin	Medeiros et al. (2007)
38 patients with PD without dementia and with complaints on sleep disorders	Open-label trial	6 weeks	Group 1 ($n=20$) received 3 mg melatonin in addition to the previous dopaminergic group 2 ($n=18$) received clonazepam 2 mg at night	Polysomnography (PSG) at baseline and at the end of the trial. Subjective evaluation by the PD sleep scale (PDSS) and the Epworth Sleepiness Scale (ESS). Neuropsychological testing using MMSE, five-word test, digit span, and the Hamilton scale	Compared to baseline, melatonin and clonazepam reduced sleep disorders in patients. The daytime sleepiness (ESS) was significantly increased in the clonazepam group. Patients treated with melatonin had better scores on the MMSE, five-word test, Hamilton scale at the end of the study period as compared with the clonazepam group. Changes in total point scores on the PSG at the end of week 6 were in favor of the group treated with melatonin	Litvinenko et al. (2012)
1 RBD patient	Case report	5 months	3 mg melatonin p.o./ daily at bedtime	Actigraphy, PSG	Significant reduction of motor activity during sleep, as measured by actigraphy. After 2 months' treatment, PSG showed no major changes except an increase of REM sleep	Kunz and Bes (1997)

(continued)

Table 9.3 (continued)

Subjects	Design	Study's duration	Treatment	Measured	Results	Reference(s)
6 consecutive RBD patients	Open-label prospective case series	6 weeks	3 mg melatonin p.o./daily at bedtime	PSG	Significant PSG improvement in 5 patients within a week which extended beyond the end of treatment for weeks or months	Kunz and Bes (1999)
14 RBD patients	Open-label prospective case series	Variable	3–9 mg melatonin p.o./daily at bedtime	PSG	Thirteen patients and their partners noticed a suppressing effect on problem sleep behaviors after melatonin administration. % tonic REM activity in PSG findings was decreased after melatonin administration. Melatonin concentrations in 10 RBD patients were under 30 pg/mL at maximal values; their mean 33.5 pg/mL RBD patients with low melatonin secretion tended to respond to melatonin therapy	Takeuchi et al. (2001)
14 RBD patients	Retrospective case series	14 months	3–12 mg melatonin p.o./daily at bedtime	PSG	8 patients experienced continued benefit with melatonin beyond 12 months of therapy	Boeve et al. (2003)
39 RBD patients	Retrospective case series		All initially treated with clonazepam. When melatonin was used, it was given at a 10 mg p.o./daily at bedtime		21 patients continued to take clonazepam, 8 used another medication, and 4 required a combination of medications to control symptoms adequately. Zopiclone was used in 11 patients either alone or in combination. Two patients used melatonin (10 mg) and both found it effective. Combination therapy (clonazepam/gabapentin/melatonin) was used in one patient	Anderson and Shneerson (2009)
25 RBD patients	Retrospective case series	27–53 months	6 mg melatonin p.o./daily at bedtime		As compared to clonazepam-treated RBD patients ($n=18$), patients receiving melatonin reported significantly reduced injuries and fewer adverse effects	McCarter et al. (2013)

compared to control subjects (Fertl et al. 1993). Similar findings were noted in studies in which a phase advance of about 2 h in plasma melatonin secretion was seen in PD patients receiving dopaminergic treatment when compared to untreated patients (Bordet et al. 2003). This study also confirmed previous findings that L-DOPA treatment influenced melatonin secretion rhythmicity. An increase in daytime melatonin secretion was also noted in L-DOPA-treated patients. An increase in melatonin secretion may be one of the adaptive responses to neurodegeneration (Bordet et al. 2003) and could play a neuroprotective role through an antioxidant effect.

The occurrence of motor fluctuations in PD was related to fluctuations in serum melatonin levels, a finding that was attributed to interactions of monoamines with melatonin in the striatal complex (Escames et al. 1996). Melatonin may exert direct motor effects through its interactions with DA and serotonin. Changes in levodopa-related motor complications may be related to changes in melatonin secretion pattern. L-DOPA-related motor complications occur in nearly half of the patients with PD on completion of the first 5 years of treatment (Koller 1996), and as noted above, results on experimental parkinsonism in mice support the use of melatonin as an adjuvant to L- to bring down the therapeutic doses of L-DOPA in PD (Naskar et al. 2013).

The hypothesis that melatonin has an inhibitory motor effect which is probably involved in wearing-off episode (i.e., the progressively shorter intervals during which symptoms remain adequately controlled as if the effects of medication would start to "wear off") has been supported by some therapeutic studies. Stimulation of globus pallidus inhibited an increase in daytime plasma melatonin levels in parkinsonian patients as compared to healthy subjects (Catala et al. 1997) and was also reported to improve motor symptoms and complications in patients with PD (Olanow et al. 2000). Melatonin may be useful in halting or retarding the progressive degeneration of PD and may hold further promise for inhibiting the L-DOPA-related motor complications.

Because of the lower rates of cancer mortality/incidence in patients with PD, speculations about risk or preventative factors common to both diseases, including lifestyle factors (such as smoking) and genetic susceptibility, have been entertained. Relevant to the subject of the present review is that preliminary epidemiological evidence suggests that longer years of working night shifts are associated with reduced melatonin levels and reduced risk of PD among, whereas longer hours of sleep appear to increase their risk (Schernhammer and Schulmeister 2004). While lower melatonin concentrations may predict a higher cancer risk, there is also some evidence that they may be associated with a lower risk of PD.

The finding that a reduced expression of melatonin MT_1 and MT_2 receptors occurs in amygdala and substantia nigra in patients with PD (Adi et al. 2010) indicates that there is a possibility that the melatonergic system is involved in the abnormal sleep mechanisms seen as well as in its overall pathophysiology. Melatonin has been used for treating sleep problems, insomnia, and daytime sleepiness in PD patients. In a study undertaken on 40 patients (11 women, 29 men; range 43–76 years) melatonin was administered for a treatment period of 2 weeks, in doses ranging from 5 mg to 50 mg/day (Dowling et al. 2005). To avoid the possibility of producing a circadian shift, melatonin was administered 30 min before bedtime (circadian

shifts can occur if administered melatonin is administered at any other time). All subjects were taking stable doses of antiparkinsonian medications during the course of the study. Relative to placebo, treatment with 50 mg of melatonin significantly increased nighttime sleep, as revealed by actigraphy. As compared to 50 mg or placebo, administration of 5 mg of melatonin was associated with significant improvement of sleep in the subjective reports. The study also found that the high dose of melatonin (50 mg) was well tolerated (Dowling et al. 2005).

In another study 18 PD patients were randomized after performing a basal polysomnography to receive melatonin (3 mg) or placebo 1 h before bedtime for 4 weeks (Medeiros et al. 2007). Subjective sleep quality was assessed by the Pittsburgh Sleep Quality Index and daytime somnolence by the Epworth Sleepiness Scale. All measures were repeated at the end of treatment. On initial assessment, 14 patients (70 %) showed poor-quality sleep and 8 (40 %) excessive diurnal somnolence. Increased sleep latency (50 %), REM sleep without atonia (66 %), and reduced sleep efficiency (72 %) were found in PSG. Sleep fragmentation tended to be more severe in patients on lower doses of L-DOPA, although melatonin significantly improved subjective quality of sleep. The objective abnormalities remained unchanged. Motor dysfunction was not improved by the use of melatonin (Medeiros et al. 2007).

Exposure to light of 1,000–1,500 lx intensity for 1–1.5 h, 1 h prior to bedtime for 2–5 weeks, has been found to improve the bradykinesia and rigidity observed in 12 PD patients (Willis and Turner 2007). A reduction in agitation and psychiatric side effects was also reported in this study. The authors suggested that activation of the circadian system by antagonizing melatonin secretion with bright light has a therapeutic value for treating the symptoms of PD (Willis 2008).

However, bright light has been employed in a number of studies for treating depressive symptoms, and the view has been advanced that suppression of melatonin secretion is not the likely mechanism by which artificial light exerts its therapeutic effect (Rosenthal et al. 1984). Two possible mechanisms have been proposed for the therapeutic effect of bright light. Firstly, bright light could reset the phase of abnormal circadian rhythms seen in depressed patients (Lewy et al. 1984). Secondly, although evening bright light exposure produces a momentary suppression of melatonin, it actually causes a rebound increase in melatonin secretion late in the night (Beck-Friis et al. 1985). The fact that bright light exposure ultimately facilitates melatonin secretion rather than suppressing it is said to be responsible for the therapeutic efficacy of bright light in affective disorders. Hence in the case of PD, bright light may improve the symptoms of PD, not by antagonizing melatonin secretion but by increasing it through a rebound effect.

Indeed, the bright light effect may be indicative of circadian changes in PD. This is supported by the reduced *Bmal1* mRNA expression in leukocytes (Cai et al. 2010), although effects in peripheral oscillators do not necessarily allow conclusions on changes in the hypothalamic master clock. The finding that the mouse striatal DA receptors D1R and D2R are under circadian control (Cai et al. 2010), can be seen as an interesting facet in this context, although circadian variations in receptor expression are by no means exceptional features.

9.7 Conclusions

As melatonin exhibits both hypnotic and chronobiotic properties, it has been therapeutically used for treatment of age-related insomnia as well as of other primary and secondary insomnia (Leger et al. 2004; Zhdanova et al. 2001). A recent consensus of the British Association for Psychopharmacology on evidence-based treatment of insomnia, parasomnia, and circadian rhythm sleep disorders concluded that melatonin is the first-choice treatment when a hypnotic is indicated in patients over 55 years (Wilson et al. 2010).

As shown by the binding affinities, half-life, and relative potencies of the different melatonin agonists in the market, it is clear that studies using 2–5 mg melatonin/day are unsuitable to give appropriate comparison with the effect of the abovementioned compounds, which, in addition to being generally more potent than the native molecule, are employed in considerably higher amounts (Cardinali et al. 2011b). Melatonin has a high safety profile and it is usually remarkably well tolerated. In some studies melatonin has been administered to patients at large doses (Weishaupt et al. 2006; Chahbouni et al. 2010; Waldhauser et al. 1984; Voordouw et al. 1992). Therefore, further studies employing melatonin doses in the 100 mg/day are needed to clarify its potential therapeutical implications in humans. From animal studies it is clear that a number of preventive effects of melatonin, like those in neurodegenerative disorders, need high doses of melatonin to become apparent (Cardinali et al. 2010; Srinivasan et al. 2011a, b). If one expects melatonin to be an effective neuroprotector, especially in aged people, it is likely that the low doses of melatonin employed so far are not very beneficial.

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Conflict of Interest None is declared.

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Chapter 10
Tryptophan Metabolism and Sleep

Oguz Kokturk and Asiye Kanbay

Abstract Sleep and sleep medicine are in the limelight of the researchers for thousands of years. The definition of sleep started to change in the light of scientific experiments performed in the twentieth century, and the number of investigations on sleep medicine increased tremendously. Effects of nutritional factors on the regulation of the sleep-wake cycle and central nervous system triggered new developments in sleep medicine. Tryptophan is an essential amino acid and a precursor of serotonin, melatonin, and nicotinamide. More recently, the role of tryptophan played in the sleep-wake rhythm of newborns has been an interest of research. High levels of tryptophan are associated with the improvement in the total hours and the efficiency of sleep and increase in the duration of nocturnal immobility and decrease in both the number of nocturnal awakenings and the sleep latency of newborns. Serotonin is named as "neurohormone of sleep" after understanding the key role of serotonin in the mechanisms of the sleep-wake cycle. Inhibition of tryptophan hydroxylase enzyme decreases both the synthesis of serotonin from tryptophan and serotonin levels significantly leading to insomnia. The serotonergic system has a crucial impact on sleep and airway stabilization. Majority of investigations on serotonin have shown that serotonergic system is related to obstructive sleep apnea syndrome (OSAS). Melatonin is secreted by the pineal gland. It is synthesized from 5-HT by pinealocytes and then released into the blood and cerebrospinal fluid. It is well known that melatonin affects sleep, circadian rhythm, puberty, antioxidant status, aging, and blood pressure. Contrary to other sleep disorders, scarce data on the involvement of melatonin in OSAS are available. Investigators have shown that plasma melatonin levels are low in patients with newly diagnosed OSAS. Elaborate prospective studies should be conducted while bearing in mind the drawbacks of aforementioned studies to better delineate the role of tryptophan and its products in

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the pathogenesis, adverse outcome of sleep disorders and to answer whether consumption of tryptophan-rich foods and its products might be a novel pharmacological treatment or not in sleep medicine.

Keywords Sleep • Tryptophan • Melatonin • Serotonin • Apnea

10.1 Sleep

Sleep is a natural periodic state of rest for the mind and body during which the eyes are usually closed and consciousness is completely or partially lost. In addition, there is a decrease in bodily movement and responsiveness to external stimuli. Hippocrates postulated a humoral mechanism for sleep and asserted that sleep is caused by the retreat of the blood and related warmth into the inner parts of the body (Walsh 1986). Two thousand years ago, Lucretius delineated sleep as an "absence of wakefulness" (Moruzzi 1964). Several theories for the function of sleep have been presented, such as to restore, to conserve energy to consolidate and reinforce memory, thermoregulation, and to maintain the integrity of the synaptic and neuron networks, but none of them adequately describe the main biological functions of sleep (Chokroverty 2009). Researchers today are still asking the question: "Why do we sleep every day?" After understanding that the sleep is essential for human life, research on sleep has accelerated. In the past three decades, laboratory studies with human volunteers have disclosed new information about different types of sleep. They have learned about the cyclical patterns of sleep and their relationships to breathing, heart rate, brain waves, and other physical functions. Using electroencephalography (EEG), electrooculography, and electromyelography, researchers have determined that sleep is divided into two main stages: non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. NREM sleep is divided to three stages (Chokroverty 2003). In Stage 1, NREM sleep occurs while an individual is falling asleep. In Stage 2, NREM sleep is the start of real sleep. In this stage, EEGs have shown different forms of waves, which are called sleep spindles and K complexes. In Stage 3, delta or slow-wave sleep occurs; these are the deepest levels of human sleep and are prominent during the first 30–50 % of a sleeping period. REM sleep accounts for approximately 25 % of one's total sleep time. It usually begins about 90 min (REM latency) after a person falls asleep. This stage is prominent in the second half of the night. Sleep disorders are a group of syndromes characterized by disturbances in the amount, quality, or timing of sleep or by behaviors or physiological conditions that negatively affect sleep. Recently, sleep disorders have been divided into seven major categories such as insomnia, sleep-related breathing disorders, central disorders of hypersomnolence, circadian rhythm sleep-wake disorders, parasomnias, sleep-related movement disorders, and other sleep disorders. These major groups include over 80 specific diagnoses (American Academy of Sleep Medicine 2014).

10.2 Tryptophan and Sleep

Tryptophan is an essential amino acid derived from food. It has a direct effect on the homeostatic regulation of sleep (Yao et al. 2011). This amino acid is the precursor of serotonin and melatonin, which play roles in the regulation of circadian rhythms (Hajak et al. 1991a; Garau et al. 2012). Tryptophan hydroxylase is the rate-limiting enzyme for the production of serotonin which involves in the conversion of tryptophan to 5-hydroxytrptophan (5-HT). The production of this enzyme can be inhibited by insulin resistance, stress, magnesium, or vitamin B6 deficiency, and its levels decrease with aging (Hussain and Mitra 2000) (Fig. 10.1). The main sleep regulation product of tryptophan is 5-HT, which affects sleep in several ways, and it is converted into serotonin by decarboxylation on the presence of the active form of vitamin B6. Serotonin is then converted into melatonin by S-adenosyl-L-methionine (Lucini et al. 1996). Nocturnal administration of tryptophan has been shown to increase physiological concentrations of serotonin and melatonin (Estaban et al. 2004). Darkness signals the secretion of melatonin from 5-HT in the pineal gland (Vaneck 1998). In other words, in the evening, the synthesis of melatonin is activated, and serotonin is converted into melatonin. Melatonin has an indolamine base. Specialized photoreceptive cells in the retina detect light, and this information goes directly to the suprachiasmatic nuclei through the retinohypothalamic tract and indirectly through the geniculohypothalamic tract. Activation of N-acetyltransferase system by this information is the limiting step in melatonin synthesis (Sánchez et al. 2008). Production of melatonin is increased during the night in the pineal gland, and it is inhibited by light. The circadian rhythm of melatonin release from the pineal gland in humans is highly synchronized with habitual hours of sleep. It has been shown that the secretion of melatonin is positively correlated with the onset of nocturnal sleepiness (Shochat et al. 1998). The effect of tryptophan on sleep was first

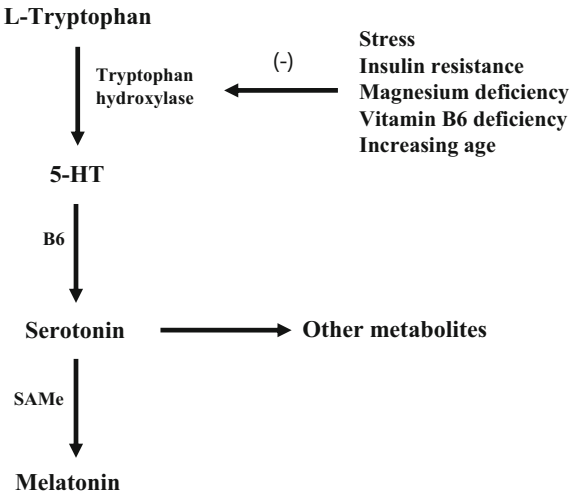

Fig. 10.1 Tryptophan metabolism. Abbreviations: *5-HT* 5-hydroxytryptophan, *SAMe* S-adenosyl-L-methionine

noted by Oswald et al. in 1966. They reported that 5–10 g of tryptophan decreased the time interval up to the onset of REM sleep in healthy adults (Oswald et al. 1966). Between 1980 and 1986, three studies reported positive effects of tryptophan on sleep (Cole 1980; Hartmann and Greenwald 1984; Young 1986). This remarkable finding encouraged many people to consume this hormone on daily basis. Unfortunately, because consumption of excessive amounts of tryptophan used to treat sleep disorders resulted in development of a deadly autoimmune illness called eosinophilia-myalgia syndrome, uncontrolled use of this product was banned in the United States (Kilbourne et al. 1996). Today, tryptophan is used only in infant formulas, enteral feeding products, and for investigational purposes. Tryptophan and 5-HT cross the blood-brain barrier to affect the homeostatic regulation of sleep. It has been reported by several researchers that this transport diminishes with aging (Tang and Melethil 1995; Porter et al. 2005). Furthermore, body stores of tryptophan hydroxylase, which catalyzes the rate-limiting footstep in the biosynthesis of melatonin and serotonin in cells, are depleted with age due to oxidation by reactive oxygen species and alterations in the phosphorylation cascade that modulates enzyme activity (Hussain and Mitra 2004). The increased frequency of sleep disorders in the elderly may be secondary to these mechanisms. In this chapter, we describe the effect of tryptophan and its products on sleep and its use in the treatment of sleep disorders.

10.2.1 Effect of Tryptophan Administration on Sleep Parameters in Healthy Populations

For healthy subjects who fall asleep normally, tryptophan loading has minimal hypnotic effects. Studies have shown that 500 mg, 1 g, 1.2 g, 2.4 g, and 4 g doses of tryptophan significantly increase subjective sleepiness scores and decrease sleep latency in healthy adults during day and night hours (Chauffard-Alboucq et al. 1991; George et al. 1989; Spinweber et al. 1983). Various researchers have found a negative correlation between sleep latency and tryptophan loading at 0, 60, and 120 min following doses of 1.2 and 2.4 g administered to healthy volunteers. Hajak et al. found a dose-dependent increase in the amount of sleep during Stages I and II during the day (Hajak et al. 1991b). In the same study, nighttime loading doses of 1.3 and 5 g of tryptophan decreased sleep latency and improved sleep efficiency in healthy males. It was remarkable that tryptophan loading has significantly increased plasma melatonin synthesis. However, the effect of tryptophan on the stages of sleep has been contradictory. A loading dose of 4 g tryptophan increased slow-wave sleep in healthy males (Nicholson and Stone 1979). On the other hand, loading dose of 4 g tryptophan did not affect sleep stages during daytime sleep (Spinweber et al. 1983).

10.2.2 Effect of Tryptophan Administration on Sleep Parameters in Insomnia Patients

Several studies have found that a low dose (e.g., 1 g) of tryptophan significantly decreases sleep latency and increases subjective sleepiness scores in individuals with insomnia (Brown et al. 1979). Distribution of sleep stages varies with different doses of tryptophan. A dose of 250 mg of tryptophan has been found to increase the duration of slow-wave sleep significantly, but doses higher than 250 mg (e.g., 500 mg to 1 g) did not affect the sleep stages in insomniacs (Cole 1980; Spinweber et al. 1983). Surprisingly, the effect of continued doses of tryptophan loading (e.g., four to six nights) on sleep latency has been found to be similar to a placebo.

10.2.3 Effect of Subchronic Tryptophan Administration on Sleep

Several studies have demonstrated improved quality of sleep and decreased sleep latency for several nights following tryptophan loading in patients with chronic insomnia (Cole 1980; Demisch et al. 1987; Schneider-Helmert 1981). These results are consistent across different times of the treatment period. For instance, after administration of 2 g tryptophan for three nights, the quality of sleep improved statically significant compared to the pre-tryptophan baseline (Schneider-Helmert 1981).

10.2.4 Effect of Tryptophan Administration on Sleep and Cognition

Studies have demonstrated that tryptophan loading does not impair next-day performance (Johnson and Chernik 1982; Vermeeren 2004). They also revealed that tryptophan loading together with intake of α-lactalbumin protein decreased feelings of sleepiness in the morning and improved morning alertness and attention both with and without mild sleep complaints (Markus et al. 2005). Based on these findings, tryptophan has an indirect effect on cognitive function by improving quality of sleep.

10.2.5 Effect of Tryptophan Administration on Sleep in Infants and Children

Human milk contains different amounts of tryptophan depending on the age of the infant, duration of the feeding episode, and time of the day (Cubero and Valero 2005). Not surprisingly, levels of tryptophan in the breast milk have been shown to

be higher during the night. Up to 2000s, commercial infant formula contained very little tryptophan compared to breast milk (Heine 1999). Researchers found that infants consuming increased amount of tryptophan had shorter sleep latencies, little or no motor activity, and slower respiratory rates. These infants were also less alert and spent less time crying. In the light of this information, tryptophan was added to the commercial formulas to modulate sleep latency and wakefulness in infants. More recently, Aparicio et al. showed that in infants who were given different infant milk formulas with tryptophan contents adjusted to light-dark changes, tryptophan levels ameliorate their circadian sleep-wake cycles (Aparicio et al. 2007).

10.3 Serotonin

Serotonin is a central nervous system neurotransmitter. After the key role of serotonin in the mechanisms of the sleep-wake cycle was discovered, it was nicknamed the "neurohormone of sleep." Physiological functions, such as regulation of appetite and body temperature, hormone secretion, pain perception, and sexual behavior, are all regulated by serotonin (Deneris 2011; Galfi et al. 2005; Chan et al. 2011). Neurochemical, neuropharmacological, and electrophysiological studies have shown that serotonergic activation triggers waking and inhibits slow-wave sleep and/or REM sleep (Monti and Jantos 2008). Rapid eye movement is often seen in serotonin reuptake inhibitor users in all NREM stages. This phenomenon is called "prozac eyes" (Peigneux et al. 2001). In addition, serotonin is one of the best established mediators of wake-related activation of hypoglossal (XII) motor neurons that innervate the muscles (genioglossus, hypoglossus, and geniohyoid) of the tongue (Volgin et al. 2013). Thus, serotonergic system plays a functional role in the maintenance of the patency of upper respiratory tract, the disorganization of which could contribute to the pathophysiological mechanism of obstructive sleep apnea (Wu et al. 2013).

10.4 Melatonin

Melatonin is secreted by the pineal gland. It is synthesized from 5-HT by pinealocytes and then released into the blood and cerebrospinal fluid. Melatonin transmits signals to target organs, primarily the brain, and initiates the synthesis of second messengers to affect sleep and other circadian rhythms. In the 1970s, investigators have shown that nighttime melatonin production is at least ten times higher than daytime production in humans (Lynch et al. 1975). It has been emphasized that this circadian rhythm does not accommodate itself to the light-dark cycle of shift workers (Lynch et al. 1978). Since the relationship between melatonin release and the circadian rhythm was discovered, much pineal research has focused on the brain's responses to melatonin rhythms. The indolamine is the core structure of melatonin.

Specialized photoreceptive cells in the retina detect light, and this information goes directly to the suprachiasmatic nuclei through the retinohypothalamic tract and indirectly through the geniculohypothalamic tract. Activation of the N-acetyltransferase system by this information is the limiting step in melatonin synthesis (Sánchez et al. 2008). The production of melatonin in the pineal gland is increased during the night and inhibited by light. The circadian rhythm of melatonin being released from the pineal gland in humans is highly synchronized with the habitual hours of sleep. It has also been shown that the secretion of melatonin is positively correlated with the onset of nocturnal sleepiness (Shochat et al. 1998).

Today, it is well known that melatonin affects sleep, circadian rhythm, puberty, antioxidant status, aging, and blood pressure. The European Food Safety Authority has approved the use of 1 mg of melatonin at bedtime or after nocturnal awakening for reducing sleep latency in normal sleepers or people with insomnia (European Food Safety Authority 2011). Nevertheless, in the United States, the Food and Drug Administration's Dietary Health and Education Act has approved melatonin as a dietary supplement and not as a drug. Melatonin has three major receptors that demonstrate their effects on organisms. These macromolecules are localized in the suprachiasmatic nucleus of the hypothalamus, pars tuberalis of the pituitary, cardiac blood vessels (MT1), retina and hippocampus (MT2), kidney, brain, and various peripheral organs (MT3) (Witt-Enderby et al. 2003). After understanding the effects of melatonin on circadian rhythm sleep, supraphysiologic concentrations (10–80 mg) of melatonin have been used and investigated, and numerous biological effects of melatonin involving daytime sleepiness, impaired mental and physical performance, hypothermia, and hyperprolactinemia were noted (Atkinson et al. 2005; Deacon and Arendt 1995; Waldhauser et al. 1987). Based on these findings, researchers started to investigate lower doses (0.1–10 mg) of melatonin. Dollins et al. found that oral doses between 0.1 and 0.3 mg caused dose-related decreases in sleep latency and increases in sleep duration and self-reported sleepiness and fatigue without reducing body temperature or elevating plasma melatonin concentrations above their normal nocturnal range (Dollins et al. 1994). This finding suggested that nocturnal melatonin secretion, which produces plasma melatonin concentrations similar to those seen after the 0.3 mg dose, has a physiologic effect on sleep. This study also identified the dose range that clinicians and investigators should use if they want to examine physiologic effects of melatonin.

10.4.1 Effect of Melatonin on Sleep

It has been found that melatonin affects the parameters of sleep by decreasing sleep latency and increasing sleep efficiency and total sleep time. Currently, investigators demonstrated that a physiologic dose of melatonin administration for individuals with age-related insomnia and normal healthy volunteers has shortened the sleep latency (Zhdanova et al. 1995, 1996, 2001). Three meta-analyses have been published in recent years regarding the effects of exogenous melatonin on sleep. In a

meta-analysis of 17 studies with a total study population of 284 participants, sleep onset latency, total sleep duration, and sleep efficiency were selected as the outcome measures (Brzezinski et al. 2005). Melatonin administration was found to cause a significant increase in sleep efficiency of 2.2 % (95 % CI 0.2–4.2), a significant decrease in sleep latency of 4 min (95 % CI 2.5–5.4), and a significant increase in total sleep duration of 12.8 min (95 % CI 2.9–22.8). The second meta-analysis consisted of 14 studies on primary sleep disorders. Melatonin was found to decrease sleep-onset latency, particularly in people with delayed sleep-phase syndrome (Buscemi et al. 2005). The third meta-analysis which consisted of only patients with secondary insomnia showed that melatonin is ineffective for this particular disorder. These patients were categorized in two groups as insomniacs with neurologic or psychiatric diseases and those whose insomnias were related to jet lag and shift work. Unfortunately, melatonin was found to be ineffective for treating secondary sleep disorders or sleep disorders accompanying sleep restrictions, such as those caused by jet lag and shift work (Buscemi et al. 2006). These inconsistent results arose from the administration times of the melatonin administration in different periods of the night. Melatonin has a short elimination half-life (20–30 min). This may lead to accurate dose adjustments for exogenously administered melatonin, as the pineal gland is continuing to release unimportant, negligible amounts of endogenous melatonin.

10.4.2 Effect of Melatonin on Sleep Architecture

Several studies have found that melatonin appears to decrease sleep latency but has no consistent effect on sleep architecture. In contrast to hypnotic drugs, it does not affect the REM phase of a sleep period (Zhdanova et al. 1995, 1996).

10.4.3 Melatonin and Age

Transport of melatonin through blood-brain barrier is decreased with increasing age. The increased frequency of sleep disorders in the elderly may be explained by this impaired melatonin transport through the cerebrospinal fluid and blood.

10.4.4 Melatonin and Insomnia

A recently published meta-analysis in 2013 included 19 studies involving 1683 subjects. Fourteen studies investigated the effects of exogenous melatonin on sleep. Sleep onset latency, total sleep time, sleep quality, age, and dose and duration of melatonin were selected as the outcome measures. Weighted mean differences (WMD) between the melatonin and placebo were used to evaluate sleep latency and

total sleep time. Melatonin demonstrated significant efficacy for reducing sleep latency (WMD = 7.06 min) and increased total sleep time (WMD = 8.25 min [95 % CI 1.74–14.75]). Melatonin doses of 0.3, 2, 3, and 5 mg had greater effects on decreasing sleep latency and increasing total sleep time. In addition, sleep quality was significantly improved in the melatonin group (difference = 0.22 [95 % CI: 0.12–0.32]) compared to a placebo. Ferracioli-Oda et al. emphasized that melatonin decreases sleep onset latency, increases total sleep time, and improves overall sleep quality (Ferracioli-Oda et al. 2013). The effects of melatonin on sleep are modest but do not appear to dissipate with continued melatonin use. Although the absolute benefit of melatonin compared to placebo is smaller than other pharmacological treatments for insomnia, melatonin may have a role in the treatment of insomnia given its relatively benign side effect profile compared to other agents.

10.4.5 Melatonin and Jet Lag

A review by Cochrane examined randomized trials in which travelers took placebo or melatonin (0.5–5 mg) at bedtime of their destination after crossing five or more time zones (Herxheimer and Petrie 2002). Of ten trials, eight showed significant reductions in the jet lag score (an improvement in visual analog scale scores from 0 to 100). A score of ≥60 was detected in 67 % of the controls but in only 17 % of the melatonin recipients (number needed to treat = 2). The benefit was greater when flying eastward and crossing more than one time zone. Therefore, using melatonin to treat jet lag is recommended (Sack 2010).

10.4.6 Melatonin Agonists

Ramelteon is a selective melatonin MT_1 and MT_2 agonist with a 2.6-h elimination half-life that has been approved in the United States for the treatment of insomnia. Agomelatine is a potent melatonin receptor agonist and 5-HT_{2C} antagonist with a 1- to 2-h elimination half-life. It has antidepressant, anxiolytic, and sleep-promoting features.

10.5 Obstructive Sleep Apnea Syndrome (OSAS)

Obstructive sleep apnea syndrome (OSAS) is characterized by an intermittent, complete, or partial upper airway obstruction during sleep that causes hypoxia, sleep disruption, daytime sleepiness, and mental and physical adverse effects. OSAS is the second most common respiratory system disease, and it affects approximately 5–30 % of adults in the world (Young et al. 1993; Tufik et al. 2010). Intermittent

hypoxia, sleep fragmentation, and sympathetic activation are the principal causes of inflammation, insulin resistance, hypertension, and other cardiovascular events. There is growing strong evidence related to an independent contribution of OSAS to the development of cardiovascular diseases and associated risk factors such as inflammation, diabetes mellitus, hypertension, and chronic kidney disease (Shahar et al. 2001; Peker et al. 2002). As a result of the complexity of the neurochemical control, neuromodulation of the central respiratory drive, and the upper airway motor output, limited number of pharmacologic treatment modalities exists for OSAS (Smith et al. 2006; Carley and Radulovacki 2008).

10.5.1 Effects of Tryptophan and Its Metabolites on OSAS

Because of poor tolerance to long-term adherence to continuous positive airway pressure (CPAP) therapy, which is the current gold standard treatment for OSAS, the discovery of novel well-tolerated therapeutic alternatives has conveyed importance (Weaver and Grunstein 2008). In a previous study, tryptophan demonstrated significant improvement in OSAS contrary to central sleep apnea (Schmidt 1983). Contrary to other sleep disorders, limited data are available on the role played by melatonin in OSAS. Investigators have shown that plasma melatonin levels are low in patients with newly diagnosed OSAS. In this study, the patients underwent diagnostic polysomnography by drawing blood at 2-h intervals for 24 h. After 3 months of CPAP therapy, OSAS patients' melatonin peaked at 6:00 a.m. in comparison to the 2:00 a.m. peak of healthy controls. They demonstrated that OSAS patients have an altered circadian rhythm of melatonin in the night toward the morning hours (Zirlik et al. 2013).

Melatonin should be analyzed in further larger prospective studies. OSAS is considered to be a multifactorial disease that is affected by environmental factors, anatomic features, and genetic disorders. The serotonergic system has a crucial impact on sleep and airway stabilization (Douse and White 1996; Kubin et al. 1992). So outstanding investigations on serotonin are those related to obstructive sleep apnea. One meta-analysis exhibited candidate genes associated with OSAS. Serotonin 2A (5-HT2A) receptor was found to be a key region for motor neurons that affected upper airway stability. Extensive evidence has demonstrated that the 5-HT2A receptor has significant properties on central respiratory neuronal system. 5-HT2A might influence the control of the upper airway at multiple levels (Varvarigau et al. 2011). In addition, many studies have found significant associations between OSAS risk and 5-HT2A receptor genes. In addition, melatonin and serotonin might be key modulators for the coordination of the circadian mechanism and upper airway maintenance mechanism of sleep due to the aforementioned mechanisms. Elaborate prospective studies should be conducted while keeping in mind the drawbacks of aforementioned studies to better delineate the role of tryptophan and its products in the pathogenesis and adverse outcome of OSAS and to determine whether consumption of tryptophan-rich foods and products might be a novel pharmacological treatment for OSAS.

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Chapter 11
Tryptophan in Molecular Hematopoiesis

Ibrahim C. Haznedaroglu

Abstract The localizations of tryptophan residues are evident in membrane-binding proteins that are functional in hematopoiesis. Tryptophan at the transmembrane–cytosolic junction modulates the main cytokine of megakaryothrombocytopoiesis, the thrombopoietin receptor (TpoR), dimerization, and activation. Tryptophan is absolutely required at juxtamembrane position 515 to maintain the unliganded TpoR inactive. Tryptophan is located in the bone marrow microenvironment for the modulation of hematopoiesis. Likewise, endonexin also has a tryptophan residue that interacts strongly with membrane phospholipids. Tryptophan and tryptophan metabolism could have a role in the development of hematological neoplastic disorders. For instance, modulation of the tryptophan catabolism by human leukemic cells results in the conversion of CD25– into CD25+ T regulatory cells. The expression of indoleamine 2,3-dioxygenase (IDO), which is induced by interferon-gamma (IFN-gamma) and catalyzes the conversion from tryptophan to kynurenine, has been identified as a T-cell inhibitory effector pathway in professional antigen-presenting cells in the marrow stroma. Human acute monoblastic leukemia (AML-M5) and acute lymphoblastic leukemia (ALL) express IDO, and both can be treated by 1-methyltryptophan in mice. Tryptophan metabolism is deregulated in the pathobiology of numerous hematological disorders including myeloid leukemia, plasma cell myeloma, lymphoma, immune thrombocytopenic purpura (ITP), and graft-versus-host disease (GVHD) following hematopoietic stem cell transplantation (HSCT). The aim of this chapter is to outline the status of tryptophan and tryptophan metabolism in normal and neoplastic hematopoiesis. Pharmacological and cellular therapeutics are being developed for the modulation of tryptophan metabolism for the better management of the patients with hematological neoplastic diseases.

Keywords Tryptophan • Hematopoiesis • Signaling • Indoleamine 2,3-dioxygenase • Leukemia • Graft-versus-host disease

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11.1 Introduction

Tryptophan is an amphipathic amino acid that has both polar and hydrophobic traits. This dual nature of the tryptophan makes the molecule well suited for participating in protein–protein interactions. Tryptophan is the amino acid required by all life forms for protein synthesis, metabolic functions, and the synthesis of the essential cellular factors and mediators (Curti et al. 2009). Localizations of the tryptophan residues are evident in membrane-binding proteins that are functional in normal and neoplastic hematopoiesis. XRCC1 (the gene of X-ray repair complementing defective repair in Chinese hamster cells 1) 194Trp allele may be associated with a protective effect against the development of childhood B-cell lymphoma (Baris et al. 2009). The possible role of the tryptophan in blood development was first studied in 1933 (Alcock 1933). *Robert Saxelby Alcock* proposed in his historical Blood article in 1933 that "The tryptophan exerts its influence by actually forming part of the building material for the new blood. The other is that tryptophan has a stimulating influence on the blood-forming organs without necessarily being used up in the process" (Alcock 1933). Those statements are still true today particularly in the context of cytokine signaling, tryptophan metabolism, and genomics. The aim of this chapter is to outline the status of tryptophan and tryptophan metabolism in the molecular hematopoiesis and related pathological states.

11.2 Molecular Hematopoiesis and Tryptophan

Hematopoietic cytokine receptors have significant homology in that the extracellular regions contain a common domain with four conserved cysteines in the N-terminal segment and a tryptophan–serine doublet near the C-terminal end. Point mutations, chromosomal translocations, and other genetic alterations targeting the tryptophan may modify the protein structure and function to cause hematological neoplastic diseases. For instance, in a study (Pardanani et al. 2006), *JAK2V617F* (Janus kinase2 V617F mutation)-negative patients with essential thrombocythemia (ET) or myelofibrosis (MF) were examined for the mutations in the cytokine receptors that bind JAK2 (Janus kinase2). Many of the patients had a tryptophan-to-leucine substitution at codon 515 on the thrombopoietin receptor (*MPL*). *MPLW515L/K* is an acquired somatic mutation resulting in massive thrombocytosis and megakaryocytic hyperplasia. The mutation is present in 10 % of patients with primary MF and in 5–8 % of patients with ET (Pardanani et al. 2006). Homozygosity for the VHL (von Hippel–Lindau tumor suppressor) Arginine 200-Tryptophan mutation is the cause of Chuvash polycythemia, an autosomal recessive polycythemic disorder characterized by elevated serum erythropoietin (Epo) and hypersensitivity of erythroid cells to Epo (Pastore et al. 2003). Likewise, G-CSF (granulocyte colony-stimulating factor)-induced activation of signaling complexes depends on the function of the membrane-proximal cytoplasmic region of G-CSF-R.

A G-CSF-R mutant was constructed in which tryptophan 650 was replaced by arginine (Barge et al. 1996).

Mutations involving tryptophan exhibit distinct pathobiological states in the clinical hemostasis. For instance, C3178T encodes an arginine to tryptophan (R1060W) substitution in the TSP(thrombospondin)1–7 domain of ADAMTS-13 (a disintegrin-like and metallopeptidase with thrombospondin type 1 motif), an enzyme that is very important in the pathobiology of thrombotic thrombocytopenic purpura (Camilleri et al. 2008). Likewise, arginine 352 substituted by tryptophan lead to amino acid substitutions in the serine protease domain of the mature protein C in hereditary protein C deficiency (Doig et al. 1994). The mutations in the A2 domain at position 834 in which arginine (R) was substituted for glutamine (R834Q) or tryptophan (R834W) have been identified in the von Willebrand's disease (vWD) type 2A (Lankhof et al. 1999). The mutations in the A1 domain at position 543 in which arginine (R) was replaced by glutamine (Q) or tryptophan (W) can cause platelet aggregation, which in vivo is followed by removal of the aggregates leading to the loss of high molecular weight multimers and thrombocytopenia (Lankhof et al. 1997). The point mutation in the GPIX coding region that changes the codon for tryptophan 126 (TGG) to a nonsense codon (TGA) has been detected in the Bernard–Soulier syndrome (Noda et al. 1995). Point mutation at the nucleotide 8812 in exon 10 results in the replacement of tryptophan-357 (TGG) by cysteine (TGT) in the prothrombin gene, leading to hemorrhagic diathesis (Poort et al. 1997). The arginine to tryptophan mutation at position 527 is the cause of the reduced activity of factor VIII Leiden (Pieneman et al. 1998). The localization of tryptophan residues is very critical in the membrane-bound hemostasis-related annexins (Meers 1990). Moreover, tryptophan (2063) and tryptophan (2064) play a critical role in the high-affinity binding of coagulation factor Va to the membrane phosphatidylserine membranes (Peng et al. 2004).

Tryptophan metabolism is involved in normal and neoplastic cellular development. The metabolic pathway induced in response to T-cell activation is degradation of the essential amino acid tryptophan by indoleamine 2,3-dioxygenase (IDO). Indoleamine 2,3-dioxygenase (IDO)-induced tryptophan catabolism along the kynurenine pathway is depicted in Fig. 11.1 (Lob et al. 2009). Tryptophan starvation by IDO consumption inhibits T-cell activation, whereas products of tryptophan catabolism, such as kynurenine derivatives and O_2-free radicals, regulate T-cell proliferation and survival (Curti et al. 2009). Increased IDO activities may have an impact on negative clinical outcome in neoplastic disorders (Chamuleau et al. 2008; Rutella et al. 2009). *Chamuleau and coworkers* have investigated whether the expression of *INDO* (the gene encoding for IDO) correlates to clinical outcome of patients with acute myeloid leukemia (Chamuleau et al. 2008). They determined *INDO* expression in myeloid leukemic blasts of 285 patients by gene-expression analyses and in 71 patients by real-time quantitative polymerase chain reaction (qPCR) and correlated these data to relevant known prognostic factors and survival. In their study, correlation of *INDO* expression to survival data of 262 patients revealed that all patients with prolonged overall survival had low *INDO* expression (Chamuleau et al. 2008). The authors suggested that measuring *INDO* levels provides useful

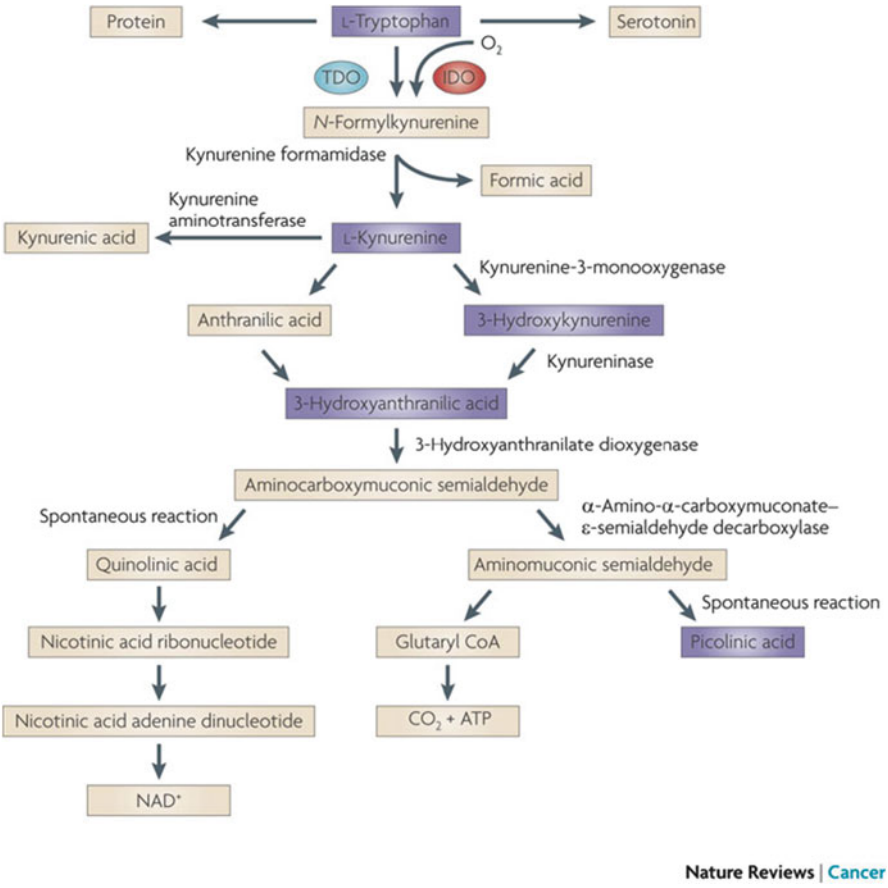

Fig. 11.1 Indoleamine 2,3-dioxygenase (IDO)-induced tryptophan catabolism along the kynurenine pathway (Reprinted with permission; Löb et al. (2009))

prognostic information, as a high *INDO* expression level is correlated to a lower complete remission rate and shorter overall and relapse-free survival of patients with acute myeloid leukemia (Chamuleau et al. 2008). Furthermore, inhibition of IDO by orally available inhibitors like 1-methyltryptophan is effective in mice and synergistic with chemotherapy (Hou et al. 2007).

11.3 Cytokine Signaling, Tryptophan, and Hematopoiesis

Hematopoiesis is regulated by many cytokines and growth factors that support the proliferation and differentiation of progenitor cells in the bone marrow. A large number of pleiotropic hematopoietic growth factors have been reported to positively

or negatively influence various phases of cellular development in the hematopoiesis, including granulocyte–macrophage colony-stimulating factor (GM-CSF); stem cell factor (SCF, also known as steel factor or c-kit ligand); IL-3, IL-6, and IL-11; leukemia inhibitory factor (LIF); EPO; and thrombopoietin (Tpo) (Haznedaroglu et al. 2002). JAK-STAT pathway is a commonly shared intracellular signal transduction system in hematopoiesis. The pathway is utilized by numerous cytokines, growth factors, and hormones for gene expression and a variety of biological activities. JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway arises as the most common signaling cascade of a wide range of cytokines and/or growth factors in propagation of physiological and pathological/neoplastic hematopoiesis (Haznedaroglu et al. 2000). The essential amino acid tryptophan is important in JAK-STAT signaling within the context of hematopoiesis. Mutational analysis of EPO receptor (EPO-R) showed that tryptophan residue (W282) located between box 1 and box 2 is essential for JAK2 activation and proliferative signaling of EPO-R. This tryptophan is conserved in several proteins of the cytokine receptor superfamily, e.g., IL-2-R *p*chain, IL-4-R, G-CSF-R, and gp130 cytokines (Barge et al. 1996). G-CSF-R is crucial for proliferative signaling and activation of JAK2 and STATs, and also required for activation of the p21ras route, which occurs via the membrane-distal region of G-CSF-R. In the study by Barge et al., a G-CSF-R mutant was constructed in which tryptophan 650 was replaced by arginine and expressed in BAF3 cells (cell line derived from BALB/c mouse strain) (BAF/W650R). In contrast to BAF3 cell transfectants expressing wild-type G-CSF-R, BAF/W650-R cells did not proliferate and did not show activation of JAK2, STAT1, or STAT3 in response to G-CSF (Barge et al. 1996). The mutation of the two conserved tryptophan residues of the WW-like domain has opposing effects on TELPDGFbetaR (TEL-platelet-derived growth factor-beta receptor) kinase activation in chronic myelomonocytic leukemias. WW-like domain in the context of TELPDGFbetaR may have both positive and negative regulatory roles in kinase activation (Chen et al. 2004). The truncation of PDGFRalpha between two conserved tryptophan residues in the juxtamembrane (JM) domain is required for kinase activation and transforming potential of FIP(fertility inhibition protein)1L1-PDGFRalpha in myeloproliferative disorders (Stover et al. 2006).

Tryptophan at the transmembrane–cytosolic junction modulates the main cytokine of megakaryothrombocytopoiesis, the thrombopoietin receptor (TpoR), dimerization, and activation. Thrombopoietin acts on the TpoR to modulate thrombocytopoiesis (Haznedaroglu et al. 2002). Tryptophan is absolutely required at juxtamembrane position 515 to maintain the unliganded TpoR inactive (Defour et al. 2013). Tryptophan at central positions in single-pass membrane proteins may mediate helix association in the physiology of TpoR. Tryptophan exhibits a strong preference for the ends of transmembrane helices in single-pass membrane proteins and has the largest free energy for partitioning into the head group region of membrane bilayers (Defour et al. 2013). Myeloperoxidase (MPO) is present in the azurophilic granules of polymorphonuclear leukocytes. The point mutation at codon 569 in exon 10, resulting in arginine to tryptophan substitution of MPO gene, represents one molecular form of MPO deficiency (Kizaki et al. 1994).

Tryptophan residues may also have a role in the neoplastic hematopoiesis. For instance, acute myeloid leukemia (AML) may be associated with the mutations occurring at the exon 12 of the nucleophosmin (NPM) gene. Falini and coworkers (2006a) determined the mutated NPM alleles encoding abnormal proteins which have acquired at the C-terminus a nuclear export signal (NES) motif and lost both tryptophan residues 288 and 290 (or only the residue 290) which determine nucleolar localization. The alterations in the tryptophan residues are crucial for the NPM mutant export from nucleus to cytoplasm. The cytoplasmic accumulation of NPM was blocked by reinsertion of tryptophan residues 288 and 290, which respectively relocate NPM mutants in the nucleoplasm and nucleoli in their study (Falini et al. 2006a). NPM leukemic mutants in turn recruit the wild-type NPM from nucleoli to nucleoplasm and cytoplasm. The new NES motif and the mutated tryptophan(s) at the NPM mutant C-terminus are thus crucial in the pathobiology of AML (Falini et al. 2006a). The alterations at C-terminus of leukemic NPM mutants are similar. Immunohistochemistry analyses could detect all exon 12 NPM mutations involving tryptophan, which represent a valuable tool in the diagnostic–prognostic work-up of the patients with AML with normal karyotype (Falini et al. 2006b). Immunohistochemical detection of cytoplasmic NPM predicts NPM1 mutations and helps rationalize cytogenetic/molecular studies in AML. NPM1 mutations in the absence of FLT3-ITD identify a prognostically favorable subgroup in the heterogeneous AML- normal karyotype category (Falini et al. 2007).

11.4 Tryptophan Metabolism, IDO, and Blood Cells

The enzyme IDO regulates immune responses through the capacity to degrade the essential amino acid tryptophan into kynurenine and other downstream metabolites that suppress effector T-cell function and favor the differentiation of regulatory T cells. IDO can be expressed by dendritic cells, by tumor cells or by surrounding stromal cells, either within proximity of the tumor or at distal sites (Rutella et al. 2009). The role of IDO, induction of immunologic tolerance, affects also the pathobiology of the hematologic malignancies. IDO is one of the key players involved in the inhibition of cell proliferation, including that of activated T cells (Curti et al. 2009). Tryptophan can be catabolized by IDO1 but also by the related enzyme IDO2 and by tryptophan 2,3-dioxygenase (TDO), which is mainly active in the liver. Catabolism of tryptophan by IDO1 plays an important role in the control of immune responses (Barnes et al. 2009). IDO activity may be detected in draining lymph nodes. BLIMP-1 (the transcriptional repressor B lymphocyte-induced maturation protein-1) binds to IFN (interferon)-responsive sites in the IDO1 promoter and represses IFN-dependent IDO1 activation (Barnes et al. 2009). Likewise, there is a possible link between the IDO-like protein IDO2 and cancer. IDO2 expression has been described in human tumors, including renal, gastric, colon, and pancreatic tumors. The selective inhibition of IDO2 by the D-stereoisomer of the IDO blocker 1-methyltryptophan (1-MT) suggested that IDO2 may be important to sustain

immune escape and growth of tumors. IDO2-specific T cells are cytotoxic effector cells that recognize and kill tumor cells. Thus, IDO2 might be a useful target for anticancer immunotherapeutic strategies (Sorensen et al. 2011). For instance, the inhibition of IDO expressed by myeloid leukemic blasts may result in the breaking of immune tolerance. That situation offers new therapeutic options for the patients with AML (Chamuleau et al. 2008). Tumor escape from immune effector cells, such as cancer vaccines and adoptive T-cell infusions, represents a great challenge to the clinical strategies of antitumor immunity. The blockade of tryptophan-catabolizing enzyme IDO may increase the efficacy of tumor-specific T-cell therapies in cancer patients (Gajewski 2004). Human dendritic cells express both IDO-1 and IDO-2, but that only IDO1 mediates tryptophan catabolism; furthermore, its activity is blocked by levo-1MT, whereas IDO2-inhibitor dextro-1-methyltryptophan is inefficient (Lob et al. 2008).

IDO is immunosuppressive and is induced by inflammation in macrophages and dendritic cells. Hemoglobin (Hb) has also been shown to increase IDO activity in healthy dendritic cells, and the kynurenine/tryptophan ratio is elevated during hemolytic anemia (Ogasawara et al. 2009). Tryptophan is also involved in hemolytic anemia due to red blood cell (RBC) membrane abnormalities. Band 3 Prague III is a codon 870 CGG-->TGG point mutation, replacing an arginine with a tryptophan in the transmembrane region of band 3 (Bracher et al. 2001). Hemoglobin, but not hemin or heme-free globin, has induced IDO expression in the bone marrow-derived myeloid dendritic cells. Hb-induced IDO expression was inhibited by inhibitors of PI3 kinase (phosphatidyl-inositol-3-kinase) (PI3K), PKC (protein kinase C), and nuclear factor (NF)-kappaB. Hb translocated both RelA and p52 from the cytosol to the nucleus and induced the intracellular generation of reactive oxygen species (Ogasawara et al. 2009). Tryptophan levels in the cerebrospinal fluid (CSF) of cerebral malaria children were linked to the hematological red blood cell parameters (Ebuehi et al. 2009). 4-Pyridone-3-carboxamide ribonucleoside triphosphate accumulating in erythrocytes in end-stage renal failure originates from tryptophan metabolism (Laurence et al. 2007).

Tryptophan catabolism mediated by IDO plays a central role in the regulation of T-cell-mediated immune responses. The maturation of dendritic cells in the presence of PGE2 (prostaglandin E2) results in upregulation of IDO providing a potential mechanism for the development of dendritic cell-mediated T-cell tolerance. T-cell proliferation and cytokine production could be inhibited, which was mediated mainly by IDO-induced tryptophan depletion (von Bergwelt-Baildon et al. 2006). Tryptophanyl-tRNA synthetase (TTS) can protect T cells from IDO-mediated cell injury. Impaired IDO-mediated tryptophan catabolism has been observed in some autoimmune diseases (Wang et al. 2011). Human plasmacytoid dendritic cells (PDC) express high levels of IDO, the intracellular enzyme that catabolizes tryptophan degradation (Chen et al. 2008). PDC can drive naive, allogeneic CD4(+) CD25(−) T cells to differentiate into CD4(+)CD25(+)Foxp3(+) regulatory T cells (Tregs). IDO pathway is essential for PDC-driven Treg generation from CD4(+) CD25(−) T cells (Chen et al. 2008). Natural killer (NK) cell function can be influenced by IDO, as well. For instance, l-kynurenine, a tryptophan-derived catabolite

resulting from IDO activity, was found to prevent the cytokine-mediated upregulation of the expression and function of specific triggering receptors responsible for the induction of NK cell-mediated killing (Chiesa et al. 2006). IDO(+) dendritic cells stimulated with CTLA-4-Ig (cytotoxic T-lymphocyte-associated antigen 4 immunoglobulin) inhibited immune responses by an IDO-dependent mechanism (Xu et al. 2012). Thus, increasing the expression and activity of IDO in dendritic cells might be a promising therapeutic approach for immune thrombocytopenic purpura (ITP) (Xu et al. 2012). The activity of IDO enzyme is insufficient in ITP patients. Increased TTS expressions from CD4(+) and CD8(+) T cells might link to a pathogenic mechanism involved in increasing survival of autoreactive T cells in ITP patients (Wang et al. 2011). IDO expression in dendritic cells and its role as a potent inducer of T regulatory cells are critical in the induction of immune tolerance during infections, pregnancy, transplantation, autoimmunity, and cancer (Trabanelli et al. 2011). Inhibiting IDO after chemotherapy would result in better outcomes since it persistently breaks the tolerance to tumors, thus eliciting an effective immune response (Curti et al. 2009).

The depressed T-cell immune responses after human hematopoietic bone marrow stem cell transplantation (HSCT) are clinically important. T-cell dysfunction (monocyte-mediated T-cell suppression) after HSCT is ascribed to the monocyte tryptophan catabolism into kynurenine by IDO. Blockade of the tryptophan catabolism could improve the proliferative responses in HSCT (Hainz et al. 2005). The amount of IDO expression and activity is one feature to govern the type of response of stimulated T cells. The immunosuppressive role of IDO has been widely investigated for the induction of graft tolerance in HSCT (Curti et al. 2009). Human dendritic cells can be induced to display high levels of IDO expression to modulate allogeneic T-cell responses toward tolerance by eliminating alloreactive T cells. This action is made through apoptosis and augmentation of their regulatory effects. This mechanism could be employed to use dendritic cells for the generation of alloantigen-specific tolerance in the setting of HSCT (Heitger et al. 2006). Graft-versus-host disease (GVHD) is the main cause of morbidity and mortality after HSCT (Goker et al. 2001). GVHD-dependent induction of IDO was suggested via the demonstration of the increased expression of IDO mRNA in intestinal biopsies from patients with severe GVHD following HSCT. Likewise, kynurenine levels were directly correlated with severity and clinical course of GVHD (Landfried et al. 2011). Furthermore, IDO mRNA was expressed in the blood mononuclear cells of patients with acute GVHD. Plasma IDO activity was elevated in the acute GVHD patients and was correlated with the severity of the GVHD (Xu et al. 2013). In combination with plasma IFN-gamma, IDO activity may represent a potential biomarker for the diagnosis and evaluation of acute GVHD after allo-HSCT. Intervention of the IDO pathway may also represent an alternative way to overcome steroid-resistant acute GVHD (Xu et al. 2013). Exposure to vitamin D can increase the expression of IDO, which is upregulated in the tolerizing dendritic cells. Vitamin D may therefore have a clinical role in the prevention of GVHD following HSCT (Rosenblatt et al. 2010). IDO is the underlying molecular mechanism in the pleiotropic effects of the human multipotent mesenchymal stromal cells (MSCs)

exhibiting multi-lineage differentiation potential, supporting hematopoiesis, and inhibiting the proliferation and effector function of various immune cells (Meisel et al. 2011). Tryptophan could restore the allogeneic T-cell proliferation, thus identifying IDO-mediated tryptophan catabolism as a novel T-cell inhibitory effector mechanism in human MSCs. As IDO-mediated T-cell inhibition depends on MSC activation, modulation of the IDO activity might alter the immunosuppressive properties of MSCs in different therapeutic applications (Meisel et al. 2004). For instance, cytokine stimulation of MSCs specifically induced IDO, which mediated a marked sensitivity of proximal multiple myeloma cells to tryptophan depletion in the microenvironment, suggesting that selective measures to regulate its availability could be a useful strategy to achieve myeloma growth inhibition and apoptosis (Pfeifer et al. 2012).

11.5 Tryptophan Metabolic Pathways in the Leukemic Neoplastic Hematopoiesis

The immunoregulatory enzyme IDO, which catalyzes the conversion of tryptophan into kynurenine, is expressed in a significant subset of patients with AML, resulting in the inhibition of T-cell proliferation and the induction of regulatory T cells (Curti et al. 2010). The role of IDO in the induction of immunologic tolerance is prominent in the hematologic malignancies. The constitutive expression of IDO protein is associated with the leukemic transformation and may play a role in leukemia development. Sun and coworkers investigated the function of IDO in leukemia (Sun et al. 2007). The IDO expressions in human acute monoblastic leukemia (AML-M5) and acute lymphoid leukemia (ALL) were detected by immunofluorescence staining in the leukemic mice model. Constructed leukemia mouse model was also used to observe whether the IDO inhibitor, 1-MT, has any effect in treating leukemia. The experimental group was fed with 1-MT solution every day, while the mice in control group had no further treatment. IDO expression level in leukemia cells was significantly higher than that of normal mononuclear cells. The average survival time in the experimental group was 42.3 days, while the mice in control group only lived 15.1 days in average. The difference was statistically significant in the study ($P<0.05$). The authors concluded that AML-M5 and ALL express IDO, and both can be treated by 1-MT in mice (Sun et al. 2007). AML cells induce T-cell tolerance by directly converting CD4+CD25- T cells into CD4+CD25+ T(reg) cells through an IDO-dependent mechanism (Curti et al. 2007). Tryptophan catabolism is associated with the clinical outcome of the patients with malignant lymphoma (Ninomiya et al. 2010). IDO is highly expressed in adult T-cell leukemia/lymphoma, and the IDO-initiating tryptophan catabolism could change with the antileukemic chemotherapy (Hoshi et al. 2009). 1-Methyl-d-tryptophan reverses the immunosuppressive effect of IDO, and it is currently being developed both as a vaccine adjuvant and as an immunotherapeutic agent for combination with chemotherapy (Jia et al. 2008).

Therefore, IDO is constitutively expressed by tumor cells as part of the genetic changes involved in malignant transformation and provides a platform to investigate the molecular link between tumor cell transformation and the interaction of tumor cells with the host immune system (Curti et al. 2009).

IDO is a novel immunosuppressive agent expressed in some subsets of normal and neoplastic cells, including leukemic myeloid blast cells. AML cells express an active IDO protein that converts tryptophan into kynurenine and inhibits allogeneic T-cell proliferation (Curti et al. 2009). IDO expression correlates with increased circulating CD4+CD25+FOXP3+ T cells in patients with AML at diagnosis. IDO+ AML cells could increase the number of CD4+ CD25+ T cells expressing surface CTLA-4 and FOXP3 mRNA, and this effect is completely abrogated by the IDO inhibitor, 1-MT (Curti et al. 2007). Tryptophan/kynurenine metabolism in human leukocytes is independent of superoxide and maintained in the chronic granulomatous diseases (CGDs). The lack of reduced superoxide anion-dependent interferon-gamma (IFN-gamma)-induced synthesis of kynurenine, an anti-inflammatory tryptophan metabolite produced by IDO, was proposed as a cause of hyperinflammation in CGD, and this pathway has been considered for clinical intervention (De Ravin et al. 2010). IDO-dependent formation of other tryptophan metabolites is an important component of IFN-γ actions. IFN-γ could increase the concentration of kynurenine and decrease that of tryptophan in normal human monocytes in vitro (De Ravin et al. 2010). Multipotent mesenchymal stromal cells (MSCs) inhibit proliferation, helper, and effector functions in most peripheral blood mononuclear cell (PBMC) subpopulations in vitro. The molecular mechanism is ascribed to the tryptophan degradation by the IFN-gamma-induced expression of IDO (Gieseke et al. 2007).

Tryptophan catabolites are potentially mutagenic. Genomic analyses revealed a deregulated tryptophan metabolism in the cases with mutated isocitrate dehydrogenase genes in ALL (Damm et al. 2011). Moreover, tryptophan-catabolizing enzymes have been identified as important factors in immuno-escape and tumor growth in a variety of cancers, including AML, where IDO activity was detected in patient sera and blast cells (Berthon et al. 2013). IDO-mediated catabolism has been suggested as a tolerogenic mechanism exerted by leukemic dendritic cells that have clinical implications for the use in the active immunotherapy of leukemia (Curti et al. 2010).

IDO or another tryptophan-catabolizing enzyme may be indirectly induced in patients with myelodysplastic syndrome (MDS) via the cytokines that are overproduced by the MDS clone. Berthon and coworkers (2013) assessed the tryptophan catabolism in a cohort of MDS patients by measuring kynurenine, tryptophan, and downstream metabolites in the patients' sera. They indicated that tryptophan catabolism is detectable in many MDS patients and correlates with cytopenias (Berthon et al. 2013). Moreover, the metabolites of primary and secondary tryptophan catabolism show inhibitory effects on hematopoietic progenitor amplification, suggesting that tryptophan catabolism may contribute to cytopenias in MDS. IDO metabolites inhibited hematopoietic stem cell expansion, as well. The authors detected substantial IDO activity in the MDS patient sera using HPLC (high-performance liquid

chromatography). In their study, the levels of kynurenine and tryptophan were comparable, and the kynurenine/tryptophan ratios are higher in MDS when compared to those in AML. Moreover, secondary metabolites that are downstream of kynurenine were also elevated, confirming that the entire chain of tryptophan catabolism is deregulated in MDS (Berthon et al. 2013).

Tryptophan and arginase are involved in the critical metabolic immune pathways (Nowak et al. 2012). The tumor microenvironment is hostile to T-cell function due to the expression of enzymes, which deplete the amino acids tryptophan and arginine (Hadrup et al. 2013). Mussai and coworkers brilliantly described the arginase-dependent pathways of leukemogenesis in the bone marrow microenvironment (Mussai et al. 2013). Arginase misregulation plays a key role in the pathophysiology of hypertension (Bagnost et al. 2010). Elevated arginase activity has been associated with systemic hypertension (Shatanawi et al. 2011). Likewise, arginase has been linked to the hypertensive models associated with elevated levels of angiotensin II. The central role for arginase in diseases, in which vascular endothelial pathology is linked to the elevated levels of angiotensin II, has been described (Shatanawi et al. 2011). Angiotensin II enhances vascular arginase activity in endothelial cells. Angiotensin II-activated p38 MAPK (mitogen-activated protein kinase) signaling pathway is associated with the increased arginase activity and expression in macrophages (Shatanawi et al. 2011). Mussai et al. truly indicated that the effects of macrophages for promoting the retention of progenitor cells in the bone marrow niche may be particularly important in the early development of AML, in which surrounding monocytes can be co-opted in the bone marrow to create an immunosuppressive niche (Mussai et al. 2013). We have recently reviewed the autocrine, paracrine, and intracrine effects of the local bone marrow renin–angiotensin system (RAS) in the bone marrow microenvironment, modulating the primitive and neoplastic leukemic hematopoiesis (Haznedaroglu and Beyazit 2013). We had set the provocative hypothesis of "leukemia is the hypertension of bone marrow" based on the microenvironmental pathobiological effects of angiotensin peptides in leukemia (Haznedaroglu and Beyazit 2010). On the other hand, Mussai et al. discovered that AML blasts have an arginase-dependent ability to inhibit T-cell proliferation, to modulate the polarization of monocytes, and to inhibit hematopoietic stem cells (Mussai et al. 2013). The already established interactions between arginase and angiotensin II (Shatanawi et al. 2011) shall now be searched in the leukemic bone marrow microenvironment to detect the role of local bone marrow RAS (Haznedaroglu and Beyazit 2010, 2013) in the tryptophan and arginase-dependent (Mussai et al. 2013) pathways of leukemogenesis.

IDO protein is critically involved at the tumor site, where the depletion of tryptophan and the increase of immunosuppressive metabolites result in reduced effector function, clonal expansion, and survival of antigen-specific T cells (Curti et al. 2009). Moreover, IDO is located at the tumor-draining lymph node, where the population of IDO-expressing plasmacytoid dendritic cells has a tolerogenic effect on T cells during their encounter with antigen-loaded antigen-presenting cells (Curti et al. 2009).

11.6 Conclusion

Tryptophan, as an amphipathic amino acid, is required for the protein synthesis, protein–protein interactions, important metabolic functions, signal transduction, cytokine actions, and the synthesis of the essential cellular factors and mediators (Curti et al. 2009). The immunoregulatory enzyme IDO, which catalyzes the conversion of tryptophan into kynurenine, is expressed in leukemias, resulting in the inhibition of T-cell proliferation and the induction of regulatory T cells (Curti et al. 2010). The role of IDO in the induction of immunologic tolerance is prominent in the hematologic malignancies (Curti et al. 2009). Thus, tryptophan and tryptophan metabolism are involved in normal and neoplastic hematopoiesis. Thrombocytopoiesis, erythropoiesis, granulopoiesis, and lymphoid physiology need well-regulated tryptophan genomics, proteomics, and metabolomics. Furthermore, tryptophan metabolism is deregulated in the pathobiology of numerous hematological disorders including myeloid leukemia, plasma cell myeloma, lymphoma, ITP, and GVHD following HSCT. The status of tryptophan and tryptophan metabolism in the molecular hematopoiesis and related pathological states is not just academical issues. Pharmacological and cellular therapeutics are being developed for the modulation of tryptophan metabolism for the design of clinical trials in hematological disorders. It is hoped that the next decade investigations on the tryptophan will shed further light on the clinical efficacy of those drugs, cells, and vaccines for the better management of the patients with hematological neoplastic diseases.

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Chapter 12
Night Shifts and Melatonin: Relevance to Age and Breast Cancer

Atilla Engin and Ayse Basak Engin

Abstract In mammals, melatonin is synthesized not only in the pineal gland but also in many other parts of the body. The nocturnal synthesis and release of melatonin by the pineal gland are tightly controlled by the central suprachiasmatic nucleus (SCN) clock. This circadian pacemaker encodes rhythmic output in accordance with light input. Environmental light is sensed by an intrinsically photosensitive retinal ganglion cells (ipRGCs). Circadian rhythmicity in the SCN originates from the interaction of a defined set of "clock genes." Melatonin is able to alter the levels of various circadian rhythm genes by re-synchronizing a rhythmic pattern of clock gene expression. Silent mating type information regulation 2 homolog-1 (Sirt1), a nicotinamide adenine dinucleotide [NAD^+]-dependent histone deacetylase, is required for circadian clock gene expression. Altered circadian rhythm regulation plays a critical role in carcinogenesis. Melatonin significantly inhibits Sirt1 protein transcription and activity in multiple human cancer cell lines. However, light is able to either suppress or synchronize melatonin production according to the light schedule. Therefore in industrialized countries, night-shift workers are at high risk for circadian disruption. Epidemiological studies displayed that the increase in breast cancer risk in night-shift workers is associated with exposure to light at night. Furthermore age-related changes reflect a decline in pacemaker amplitude, due to alteration of SCN functions and systems under its control. Consequently dysfunction of endogenous clocks, melatonin receptor polymorphisms, and age-associated decline of melatonin synthesis are already increased risks of breast cancer.

Keywords Melatonin • Suprachiasmatic nucleus • Melanopsin • Sirt1 • Circadian clock genes • Night-shift work • Breast cancer

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12.1 Biological Clock

Synchronization of a rhythm by a repetitive signal or the light–dark cycle is referred as diurnal rhythm. "Circadian rhythms" denote those endogenous, 24-h physiologic, metabolic, or behavioral rhythms that persist under constant conditions and are driven by the molecular clockwork mechanism in the suprachiasmatic nucleus (SCN) and peripheral tissues (Ko and Takahashi 2006). Light, including artificial light, can alter human physiology when inappropriately timed. Light-induced disruption effect on circadian organization changes the daily rhythm of several hormone productions. Changes in light–dark exposure shift the timing of the circadian system. So that internal rhythms can become desynchronized from both the external environment and internally with each other (Stevens et al. 2007). Daily rhythms in physiological and behavioral processes are controlled by a network of circadian clocks which is adjusted by inputs and delivering circadian signals to the brain and peripheral organs (Pevet and Challet 2011). The SCN itself is composed of 20,000 neurons approximately. Although each of these cells contains an autonomous circadian oscillator, SCN cells are coupled together and oscillate in a coherent manner. Consequently SCN functions as a network (Herzog 2007). Thus in mammals at the top of the network, the master clock resides in the SCN of the ventral hypothalamus and mainly reset by ambient light (Pevet and Challet 2011). Eventually SCN comprises a self-sustained, biological clock that generates endogenous circadian rhythms (Kuhlman and McMahon 2006) and regulates a variety of hormonal, metabolic, and behavioral responses. Therefore, internal physiological and metabolic processes are not only in synchrony with the external environment but also are coordinated with each other (Hastings et al. 2003). The molecular events determining the functional oscillation of the SCN neurons with a period of 24 h involve recurrent expression of several clock proteins that interact in complex transcription/translation feedback loops (Richter et al. 2004). The circadian pacemaker in SCN encodes rhythmic output in accordance with light input. Consequently, SCN neurons encode clock gene output as circadian rhythms in spike frequency via ionic mechanisms, as well as cellular and molecular mechanisms (Kuhlman and McMahon 2006).

Circadian rhythmicity in the SCN originates from the interaction of a defined set of "clock genes" (Kuhlman and McMahon 2006). The core genetic apparatus of the clock mechanism appears to be based on a small number of genes (Hastings et al. 2003; Fu and Lee 2003). However, circadian "clock gene" expression capacity is widespread throughout the body (Dibner et al. 2010). Eventually a set of genes generates circadian oscillations and form autoregulatory feedback loop bases on transcriptional activators and inhibitors genes. In mammals, these include (1) transcriptional activators (clock, brain, and muscle arnt-like protein-1 (Bmal1)), and (2) transcriptional inhibitors (clock protein Period-1 (Per1), clock protein Period-2 (Per2), cryptochrome-1 (Cry1), and cryptochrome-2 (Cry2) genes) (Mohawk et al. 2012). After the "Per" and "Cry" proteins have been translated in the cytoplasm, they form heterodimeric complexes that translocate to the nucleus and inhibit their own transcription by binding to the Bmal1 promoter. Finally Bmal1 gene expression

is inhibited (Xiang et al. 2012). The "circadian intracellular proteome" of the SCN comprises 13 % of soluble proteins that are subjected to circadian regulation. Circadian proteins of the SCN depend on rate-limiting factors in metabolism such as protein trafficking and synaptic vesicle recycling (Deery et al. 2009). Indeed a prominent day–night variation of the protein levels is evident in the pineal gland. Some proteins are upregulated during the night concomitant with the melatonin secretion of the gland. Other proteins are upregulated during the day indicating a pineal metabolism not related to the melatonin synthesis (Møller et al. 2007).

Circadian control of metabolism occurs at the central SCN, as well as at local levels, and involves clocks within a number of peripheral tissues including the liver, pancreas, skeletal muscle, intestine, and adipose tissue (Bass and Takahashi 2010; Green et al. 2008). Therefore, organization of the circadian system requires a combination of autonomic innervation of peripheral tissues, endocrine signaling, temperature, and local signals (Mohawk et al. 2012). On the other hand, aging affects re-entrainment of central and peripheral circadian oscillators in a tissue-dependent manner. Age-related changes reflect a decline in pacemaker amplitude, due to alteration of SCN functions and systems under its control (Sellix et al. 2012). The SCN receives direct photic input from the photoreceptor cells of the retina. These cells are known as "intrinsically photoreceptive retinal ganglion cells" (ipRGCs) (Do and Yau 2010). Environmental light is sensed by an ipRGCs which may be the primary photoreceptors in the retina that contain the blue light-sensitive photopigment, melanopsin (Berson et al. 2002). An opsin-based action spectrum of melanopsin provides the primary retinal input of ipRGCs to the SCN (Berson et al. 2002) (Fig. 12.1). Actually melanopsin is expressed in cells of the mammalian inner retina. This light-sensitive photopigment mediates the regulation of circadian rhythm and the acute suppression of pineal melatonin (Provencio et al. 2000). While photoreception for the image-forming pathway begins at the rods and cones, ipRGCs are capable of non-image-forming light responses by expressing melanopsin (Sekaran et al. 2005). In brief, these light-sensitive retinal ganglion cells extend an expansive arbor of dendrites that seems to form a "photoreceptive network" and project to the SCN via the retinohypothalamic tract for circadian phototransduction (Gooley et al. 2001; Provencio et al. 2002). Although the light detection for circadian regulation seems to be mediated principally by the visual pigment of phototransducing retinal ganglion cells, mice lacking melanopsin still retain nonvisual photoreception. This means that rods and cones could operate the light induction in the suprachiasmatic nuclei. Thus, the mice with both outer and inner retinal degeneration exhibit complete loss of the circadian oscillator (Hattar et al. 2002; Panda et al. 2003). As a projection site of the retinohypothalamic tract, the SCN respond to retinal light information primarily by glutamatergic neurotransmission (Tischkau et al. 2003). In this case, a glutamatergic monosynaptic pathway originating from the retina regulates the clock gene expression pattern in the SCN neurons, synchronizing them to the light–dark cycle (Richter et al. 2004). Light resets the clock throughout the night via glutamate (GLU) release, glutamatergic-N-methyl-D-aspartate (NMDA) receptor activation, neuronal nitric oxide synthase (nNOS) stimulation, and nitric oxide (NO) production (Ding et al. 1994). Light/GLU phosphorylates Ca^{2+}/cyclic adenos-

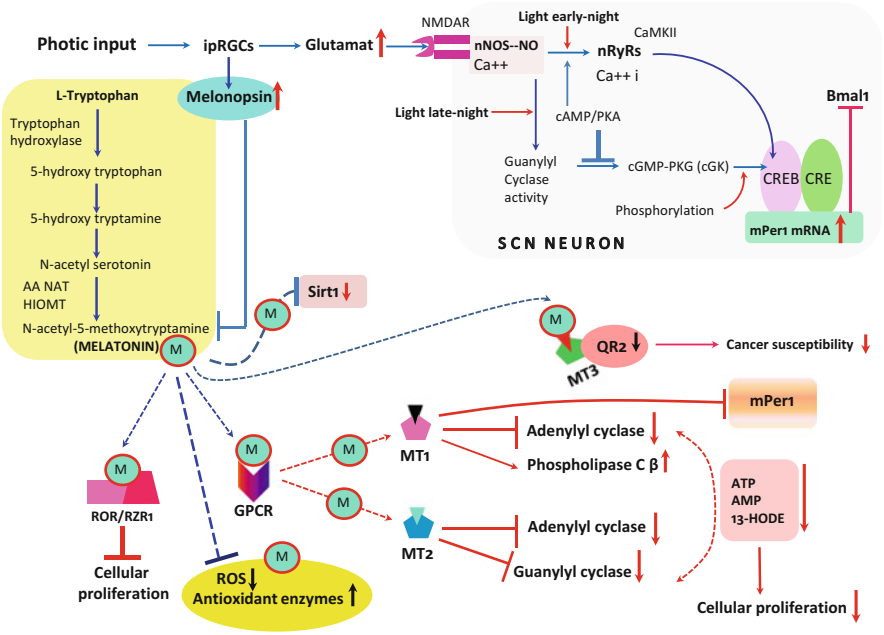

Fig. 12.1 The impact of exposure to light at night on disruption of circadian rhythm and melatonin synthesis. *ipRGCs* Intrinsically photoreceptive retinal ganglion cells, *NMDAR* N-methyl-D-aspartate receptor, *nNOS* Neuronal nitric oxide synthase, *NO* Nitric oxide, *nRyRs* Neuronal ryanodine receptors, *CaMKII* Ca^{2+}/calmodulin-dependent kinase, *cGMP* Cyclic guanosine monophosphate, *PKG* Protein kinase G, *cAMP* Cyclic adenosine monophosphate, *PKA* Protein kinase A, *CREB* Ca^{2+}/cAMP response element-binding protein, *CRE* CREB/cAMP response element, *mPer1mRNA* clock protein Period-1 gene mRNA, *SCN* Suprachiasmatic nucleus, *AA NAT* Arylalkylamine N-acetyltransferase, *HIOMT* hydroxyindole-O-methyltransferase, *Sirt1* NAD^+-dependent protein deacetylase, silent mating type information regulation 2 homolog- 1, *M* Melatonin, *ROR* Retinoic acid-related orphan receptor, *RZR1* Retinoid Z receptor-1, *GPCR* Transmembrane G protein-coupled receptor, MT1 and MT2 G protein-coupled membrane receptors of melatonin, *13-HODE* 13-hydroxyoctadecadienoic acid, *QR2* Quinone reductase II (MT3 receptors), *Bmal1* Brain and muscle arnt-like protein-1

ine monophosphate (cAMP) response element-binding protein (CREB) on the transcriptional regulatory site, Ser133, through NMDA receptor activation and NO signaling (Ginty et al. 1993; Ding et al. 1997). Ca^{2+}-dependent stimulation of cAMP response element (CRE)-mediated transcription is dependent upon activation of the mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) signaling pathway. Finally, CREB/CRE transcriptional pathway is regulated within the SCN cells (Obrietan et al. 1999). Consequently the circadian clock gates expression of light induction at night and daily as two independent rhythms related to immediate early genes expression in the SCN (Guido et al. 1999; Kornhauser et al. 1996). Light at dusk and dawn can increase clock protein Period gene (mPer) expression to synchronize the phase of the molecular clockwork to the environmental day and night conditions. However, the signal transduction pathways differ remarkably between

the day–night and the night–day transition. Light during early night delays phase by leading to intracellular Ca^{2+} release by neuronal ryanodine receptors (RyRs). Light during late night advances phase by triggering an increase in guanylyl cyclase (GC) activity (Pfeffer et al. 2009). Expression of mPer1 and mPer2 overlaps but asynchronous by 4 h. mPer1, unlike period and mPer2, is expressed rapidly after exposure to light (Albrecht et al. 1997). Clock mPer2 is strongly expressed at the constant darkness. Light exposure at the early night induces a transient increase in mPer2 transcripts with delayed onset, as compared to mPer1 mRNA induction (Takumi et al. 1998). CRE-regulated activation of mPer1mRNA is required for light/GLU-induced phase advances during the late night (Tischkau et al. 2003). However, in the early night, the light-induced state change proceeds through NO-dependent activation of a RyRs (Ding et al. 1998).

Signal transduction pathway responsible for light-induced phase advances of the circadian clock could be summarized as follows: GLU-NMDAR-Ca^{2+}–Ca^{2+}/calmodulin-dependent kinase (CaMKII)–nNOS–GC–cyclic guanosine monophosphate (cGMP)–cGMP-dependent kinase (cGK) and clock genes (Golombek et al. 2004).

12.2 Melatonin and Circadian Rhythm

The nocturnal synthesis and release of melatonin by the pineal gland are tightly controlled by the central SCN clock and inhibited by light exposure (Pevet and Challet 2011). In mammals, melatonin is synthesized not only in the pineal gland but also in many other parts of the body, including the eyes, bone marrow, gastrointestinal tract, skin, and lymphocytes (Srinivasan et al. 2011). Tryptophan (Trp) is taken up from blood and converted to melatonin (N-acetyl-5-methoxytryptamine) via the four-step pathway: 5-hydroxy-tryptophan, 5-hydroxy-tryptamine (serotonin), N-acetyl-serotonin, and N-acetyl-5-methoxytryptamine (Reiter et al. 2013). Serotonin is essential in the melatonin biosynthesis pathway; however, biological actions and degradation of melatonin are independent of serotonin (Srinivasan et al. 2011). Serum melatonin has a very short half-life and is rapidly metabolized, mainly in the liver. In this respect 60 % of melatonin is hydroxylated at position 6 and further conjugated to the hydrophilic moieties glucuronyl or sulfate groups. Approximately 15 % of plasma melatonin is metabolized by myeloperoxidase and/or by indoleamine-2,3-dioxygenase (IDO) to N1-acetyl-5-methoxy-kynurenine, and almost 25 % remains unchanged (Boutin et al. 2005). For a healthy, young adult, while daytime melatonin value is low (2–20 pg/ml), nocturnal melatonin reaches to 80–120 pg/ml during the night. Actually urinary melatonin and urinary 6-sulfatoxymelatonin (aMT6-s) levels reflect pineal function and amount of nocturnal plasma melatonin (Schernhammer et al. 2004; Reiter 1991). Both morning urinary melatonin and morning urinary aMT6-s levels account for approximately 72 % of the total nocturnal plasma melatonin (Graham et al. 1998). Melatonin easily crosses cell membranes and the blood–brain barrier and therefore participates in

diverse physiological functions and has great functional versatility related to the regulation of circadian rhythms and seasonal behavior, sexual development, retinal physiology, and tumor inhibition and has antioxidant, immunomodulatory, and anti-aging properties (Sánchez-Hidalgo et al. 2012). The circadian rhythm of melatonin levels in saliva or plasma, or of the melatonin metabolite aMT6-s in urine, is defined as the feature of SCN function. Practically the onset of melatonin secretion under dim light conditions is the single most accurate marker and the gold standard of circadian phase for assessing the function of circadian pacemaker (Pandi-Perumal et al. 2007). Moreover melatonin production is altered by aging. In elderly humans ability to retain the circadian patterns of melatonin and aMT6-s in serum is significantly depressed, and the day–night differences in pineal melatonin production and circulating melatonin levels are severely diminished due to a reduced pineal activity (Bartsch et al. 1992).

Melatonin regulates various biological functions through two distinct classes of receptor proteins. The first group is the members of the seven transmembrane G protein-coupled receptor (GPCR) superfamily, melatonin type 1 (MT1) and MT2, and the second group includes quinone reductase (QR2) enzyme family, MT3. The MT1 and MT2 receptors can couple to multiple and distinct signal transduction cascades whose activation can lead to unique cellular responses in human. These receptor subtypes are 60 % homologous at the amino acid level (Witt-Enderby et al. 2003; Danielczyk and Dziegiel 2009) (Fig. 12.1). MT1 receptor mediates adenylyl cyclase inhibition and phospholipase C beta activation. The MT2 receptor is also coupled to inhibition of adenylyl cyclase, and additionally it inhibits the soluble guanylyl cyclase (sGC) pathway. Melatonin produces a long-lasting sensitization of adenylyl cyclase and amplifies cAMP signaling even when the melatonin levels decline (Von Gall et al. 2002). Melatonin determines rhythmic expression of the circadian proteins mPer1, mPer2, and mCRY1 by the clock genes via MT1 receptors. Thus, melatonin plays a crucial role in regulating rhythmicity in hypophyseal pars tuberalis cells (Jilg et al. 2005). Actually melatonin reduces the expression of Per1 and Clock, but has no effect on Bmal1 expression. Mutation in the MT1 receptor reverses melatonin-induced changes. These findings indicate that MT1 receptor is crucial in the regulatory action of melatonin on clock genes (Imbesi et al. 2009). However, some investigators claim that melatonin indirectly regulates nuclear receptors via the MT1 membrane receptor (Dai et al. 2001; Ram et al. 2002). Melatonin may also mediate its actions through the retinoid orphan receptors (RORs)/retinoid Z receptor group (Smirnov 2001).

12.3 Sirtuins, Melatonin, and Circadian Rhythm

As it is known, mammalian circadian rhythm is controlled by a number of genes, collectively termed “clock genes,” which play a critical role in controlling the central circadian rhythm apparatus. Altered circadian rhythm regulation plays a critical role in carcinogenesis (Jung-Hynes et al. 2010a). Chen and colleagues observed that

96 % of all human breast tumors display deregulated levels of "Per" genes compared with their paired nearby noncancerous cells. Different "Per" expression patterns in different cancer cell populations in the same breast cancer tissue indicate that several asynchronized circadian clocks may be operational in the same cancer tissue (Chen et al. 2005).

Overexpression of "Per1" stimulates the DNA damage-induced apoptosis in human cancer cells; in contrast, inhibition of "Per1" precludes apoptosis (Gery et al. 2006). Mammalian "Per1" is a core clock gene, and its daily expression pattern regulates the cell proliferation. "Per1" as a tumor-suppressor gene diminishes breast cancer cell proliferation in a circadian time-dependent manner (Yang et al. 2009a). Contrarily downregulation of "Per2" also accelerates tumor growth rate by increasing cyclin D and cyclin E levels and doubles the daily amplitude of the breast cancer cell proliferation (Yang et al. 2009b). Melatonin is able to alter the levels of various circadian rhythm genes by re-synchronizing a rhythmic pattern of clock gene expression (Jung-Hynes et al. 2010b). On the other hand, silent mating type information regulation 2 homolog-1 (Sirt1), a nicotinamide adenine dinucleotide [NAD^+]-dependent histone deacetylase, is required for circadian transcription of Bmal1, ROR-gamma, Per2, and Cry1. Bmal1 and Clock activate the expression of "Per" and "Cry" genes. When "Per" and "Cry" proteins accumulate to a critical level, they repress the transcription of their own genes by constituting complexes with Bmal1–Clock heterodimers. Also Sirt1 binds Bmal1–Clock complex in a circadian manner and stimulates the deacetylation and subsequent degradation of "Per2" (Asher et al. 2008).

Actually sirtuins utilize NAD^+ to remove acetyl groups from various targets and transfer them to the 2′-OH of nicotinamide ribose, yielding 2″-O-acetyl-adenosine diphosphate (2″-O-acetyl-ADP) ribose. The nicotinamide ribosyl bond is then cleaved to add a molecule of water to nicotinamide ribose (Sauve et al. 2006). Additionally this family of histone deacetylases mediates posttranslational modifications of the N-terminal tails of the histone proteins, which package DNA into chromatin and play key roles in the regulation of gene expression (Marmorstein 2004). However, melatonin significantly inhibits Sirt1 protein transcription and activity in multiple human cancer cell lines. Thus, Sirt1 is a direct target of melatonin, and melatonin-mediated Sirt1 inhibition is accompanied with a significant decrease in the proliferative potential of tumor cells, but not of normal cells (Jung-Hynes et al. 2011). In this case melatonin shows a strong antitumor activity not only by decreasing tumor cell viability, adhesion, and migration ability but also by increasing the apoptotic index and reactive oxygen species (ROS) generation. While Sirt1 inhibitors enhance the antitumor activity of melatonin, Sirt1 activators weaken the effect of melatonin (Cheng et al. 2013). Therefore, melatonin levels may have an inverse correlation with Sirt1 and control circadian rhythm via Sirt1 (Jung-Hynes et al. 2011).

Although an inborn aging process limits human average life expectancy at birth, a rough measure of the healthy life span is about 85 years (Harman 2006). Furthermore about 20 % of breast cancers occur among women aged younger than 50 years, while 40 % occur among women aged 65 years and older (Siegel et al.

2012). DNA methylation and histone modification are essential for normal development and become altered during aging and by cancer. Interestingly decrease in sirtuins but increase in cancer cases by aging indicates the presence of a potential connection between aging and cancer (Fraga et al. 2007). Melatonin levels reach a maximum at a young age and remain relatively stable until 35–40 years of age, and thereafter they gradually decrease to the levels of similar to daytime low concentrations (Karasek 2004). Consequently, many aged individuals do not exhibit a day–night difference in melatonin levels. Loss of melatonin in the elderly may lead to deterioration of circadian rhythm and possibly an increase in cancer susceptibility (Karasek 2007). The circadian clock determines the strength of cellular responses to DNA damage. DNA repair pathways maintain genetic stability and protect DNA integrity (Robertson et al. 2009; Pardo et al. 2009). Melatonin may enhance DNA repair capacity of breast cancer cells through the upregulation of centrosomal protein of 152 kDa (CEP152), a centrosomal protein involved in the maintenance of genomic integrity and cellular response to DNA damage (Liu et al. 2013). Sirt1 is regulated by a NAD^+-dependent DNA repair enzyme, poly(ADP-ribose) polymerase-1, and subsequent NAD^+ depletion by oxidative stress may have consequent effects on inflammatory and stress responses as well as cellular senescence (Hwang et al. 2013). Furthermore DNA damage or oxidative stress-induced apoptosis or cellular senescence is inhibited by Sirt1 transcription. Eventually cell survival is increased via Sirt1 by protecting the mammalian cells against the stress response (Michan and Sinclair 2007). Sirt1 expression is associated with reduced apoptosis, whereas inactivation of Sirt1 promotes the translocation of B-cell lymphoma 2-associated X protein (Bax) from the cytosol to mitochondria (Cohen et al. 2004). Aging induces significant increases in gene and protein expressions of proapoptotic markers Bax and B-cell lymphoma 2 (Bcl-2)-associated death promoter (Bad) and a significant decrease in the expression of Sirt1. Chronic treatment with melatonin reduces the expression of Bax and Bad and increase in the mRNA expression of Sirt1 in old male rats. Bcl-2 to Bax ratio is significantly higher in young animals as compared with elderly, indicating a lower level of apoptosis with aging (Kireev et al. 2013). Melatonin supplementation increases the protein levels of Sirt1 and subsequently improves pro-survival signals and reduces pro-death signals by decreasing the levels of acetylated p53 and nuclear factor-kappa B (NF-kappaB) in senescence-accelerated mice (Gutierrez-Cuesta et al. 2008), whereas Sirt1 inhibition promotes NF-kappaB transcriptional activity by poly(ADP-ribose)-1-inducing changes in NAD^+ levels (Kauppinen et al. 2013). Contrarily melatonin inhibits hydrogen peroxide-induced activation of NF-kappaB, which is reversed by Sirt1 inhibition (Lim et al. 2012).

12.4 Night Shift

"Exposure to light at night" is a feature common in night-shift work and "disturbs the circadian system with alterations of sleep-activity patterns, suppression of melatonin production and deregulation of circadian genes involved in cancer-related

pathways" (Erren et al. 2009). Short-term disturbances of biological daily rhythms following exposures to light at unusual times are defined as "jet lag" or "shift lag." However, chronic irregularity in timely sequenced circadian rhythms or chronodisruption is a new concept (Erren et al. 2010). Eventually either increase in the duration of daily light exposure and nocturnal melatonin suppression, chronical advancement in the phasing of light exposure (chronic jet lag), or light-at-night-induced suppression of the nocturnal circadian melatonin signal causes circadian disruption (Blask 2009; Blask et al. 2005a; Filipski et al. 2004). Thus, Blask et al. asserted that daytime-collected melatonin-deficient blood-perfused human breast cancer xenografts exhibit markedly suppressed proliferative activity and linoleic acid uptake compared with nocturnal, physiologically melatonin-rich blood-perfused tumor. However, tumors perfused with melatonin-deficient blood collected following ocular exposure to light at night exhibit the daytime pattern of high tumor proliferative activity. These results confirmed the circadian disruption hypothesis and demonstrated that the tumor growth response to human nocturnal, circadian melatonin signal is abolished by short-term ocular exposure to light at night (Blask et al. 2005b). Twenty percent of people worldwide are engaged in some type of work at unusual times, including the night (Erren and Reiter 2009). Therefore in industrialized countries, night-shift workers are at high risk for circadian disruption. Melatonin as a primary output signal of the central circadian pacemaker synchronizes the internal metabolic environment to the light–dark cycle of the external environment. Actually melatonin acts as a chemical code for the night. Furthermore duration of melatonin secretion is correlated with the length of the night (Claustrat et al. 2005). Light is able to either suppress or synchronize melatonin production according to the light schedule. However, synthesis of melatonin during the daytime is almost negligible (Claustrat et al. 2005). Eventually chronodisruption, "the maladjustment of time," is the alterations in the temporal organization of order of biological rhythmicity over days and seasons (Erren and Reiter 2009). In this case failure to coordinate peripheral rhythms via the suprachiasmatic nuclei can lead to circadian or chronodisruption. Disturbances of the circadian organization of physiology, endocrinology, metabolism, and behavior are connected with the shift work, duration of light exposure, and biological 24-h rhythms. For instance, relative risks of breast cancer increase 70 % and 40 % in flight personnel and shift workers who are exposed to chronodisruption via transmeridian, time zone travel, and shift work conditions, respectively (Erren et al. 2008). Epidemiological studies displayed that the increase in breast cancer risk in night-shift workers is associated with exposure to light at night. Eventually International Agency for Research on Cancer (IARC) defined the shift work involving circadian or chronodisruption as probable human carcinogen, group 2A. In addition to length of night, "dose of chronodisruption" is also important. Thus, significant difference in the breast cancer risk between female flight personnel and women who do shift work is attributed to the higher "dose of chronodisruption" among the former (Erren et al. 2009). Changes in either the length of the day or the timing of light exposure due to night shift or transmeridian travel can compromise SCN activity and/or the pineal gland production of melatonin in circadian disruption. In suppression of melatonin synthesis, internal rhythms may become desynchronized from both the external environment and internally

with each other (Stevens et al. 2007). Consequently dysfunction of endogenous clocks, melatonin receptor polymorphisms, and age-associated decline of melatonin synthesis probably contribute to numerous diseases including cancer (Hardeland et al. 2012). As mentioned already, an increased risk of breast cancer has been observed in night-shift workers. In this case exposure to artificial light at night and thereupon disruption of the endogenous circadian rhythm suppress the melatonin synthesis (Peplonska et al. 2012). Furthermore the decline in the production of melatonin with age has been suggested as one of the major contributors to immunosenescence and development of neoplastic diseases in addition to night shift (Srinivasan et al. 2011). In fact appropriate melatonin concentration is necessary in suppressing neoplastic growth in a variety of tumors like melanoma, breast and prostate cancers, and ovarian and colorectal cancers (Srinivasan et al. 2011). In nondividing tissues, nonproliferative metabolic and physiological processes are controlled by the regulated expression of clock-controlled genes (Canaple et al. 2003). Growth control might be influenced by circadian rhythm, and alterations in circadian rhythm can be associated with cancers in humans (Mormont and Lévi 1997).

12.4.1 Night Shift and Breast Cancer

It has been postulated that light exposure at night represents a unique risk factor for breast cancer in industrialized societies via suppressing the nocturnal melatonin secretion from the pineal gland (Stevens 1987). A number of epidemiological studies demonstrated that women working night shifts have a significantly elevated risk of breast cancer presumably due to their increased exposure to light at night (Stevens 2009). Based on 13 studies, including 7 studies of airline cabin crew and 6 studies of other night-shift workers, a similar significant elevation of breast cancer risk among female airline cabin crew and female night workers were obtained (Megdal et al. 2005). Furthermore the risk of developing breast cancer is up to five times higher in industrialized nations than in underdeveloped countries. It is interestingly noted that overall, nearly 50 % of breast cancers cannot be accounted for by conventional risk factors (Stevens 1987). Since primarily life is inside the buildings in industrialized societies, electric lighting in the built environment is generally more than sufficient for visual performance but may be harmful for the maintenance of normal neuroendocrine rhythm in human. Therefore, increasingly greater numbers of people are exposed to more artificial light during the night both at home and in the workplace. Suppression of melatonin synthesis is a major untoward long-term consequence of changes in light–dark exposure (Stevens and Rea 2001; Stevens et al. 2007). Likewise it was shown that the breast cancer risk decreases among women with total visual blindness before age of 65 (Kliukiene et al. 2001). Blind women with no perception of light appear to have a lower risk of breast cancer, compared to blind women with light perception (Flynn-Evans et al. 2009). Short-wavelength lighting (between 470 and 525 nm) is associated with an increased risk of cancer. Furthermore preventing short-wavelength light from reaching the retina

may protect shift workers from suppression of melatonin and increased cancer rates (Kayumov et al. 2007).

More recently a population-based case–control study on breast cancer, GENICA (gene–environment interaction and breast cancer), reported detailed information on shift work from 857 breast cancer cases and 892 controls. This study showed that night work for more than 20 years is associated with a significantly elevated risk of estrogen receptor (ER)-negative breast cancer [odds ratio (OR) 4.73, 95 % confidence interval (95 % CI) 1.22–18.36] (Rabstein et al. 2013).

As pointed out in an earlier study, women who had worked 30 or more years on rotating night shifts have a 36 % greater risk of breast cancer compared with never workers. Longer duration in rotating night shifts is associated with 23 % increase in estrogen receptor-positive breast cancer risk, particularly for premenopausal women. The risk of hormone receptor-negative breast cancer is not elevated after 30 or more years of rotating night shifts (Schernhammer et al. 2001). In fact serum melatonin levels diminish significantly by the fifth and sixth decades of life as the incidence of breast cancer increases. Thus, enhanced mammary tumor growth is associated with old age and diminished levels of melatonin and MT1 receptor expression. Therefore, growth-suppressive actions of exogenous melatonin are reduced by aging (Hill et al. 2011a).

For the first time, in 2007 IARC, an arm of the World Health Organization (WHO), classified "shift work that involves circadian disruption" as a probable human carcinogen (Stevens et al. 2011).

A case–control study of Norwegian nurses comprising 563 breast cancer cases and 619 controls was carried out within a cohort of 49 402 Norwegian nurses, aged 35–74 years. Significant and noteworthy associations between several polymorphisms in circadian genes, night work, and breast cancer risk were found among nurses who had greater than or equal to three consecutive night shifts worked for at least 5 years (Zienolddiny et al. 2013). However, the most significantly increased risks were seen in nurses who worked more than or equal to 5 years with more than or equal to six consecutive night shifts (Lie et al. 2011). The effect of night work on the risk of ER- and progesterone receptor (PR)-defined breast cancers is evaluated in 513 nurses with breast cancer and in 757 frequency-matched controls, all of whom were selected from a cohort of Norwegian nurses. Statistically significant associations have been observed between breast cancer and work durations of more than or equal to 5 years with more than or equal to six consecutive night shifts. The highest risk is observed in PR-positive tumors (Lie et al. 2013).

However, melatonin counteract tumor growth via protection from oxidative stress, regulation of the ER expression, modulation of the enzymes involved in the local synthesis of estrogens, induction of apoptosis, inhibition of telomerase activity, inhibition of metastasis, prevention of circadian disruption, prevention of angiogenesis, stimulation of cell differentiation, and activation of the immune system (Mediavilla et al. 2010). In particular, a reduction in circulating levels of melatonin and/or increased levels of reproductive hormones have been primarily proposed in the development of breast cancer as a consequence of light-at-night exposure resulting from shift work (Davis et al. 2012). Thus, the shift work group has significantly

lower urinary aMT6-s levels than daytime nurses. However, night-shift napping significantly influences 17-beta-estradiol levels. Higher outcomes of estradiol are obtained in nurses who do not take a nap compared to napping group and daytime workers. These results are comparable with the increased cancer risk of night-shift workers (Bracci et al. 2013). However, breast cancer risk might not be increased with interrupted sleep accompanied by turning on a light (Davis et al. 2001). Paracrine interactions between malignant epithelial cells and proximal stromal cells are responsible for local estrogen biosynthesis in human breast cancer (Alvarez-García et al. 2013a). Melatonin is effective on the paracrine interactions between malignant epithelial cells and proximal endothelial cells by decreasing vascular endothelial growth factor (VEGF) expression in human breast cancer cells. Lower levels of VEGF around endothelial cells are important in reducing the number of estrogen-producing cells proximal to malignant cells as well as in decreasing tumoral angiogenesis (Alvarez-García et al. 2013b). In this respect firstly melatonin diminishes endothelial cell proliferation, invasion, migration, and tube formation (Alvarez-García et al. 2013c) and later inhibits aromatase activity and its expression in endothelial cells by regulating gene expression of specific aromatase promoter regions, thereby reducing the local production of estrogens (Alvarez-García et al. 2013a). Eventually melatonin suppresses the development of endocrine-responsive breast cancers by interacting with the estrogen signaling pathways (Alvarez-García et al. 2013a). Actually there is an important mutual effect between the melatonin and estrogen signaling pathways in malignant cells. Melatonin could also inhibit the estrogen-induced proliferation of ER-alpha-positive human breast cancer cells (Girgert et al. 2003). Overexpression of the MT1 receptor mediates the antitumor activity of melatonin in ER-alpha-positive human breast cancer cells and is coupled to G proteins such as G-alpha i2, G-alpha i3, G-alpha q, and G-alpha 11 in this cell line. This growth-suppressive effect of melatonin on breast cancer cells is blocked by nonselective MT1 and MT2 receptor antagonists (Collins et al. 2003; Yuan et al. 2002). On the other hand, melatonin also inhibits the growth of human breast cancer cells via MT1-mediated suppression of cAMP. Decline in cAMP level causes the diminishing of linoleic acid uptake and its metabolism to the mitogenic signaling molecule 13-hydroxyoctadecadienoic acid (13-HODE). Downregulation of 13-HODE reduces the malignant cell proliferation and survival (Hill et al. 2011b).

While the light intensity at night increases, dose-related tumor linoleic acid uptake, mitogenic signaling molecule 13-HODE formation, DNA content, tumor growth rates, extracellular signal-regulated kinase (ERK) 1/2 activation, and cAMP levels rise. Simultaneous suppression in nocturnal melatonin secretion provokes a light intensity-dependent stimulation of proliferative activity in human breast cancer xenograft (Blask et al. 2011). Interestingly, similar human breast cancer growth rate, linoleic acid uptake, and 13-HODE production are identified in both bright light-exposed female rats and dim light-at-night-exposed rats (Blask et al. 2002). Anticancer effect of melatonin is attributed to the inhibition of cell proliferation and stimulation of differentiation and apoptosis (Blask et al. 2005a). Indeed, melatonin causes a substantial rate of growth inhibition in breast cancer cells due to a significant rise in apoptotic rate. In this case melatonin initiates apoptotic processes by

transforming growth factor beta-1 (TGFbeta1)-caspase-independent but TGFbeta1-caspase-7-dependent manners (Cucina et al. 2009). Also, via the MT1 pathway, melatonin can repress the transcriptional activity of some mitogenic nuclear receptors including ER-alpha and ROR-alpha, while potentiating the activity of other receptors involved in differentiation, antiproliferation, and apoptosis (Hill et al. 2013).

In a total of 167 patients with ER-negative, PR-negative, human epidermal growth factor receptor (Her-2)/neu-negative phenotype aggressive triple negative group of breast carcinoma, MT1 positivity is associated with a lower stage and a smaller tumor size at diagnosis. Patients with absent or low MT1 expression display a worse prognosis with shorter tumor progression-free survival and overall survival than patients with MT1-positive tumors. Moreover family history in younger women with triple negative breast carcinoma is frequently associated with negative MT1 expression (Oprea-Ilies et al. 2013). Furthermore in 190 cases of invasive ductal breast carcinomas, not only the MT1 expression is higher than in fibrocystic breast disease but also MT1 mRNA expressions are negatively correlated with the malignancy grade of the invasive ductal cancer. Actually ER-positive and HER2-positive tumors largely have a higher MT1 expression. However, the lowest MT1 protein expression level is always noted in the triple negative breast cancer cell line when compared with ER-positive cell lines (Jablonska et al. 2013).

The variants circadian (clock) genes, neuronal PAS domain-containing protein 2 (NPAS2), Clock, Cry2, and TIMELESS are claimed to be responsible for breast cancer. Grundy et al. investigated the relationships of 100 single-nucleotide polymorphisms in 14 clock-related genes with breast cancer, as well as potential interactions with shift work history in 1,042 cases and 1,051 matched controls. However, no significant interaction with shift work and circadian disruption was established on the basis of clock gene variants and breast cancer risk (Grundy et al. 2013). Thus, Monsees et al. observed that none of the 178 common variants across 15 genes involved in the circadian system was significantly associated with breast cancer risk. However, women homozygous for the minor allele with more than 24 months of shift work had a 2.83 times higher breast cancer risk compared to homozygous women with less than 24 months of shift work (Monsees et al. 2012). In fact expression of clock genes oscillates in a circadian fashion in ER-alpha mRNA-positive breast epithelial cells but not in ER-alpha-positive breast cancer cells. Downregulation of two circadian clock genes either Per2 or BMAL1 negatively affects ER-alpha circadian oscillations and breast acinar morphogenesis (Rossetti et al. 2012a). Not only ER-alpha-negative human breast cancer cells display a defective non-circadian oscillations clock but also ER-alpha-positive breast cancer cells do not display circadian oscillations in any of the clock genes. Consequently, both tumors exhibit a disrupted inner clock. According to Rossetti et al., drugs in hormone therapies targeting ER-alpha-positive breast cancer may be less efficient because the tumor cells have insufficient estrogen signaling due to impaired accumulation of ER-alpha in a circadian manner (Rossetti et al. 2012b).

During the postmenopausal period, estrogens are locally synthesized in breast tissue from adrenal-derived androgenic precursors by the enzyme aromatase.

Melatonin interferes with the activation of the ER of mammary tumor cells and also regulates the activity of the aromatases (Cos et al. 2006). Furthermore melatonin inhibits aromatase activity by regulating the gene expression of specific aromatase promoter regions (Martínez-Campa et al. 2009). Indeed suppression of CYP19A1 mRNA gene transcription by melatonin results in a significant decrease in cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1)-related aromatase activity. Eventually lower levels of melatonin in aging women may increase the risk of progressing ER-alpha-positive breast cancer through a decreased ability to suppress CYP19A1 expression. Subsequent excess local estrogen is derived from breast adipose fibroblasts which are the main local source of estrogen in breast tumors of postmenopausal women (Knower et al. 2012). At physiologic concentrations, melatonin modulates the synthesis and transformation of biologically active estrogens in human breast cancer cells, through the inhibition of enzymes involved in the estradiol formation in breast cancer cells, sulfatase and 17beta-hydroxysteroid dehydrogenase type 1 (Gonzalez et al. 2008). While melatonin reduces the activity and expression of aromatase, sulfatase, and 17beta-hydroxysteroid dehydrogenase, it simultaneously increases the activity and expression of estrogen sulfotransferase. Thus, mammary tissue may be protected from excessive estrogenic effects (Cos et al. 2008).

12.5 Conclusion

Artificial light-induced disruption of circadian organization changes the daily rhythm of several hormone productions. As a circadian pacemaker and a projection site of the retinohypothalamic tract, the SCN responds to retinal light information and encodes rhythmic output in accordance with light input. A set of genes generates circadian oscillations and form autoregulatory feedback loop based on transcriptional activators and inhibitors genes. The nocturnal synthesis and release of melatonin are tightly controlled by the central SCN clock. However, many aged individuals do not exhibit a day–night difference in melatonin levels. Either increase in the duration of daily light exposure and chronically advance in the phasing of light exposure or light-at-night-induced suppression of the nocturnal circadian melatonin signal causes circadian disruption. Eventual deterioration of circadian rhythm possibly increases the cancer susceptibility.

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Chapter 13
Chemotherapeutic Agents in Cancer Treatment and Tryptophan Metabolism

S. Altug Kesikli and Nilufer Guler

Abstract Cancer is the uncontrolled division and accumulation of cells in any part of the body. Surgery, radiotherapy, and systemic therapies are the main treatment strategies against cancers. However, systemic chemotherapy has various side effects, including nausea, vomiting, myelosuppression, cardiotoxicity (with anthracyclines), neurotoxicity (with mitotic spindle poisons), and nephrotoxicity (with platinum analogs). Although surgery can be curative in some cases, the main purposes of cancer treatments, especially in metastatic cancer patients, are the palliation of symptoms and increasing survival with better quality of life. Increasing the efficacy and decreasing the toxicity of chemotherapeutic agents are currently the main challenges in cancer treatment, for which substantial effort has been given for years. Melatonin is a hormone produced in the pineal gland from the essential amino acid tryptophan. It is one of the strongest antioxidants identified, with additional chemopreventive, oncostatic, and tumor inhibitory effects in a variety of in vitro and in vivo experimental cancer models. In addition to melatonin synthesis, tryptophan is involved in numerous metabolic activities in the human body, including the biosynthesis of serotonin, kynurenine, and nicotinamide, thereby regulating circadian rhythm, learning, memory, sleep, behavior, mood, appetite, aging, growth, reproduction, immune system, and cancer development. Therefore, tryptophan-related metabolic pathways and catabolites have gained considerable attention, especially in improving cancer treatment and predicting disease activity as well as patient survival. This chapter mainly focuses on the relationship between tryptophan metabolism and chemotherapy; the prognostic value of tryptophan catabolites as cancer biomarkers, tryptophan metabolism, and cancer-related fatigue; the association between melatonin and cancer therapy as well as chemotherapy side effects;

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and the significance of chronochemotherapy after an initial brief introduction about chemotherapeutic agents.

Keywords Cancer chemotherapy • Tryptophan metabolism • Indoleamine 2, 3-dioxygenase • 1-Methyl tryptophan • Kynurenine • Melatonin • Serotonin • Tumor vaccine • Cancer-related fatigue • Chronochemotherapy

13.1 Introduction

Cancer is the uncontrolled division and accumulation of cells in any part of the body. It is the main cause of death in developed countries. There are different treatment strategies for various cancers. These are:

1. Surgery: It is necessary for the diagnosis (biopsy) and the local treatment of cancers such as breast cancer, gastric cancer, and colonic carcinoma.
2. Radiation therapy: It is a local treatment that targets the cancer area.
3. Systemic therapy: It means the systemic control of cancer. There are different systemic treatment strategies to control cancers: chemotherapy, hormonal therapy, biologic response modifiers, and targeted therapies (tyrosine kinase and multikinase inhibitors, differentiating agents, angiogenesis inhibitors, monoclonal antibodies, proteasome inhibitors, histone deacetylase inhibitors, gene therapy, vaccines).

Chemotherapy has many side effects. The ultimate aim in cancer chemotherapy is to decrease the related side effects and increase the efficacy of the treatment. Currently, various chemicals are investigated in many studies to achieve the ultimate aim. Melatonin, a pineal hormone produced from tryptophan, has shown promising activity to decrease the side effects and increase the effectiveness of cancer chemotherapy. However, currently available data is not sufficient enough to clearly describe the relationship between tryptophan metabolites and cancer therapy. Chemotherapeutic agents, antitumor actions of melatonin, and cancer chemotherapy as well as chemotherapy side effects and the effect of tryptophan metabolism on cancer development and progression are discussed in this chapter.

13.2 Cancer Chemotherapy

Chemotherapy is the broad term used for the treatment of many different types of cancers. There are more than one hundred chemotherapy agents. These are used either as single agents or constituents of combination chemotherapy protocols. In addition, an increasing number of targeted agents (tyrosine kinase and multikinase inhibitors, differentiating agents, angiogenesis inhibitors, monoclonal antibodies, proteasome inhibitors, histone deacetylase inhibitors, gene therapy, vaccines) have been developed within the last couple of years.

Chemotherapeutic agents are usually identified initially in laboratories as a result of extensive research studies (in cell lines and/or animal studies) (Casciato 2009; Skeel 2011; Freter and Perry 2008). Human studies are generally initiated only after the proof of benefit in various animal studies. The studies that investigate the effects of any chemotherapeutic in humans are divided into four different phases:

- *Phase I trials* determine the optimal dose, schedule, and side effects of new therapies. These cancer trials usually involve a limited number of patients who have not benefited from other known treatments.
- *Phase II trials* determine the types of cancers that respond to a specific novel drug or its combinations. Additional information regarding the side effects of the treatment is also obtained. A small number of people are included due to the possible risks and unknown side effects of the agent under investigation.
- *Phase III trials* are randomized and prospectively designed trials which compare novel treatments that already demonstrated some effectiveness in a phase II trial with either placebo or treatments that are known to show some degree of effectiveness. The survival rates and the rate of side effects in each study group are investigated in a phase III trial. These cancer trials usually include hundreds to thousands of people from different study centers.
- *Phase IV trials* are trials that are performed after the approval of a new drug or combination in the treatment of a specific cancer. Therefore, these trials are also called post-marketing studies. Acute and late toxicities of the drugs under investigation as well as the quality of life of the study subjects are noted.

13.2.1 Cell Cycle

Cancer cells, unlike other body cells, are characterized by a growth process whereby their sensitivity to normal controlling factors has been completely or partially lost (Casciato 2009; Skeel 2011; Freter and Perry 2008). The cell cycle of cancer cells is qualitatively same as that of normal cells.

Eukaryotic cell cycle is a series of sequential events which ensure eukaryotic cell division and the formation of two daughter cells. Eukaryotic cell division occurs in three steps: the interphase, mitosis, and cytokinesis. During the interphase, the longest phase, cells grow and necessary nutrients are accumulated within the cells, to provide DNA replication (Sclafani and Holzen 2007). The G1, S, and G2 phases of the eukaryotic cell cycle comprise the interphase. When the cell is ready to divide, the mitosis phase begins, during which the cell is divided into two daughter cells. Complete splitting of daughter cells occurs during the cytokinesis phase. The interphase of the cell cycle is generally described in four consecutive phases: the G1 (Gap 1, postmitotic period) phase that lasts about 18–30 h, the S (Synthesis) phase which lasts about 8–30 h, the G2 (Gap 2, premitotic period) phase that lasts from 2 to 10 h, and the M (mitosis) phase which lasts 30–90 min (Kumar and Fausto 2005). The cells that do not proliferate stay in a quiescent state, called the G0 (Gap 0)

phase. Cells spend much of their lives in this phase and cells in this phase are generally programmed to perform specialized functions. This is the longest phase of the cell cycle, lasting up to months. However, in cells preparing for division or upon receiving proliferative signals, nutrients are accumulated and cell growth begins. Biosynthetic processes and the number of organelles are upregulated. When the cells at the G1 phase grow sufficiently and the microenvironment is favorable for cell division, cells enter the S phase. During the S phase, DNA and centrosome replication is completed and two sister chromatids are produced for each chromosome, though the ploidy of the cell remains unchanged. After the completion of DNA replication, cells progress into another gap phase, called the G2 phase, during which organelle production and cell growth continue. If cellular DNA is successfully replicated, the growth of the cells is sufficient and the microenvironment is favorable for cell division; cells progress to the M phase from the end of the G2 phase. During the M phase, nuclear separation occurs, and the mitotic processes, such as the prophase, metaphase, anaphase, telophase, and cytokinesis, take place. A third checkpoint exists in the M phase, which controls the proper alignment of chromosomes on mitotic spindles during the metaphase. Only the cells that pass the metaphase checkpoint can leave the M phase. The newly divided daughter cells remain in the G0 phase until the next proliferative wave (Diaz-Moralli et al. 2013).

Progression through the phases of the cell cycle is tightly regulated by cyclins (Cyc), cyclin-dependent kinases (CDKs), and the cyclin-dependent kinase inhibitors (CDKIs) (Lim and Kaldis 2013). Upon receiving growth signals, the production of CycD1 is enhanced. CycD1 can bind to CDK4 and CDK6. Cells progress through the G1 phase with the action of the CycD1 and CDK4/6 complex. CycD2 and CycD3 can also bind to CDK4/6. The CycD1-CDK4/6 complex phosphorylates the retinoblastoma susceptibility protein (Rb), resulting in the dissociation of hyperphosphorylated Rb from the Rb/E2F/DP1 complex bound to the E2F responsive genes, rendering E2F transcriptionally active (Tu et al. 2012). Active E2F initiates the transcription of various genes including CycE, CycA, DNA polymerase, thymidine kinase, and dihydrofolate reductase. The CycE produced upon E2F activation, in turn, binds to CDK2 to drive the cell through the G1/S checkpoint, which is also called as the restriction checkpoint. Two families of CDKIs, the *cip/kip* (*CDK interacting protein/Kinase inhibitory protein*) family and the INK4a/ARF (*In*hibitor of *K*inase 4/*A*lternative *R*eading *F*rame) family, regulate the G1/S checkpoint, providing an essential task in the prevention of tumor development (Ozenne et al. 2010; Saporita et al. 2007). The *cip/kip* family includes the proteins p21, p27, and p57, which bind and inhibit CycD-CDK4/6 and CycE/CDK2. p21 protein is activated by p53 that is stimulated by the DNA-damaging factors such as irradiation, whereas p27 can be activated by transforming growth factor β (TGF β), a growth inhibitor (Garner and Raj 2008; Kim et al. 2005). The INK4a/ARF family on the other hand includes p15, p16, p18, and p19 proteins. Of note, $p16^{INK4a}$ binds to the CycD-CDK4/6 complex and arrests the cell cycle in G1 phase, whereas $p19^{ARF}$ prevents p53 degradation (Maddika et al. 2007; Sperka et al. 2012). The cells that are unable to pass through the G1/S checkpoint cannot progress to complete cell division and are eliminated by the help of the apoptotic machinery.

The CycA/CDK2 complex regulates the events necessary to progress through the S and G2 phases, whereas the G2/M checkpoint is controlled by the action of CycB/CDK1 or CycA2/CDK2 complexes, which are also called the maturation-promoting factors (MPF) (Haccard and Jessus 2006). The inhibitory phosphatases within the MPF complex are removed by the activating phosphatase Cdc25, which is under the control of ATM (ataxia telangiectasia mutated) kinase protein that phosphorylates and thereby stimulates the ubiquitination and degradation of Cdc25. However, cells with proper DNA replication pass through the G2/M checkpoint and the initiation of the M phase is triggered. A final checkpoint exists during the M phase at the metaphase, which is controlled by the inhibition of anaphase-promoting complex (APC) by CDK1. APC together with Cdc20 degrades CycB1 to break down the *securin* protein (Bardin and Amon 2001). *Securins* inhibit *separases* that degrade the *cohesins*, protein rings that hold sister chromatids together. Proteolytic degradation of *securins* results in the activation of *separases* and progression of the cell from the metaphase to anaphase (Bharadwaj and Yu 2004).

13.2.2 Classification of Chemotherapeutic Agents

There are two classification systems for chemotherapeutic agents (Casciato 2009; Skeel 2011; Freter and Perry 2008):

13.2.2.1 Classification According to the Factors Such as the Way They Work, Their Chemical Structure, and Their Relationship with Other Agents (Table 13.1)

- *Alkylating agents*: Alkylating agents directly damage DNA and are cytotoxic, mutagenic, and carcinogenic (Casciato 2009; Skeel 2011; Freter and Perry 2008). These agents are cell cycle specific but not phase specific. Alkylating agents are used to treat many different types of cancers, including leukemias, lymphomas, Hodgkin's disease, multiple myeloma, and sarcomas as well as cancers of the lung, breast, and ovary. Main toxicities of this group of agents are myelosuppression, hair loss, infertility, and gonadal dysfunction. The cisplatin group has the highest potential for developing nephrotoxicity, nausea, and vomiting.

  Alkylating agents can also cause long-term damage to the bone marrow. In rare cases, this can eventually lead to acute leukemias. The risk of developing leukemia from alkylating agents is "dose dependent," as higher doses are together with higher leukemia risk. The risk of developing leukemia after receiving alkylating agents is highest at around 5–10 years after the initial treatment. Different classes of alkylating agents are shown in Table 13.1.

Table 13.1 Classification of the cancer chemotherapeutic agents is summarized (Casciato 2009; Skeel 2011; Freter and Perry 2008)

Alkylating agents	Antimetabolites	Antitumor antibiotics	Mitotic spindle poisons	Topoisomerase inhibitors
Nitrogen mustards	5-Azacitidine	*Anthracyclines*	*Taxanes*	*Topoisomerase I inhibitors*
Mechlorethamine (nitrogen mustard)	5-Fluorouracil	Daunorubicin	Paclitaxel	Camptothecin derivatives:
	6-Mercaptopurine	Doxorubicin	Docetaxel	Topotecan
Chlorambucil	Capecitabine	Doxorubicin, liposomal	Protein-bound paclitaxel	Irinotecan
Cyclophosphamide	Pemetrexed	Epirubicin	*Epothilones*	*Topoisomerase II inhibitors*
Ifosfamide	Pentostatin	Idarubicin	Ixabepilone	Epipodophyllotoxin derivatives
Melphalan	Raltitrexed	*Actinomycin-D*	*Vinca alkaloids*	
Nitrosoureas	Thioguanine	*Bleomycin*	Vinblastine	Etoposide (VP-16)
Streptozocin	Trimetrexate	*Mithramycin*	Vincristine	Teniposide
Carmustine (BCNU)	Uracil/tegafur	*Mitomycin-C*	Vindesine	*Drugs from other classes*
Lomustine		*Mitoxantrone*	Vinorelbine	Amsacrine
Alkyl sulfonates			*Estramustine*	Mitoxantrone
Busulfan				
Triazines				
Dacarbazine (DTIC)				
Temozolomide				
Ethylenimines				
Thiotepa				
Altretamine (hexamethylmelamine)				
The platinum drugs				
Cisplatin				
Carboplatin				
Oxaliplatin				

- *Antimetabolites*: Antimetabolites are a class of drugs that interfere with the building blocks of DNA and RNA synthesis (Casciato 2009; Skeel 2011; Freter and Perry 2008). Some of them are structural analogs of normal molecules that are essential for cell growth and replication. Others inhibit enzymes that are necessary for the synthesis of essential compounds. These agents damage cells during the S phase of the cell cycle. In general, these agents are most effective when cell proliferation is rapid. They are commonly used to treat leukemias and cancers of the breast, ovary, and the intestinal tract as well as other types of cancers. Except myelotoxicity, the main toxicities of this group of agents include mucositis, stomatitis, and diarrhea. Some examples of antimetabolites are shown in Table 13.1.
- *Antitumor antibiotics*: Antitumor antibiotics are drugs that are usually derived from microorganisms. These agents are usually cell cycle-nonspecific agents which are effective in all phases of the cell cycle. Antitumor antibiotics are widely used for different type of cancers (Casciato 2009; Skeel 2011; Freter and Perry 2008). These agents are especially useful in slowly growing tumors with low growth fractions. The main dose-limiting and dose-dependent toxicity of anthracyclines is the cardiotoxicity. However, pulmonary toxicity is the main and dose-limiting toxicity of bleomycin. Examples of antitumor antibiotics are shown in Table 13.1.

  Mitoxantrone is within the anthracenedione class of compounds that are similar to doxorubicin in many ways, including the potential for cardiotoxicity, though in a lesser extent. This drug may also act as a topoisomerase II inhibitor. It is commonly used to treat prostate cancers, breast cancers, lymphomas, and leukemias.
- *Mitotic spindle agents*: These drugs bind to the microtubular proteins and inhibit microtubule assembly, resulting in the dissolution of the mitotic spindle structure (Casciato 2009; Skeel 2011; Freter and Perry 2008). This class of agents is often plant alkaloids or other compounds that are derived from natural products. Although these drugs mainly act on the M phase of the cell cycle, they can cause cell damage in all phases of the cell cycle. They are used to treat many different types of cancers, including breast cancers, lung cancers, myelomas, lymphomas, and leukemias. The two main dose-limiting toxicities of these drugs are peripheral neurotoxicity and myelosuppression. Some examples of mitotic inhibitors are shown in Table 13.1.
- *Topoisomerase inhibitors*: These drugs interfere with the enzymes called the topoisomerases, which help the strands of DNA be separated so that they can be copied (Casciato 2009; Skeel 2011; Freter and Perry 2008). They are used to treat certain leukemias as well as lung, ovarian, gastrointestinal, and other cancers.

  Treatment with topoisomerase II inhibitors increases the risk of developing a second cancer, acute myelogenous leukemia (AML) in particular. With the topoisomerase inhibitors, a secondary leukemia may be seen as early as 2–3 years after the drug is administered. Topoisomerase I and II inhibitors are shown in Table 13.1.

- *Miscellaneous chemotherapy drugs*: Some chemotherapy drugs act in slightly different ways and do not fit well into any of the other categories (Casciato 2009; Skeel 2011; Freter and Perry 2008). Examples of some of these drugs and their indications include:
  - L-Asparaginase, an enzyme often used to treat acute lymphoblastic leukemias
  - Bortezomib, a proteasome inhibitor for multiple myeloma and mantle cell lymphoma
  - Anagrelide for thrombocytosis in myeloproliferative disorders
  - Hexamethylmelamine for recurrent ovarian carcinoma
  - Interferon-alpha for cutaneous T-cell lymphoma and as an adjuvant therapy in malignant melanoma, hairy cell leukemia, etc.
  - Interleukins for metastatic renal cell carcinoma and malignant melanoma
  - Temsirolimus, an mTOR (mammalian target of rapamycin) inhibitor to treat metastatic renal cell carcinoma
  - Thalidomide for multiple myeloma and myelodysplastic syndrome (MDS)
  - Lenalidomide for multiple myeloma and MDS
  - Suramin for prostatic carcinoma
  - Bexarotene, a retinoic acid receptor (RAR) inhibitor for the treatment of cutaneous T-cell lymphoma that is refractory to at least one prior chemotherapy regimen
  - Tretinoin for acute promyelocytic leukemia
- *Hormonal agents*: Examples include (Casciato 2009; Skeel 2011; Freter and Perry 2008):
  - Adrenocorticosteroids: Steroids are natural hormones and hormone-like drugs that are useful in treating some types of cancer (lymphoma, leukemias, multiple myeloma, and breast cancers) as well as other illnesses. In addition, they are used in the treatment of allergic reactions and also as antiemetic agents.
  - Adrenal inhibitors: Mitotane for the treatment of adrenal carcinomas.
  - The anti-estrogens: Fulvestrant, tamoxifen, and toremifene for the treatment of hormone receptor-positive breast cancers.
  - Aromatase inhibitors: Anastrozole, exemestane, and letrozole for the treatment of postmenopausal hormone receptor-positive breast cancers.
  - Progestins: Megestrol acetate for the treatment of endometrial and breast cancers as well as cancer cachexia.
  - Estrogens in the treatment of hormone receptor-positive breast cancers.
  - Androgens for the treatment of hormone receptor-positive breast cancers.
  - Anti-androgens: Bicalutamide, flutamide, and nilutamide for the treatment of prostatic cancer.
  - Gonadotropin-releasing hormone (GnRH) agonists that are also known as the luteinizing hormone-releasing hormone (LHRH) analogs: Leuprolide and goserelin, for the treatment of prostatic carcinoma and premenopausal hormone receptor-positive breast carcinoma.

13.2.2.2 Classification of Chemotherapeutics According to Their Activities During the Cell Cycle

- *Phase nonspesific*: These groups of drugs generally have a linear dose-response curve, which means that the greater the amount of drug dose administered, the greater the fraction of cells killed (Casciato 2009; Skeel 2011; Freter and Perry 2008).
  - *Cycle-nonspecific drugs*: These drugs kill the nondividing cells. They are especially effective in the G0 phase (e.g., steroid hormones, antitumor antibiotics except for bleomycin)
  - *Cycle-specific, phase-nonspecific drugs*: They are effective only when the cells proceed in the cell cycle. They can inflict injury at any point of the cell cycle (e.g., alkylating agents).
- *Phase specific*: Their effect is not dose related but is a function of both time and concentration. If the drug concentration is maintained over a period of time, more cells enter the specific lethal phase of the cell cycle and are killed.
  - *Cycle-specific, phase-specific drugs*: These are effective only during a particular phase of the cell cycle (e.g., antimetabolites, antimicrotubule/antimitotic spindle agents).

The goals of chemotherapy are to cure the cancer (e.g., definitive therapy: childhood leukemias and lymphomas, gestational choriocarcinoma, germ cell tumor of the testicles), to control the disease in adjuvant settings by administering it following the resection of the primary disease (e.g., breast cancer, ovarian cancer, and colorectal cancer), or to palliate the symptoms only (Casciato 2009; Skeel 2011; Freter and Perry 2008). In metastatic cancer patients, however, the main purposes of the treatment are the palliation of symptoms, prolongation of survival, and maintenance of the quality of life at the maximum. The main adverse effects of chemotherapeutics are hair loss, nausea, vomiting, fatigue, organ toxicities, myelosuppression, and immunosuppression. A number of agents, especially the antioxidants, have been investigated to increase the efficacy and to decrease the toxicity of chemotherapeutic agents (Block et al. 2008; Greenlee et al. 2009). One of these agents is melatonin (N-acetyl-5-methoxytryptamine), one of the strongest antioxidants, derived from an essential amino acid, tryptophan. In addition to melatonin synthesis, tryptophan is involved in numerous metabolic activities in the human body, including the biosynthesis of serotonin, kynurenine, and nicotinamide, thereby regulating circadian rhythm, learning, memory, sleep, behavior, mood, appetite, aging, growth, reproduction, immune system, and cancer development. Therefore, tryptophan-related metabolic pathways and catabolites have gained considerable attention, especially to improve cancer treatment and to predict disease activity as well as patient survival.

13.3 Tryptophan Metabolism and Cancer Chemotherapy

L-Tryptophan is an important essential amino acid which is vital for growth and nitrogen balance in humans. As this indole-containing amino acid cannot be produced within the human body, it should be obtained by dietary means. Dietary tryptophan is mainly used for protein synthesis. Moreover, serotonin and melatonin biosynthesis and the oxidation of tryptophan for nicotinamide production are other relevant major pathways (Wu 2010) (Fig. 13.1). However, 99 % of dietary tryptophan that is not used in protein synthesis (excess tryptophan) is catabolized along the kynurenine pathway to quinolinic acid. Furthermore, excess tryptophan can also induce a major oxidative pathway in the liver via the enzyme tryptophan 2,3-dioxygenase (TDO). Melatonin and serotonin are important for the modulation of the circadian rhythm, learning, memory, sleep, behavior, mood, appetite, aging, growth, reproduction, immune system, and cancer development, whereas nicotinamide is incorporated into nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which are essential coenzymes in a variety of enzymatic oxidation-reduction reactions (Yao et al. 2011). Moreover, it was reported in various studies that both tryptophan depletion and kynurenine accumulation contribute to the growth and development of cancers, either directly acting on the tumor or indirectly acting on the host immune system and the cells within the tumor microenvironment (Prendergast et al. 2011). Being vital for so many human functions and metabolic processes, tryptophan and tryptophan-related pathways are therefore attractive targets for cancer treatment, and substantial efforts have been

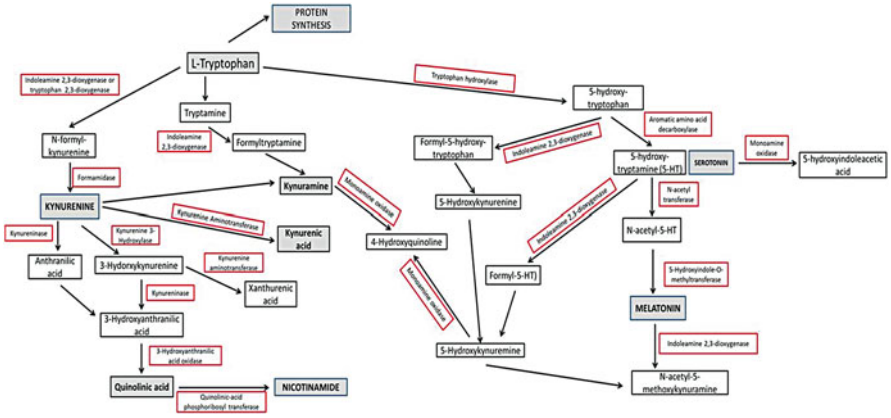

Fig. 13.1 Various metabolic pathways related to tryptophan are depicted. Dietary tryptophan can be used in one of three major pathways: (1) protein synthesis, (2) serotonin and melatonin biosynthesis (Muller et al. 2008), and (3) oxidation for the production of nicotinamide. Excess tryptophan induces the major oxidative pathway in the liver via TDO. Ninety-nine percent of dietary tryptophan that is not used in protein synthesis is catabolized along the kynurenine pathway to quinolinic acid. *Red rectangles* represent the enzymes and *rectangles* with *gray* background represent important metabolic end products (Modified and adapted from Stone and Darlington 2002; Stone et al. 2013)

given in finding the selective and efficient inhibitors of these pathways to incorporate into current cancer treatment protocols.

Indoleamine 2,3-dioxygenase is the key and rate-limiting enzyme for the catabolic pathway of tryptophan and the main metabolite of tryptophan catabolism is kynurenine. Although TDO and the two splice variant forms of the enzyme IDO (IDO1 and 2) are the rate-limiting enzymes related to the metabolic pathway for tryptophan in different tissues, various studies indicated that the plasma tryptophan to kynurenine ratio (or vice versa) could be used as an indirect measure to represent the activation status of this catabolic pathway in cancer patients (de Jong et al. 2011). Plasma tryptophan levels in humans were previously reported to change with diurnal cycle and the time of the last meal, whereas the changes in kynurenine levels were only minimal. Therefore, the variations in plasma kynurenine/tryptophan as well as kynurenine levels could largely be referred to the changes in kynurenine production (de Jong et al. 2009).

Studies related to the role of tryptophan catabolism in cancer chemotherapy can be classified in five groups (Platten et al. 2012):

1. Studies investigating the use of chemotherapeutics and/or tumor vaccines together with selective tryptophan pathway inhibitors, including 1-methyl tryptophan (1-MT)
2. Clinical studies in which the significance of the levels of tryptophan catabolites in patient sera or other biological fluids as biomarkers that represent disease activity or therapy response is investigated (prognostic biomarker studies)
3. Studies linking tryptophan metabolism to one of the most common symptoms in cancer patients, the cancer-related fatigue
4. Studies evaluating the use of melatonin in cancer treatment
5. Studies investigating the relationship between the administration of chemotherapeutics and the circadian rhythm (chronomodulated chemotherapy)

13.3.1 The Use of IDO Inhibitors with Chemotherapy and/or Tumor Vaccines

The expression of IDO in various human cancers either by the tumor cells themselves or the antigen-presenting cells in the tumor microenvironment or the tumor-draining lymph nodes were documented first by Uyttenhove et al. by employing immunohistochemistry in biopsy specimens from human cancer tissues (Uyttenhove et al. 2003). They demonstrated that IDO was expressed in glioblastoma multiforme and cancers of the lungs, esophagus, pancreas, stomach, prostate, urinary bladder, cervix, endometrium, and ovaries as well as colorectal cancers. Later, IDO expression and a role for IDO was also reported in malignant melanomas, breast cancers, basal cell carcinomas, acute myeloid leukemias, multiple myeloma, and recently in thyroid carcinomas (Munn et al. 2004; Mansfield et al. 2009; Lo et al. 2011; Curti et al. 2007a, b; Bonanno et al. 2012; Moretti et al. 2014). Moreover, a number of

studies suggested an association between IDO expression and clinical/surgical stage (endometrial, breast, and esophageal cancers), the presence of nodal or metastatic disease (colorectal cancer, malignant melanoma, endometrial cancer, pancreatic ductal adenocarcinoma, breast cancer), decreased infiltration of T cells (colorectal and endometrial cancers), and increased Foxp3-expressing regulatory T cells (myeloid leukemia, pancreas, and breast cancers) into tumor bed as well as worse survival (malignant melanoma, serous ovarian cancer, colorectal cancer, endometrial cancer, and acute myeloid leukemia) (Johnson and Munn 2012).

The idea of the use of conventional chemotherapeutics with pharmacological inhibition of IDO is based on the findings that confirm the cooperation of the immune system with chemotherapeutics to augment tumor destruction (Haynes et al. 2008). Cancer chemotherapeutics were shown to increase the release of potential cancer-specific antigens from damaged or dying tumor cells, thereby increasing the possibility of capturing and presentation by the antigen-presenting cells, eventually enhancing the chances for the activation of antitumor immune responses. However, these responses alone are never sufficient to completely eliminate the cancer (Zitvogel et al. 2008a, b). Nevertheless, the chemotherapy-induced transient lymphopenia and homeostatic recovery period are hypothesized to facilitate the breaking of tumor-induced immune tolerance, with the help of the cytokine-rich milieu (Williams et al. 2007). The depletion of tumor-promoting regulatory T cells by certain chemotherapeutics might additionally help destroy cancer cells (Ma et al. 2010). Therefore, supporting the antitumor immune responses during chemotherapy or at the post-chemotherapy period by IDO inhibitors is rational and may help to surpass tumor immune privilege.

The first identified and the most investigated pharmacological IDO inhibitor is 1-methyl tryptophan (1-MT), though there are also some other IDO inhibitors that are currently under investigation (Johnson and Munn 2012). 1-L-MT and 1-D-MT are two stereoisomers of methylated tryptophan with different biological features. 1-L-MT is the competitive inhibitor of IDO1 that is effective in in vitro and cell-free conditions, whereas 1-D-MT does not inhibit IDO1 activity. Metz et al. revealed that 1-D-MT preferentially inhibited IDO2 activity (Metz et al. 2007). However, it was repeatedly reported that 1-D-MT is more effective with concomitant chemotherapy than 1-L-MT in preclinical models and in vivo conditions, possibly due to the inhibition of immune suppression produced by IDO-expressing antigen-presenting cells in the tumor microenvironment and tumor-draining lymph nodes (Platten et al. 2012). One possible answer to this interesting paradox was recently suggested by Schmidt et al. who reported that commercially available 1-L-MT lots involve sufficient levels of tryptophan to counteract IDO-mediated tryptophan depletion in vitro and to antagonize IDO-mediated suppression of T-cell responses (Schmidt et al. 2012). Accordingly, Hou et al. demonstrated that 1-L-MT was more effective than 1-D-MT in purified enzyme and HeLa cell (human cervical cancer cell line)-based assays, whereas 1-D-MT was more beneficial in inhibiting antigen-presenting-cell-mediated T-cell suppression and as an anticancer agent in mouse melanoma and breast cancer models together with the use of cyclophosphamide, paclitaxel, or gemcitabine (Hou et al. 2007). The authors also reported that the

in vivo effects were IDO-gene dependent and concluded that 1-D-MT is the suitable choice of IDO inhibitor to be incorporated into human chemo-immunotherapy regimens. Subsequent evaluation of pharmacokinetics and toxicity of oral 1-D-MT in rats and dogs revealed that the application of 1-D-MT is safe with very little toxicity (Jia et al. 2008). Yet, Löb et al. and Qian et al. suggested that 1-L-MT is more beneficial than 1-D-MT in reversing IDO-mediated proliferation arrest and tryptophan depletion (Lob et al. 2009; Qian et al. 2009). Recently, Qian et al. raised questions about the efficiencies of both 1-L-MT and 1-D-MT in inhibiting IDO-mediated proliferation arrest (Qian et al. 2012). They demonstrated that although weaker in enzymatic activity, IDO2 could also induce tryptophan depletion and kynurenine accumulation in T cells, and 1-L-MT is a more potent inhibitor of IDO2 than 1-D-MT. Moreover, they reported that neither 1-L-MT nor 1-D-MT could reverse IDO2-mediated T-cell proliferation arrest, even at very high concentrations. These results warrant further research to identify the most optimal and potent IDO inhibitors, especially to the use in human clinical trials in combination with conventional chemotherapeutics.

Inhibition of IDO has been tested in various in vivo animal and preclinical human studies. Single-agent therapy with an IDO inhibitor in preclinical models such as Lewis lung carcinoma was usually far from being satisfactory, with only marginal benefits and slowing of tumor growth at best (Muller and Prendergast 2005). Muller et al. investigated the use of 1-MT in combination with paclitaxel in a transgenic murine breast carcinoma model that is similar to human ductal carcinoma in situ. The authors reported that in contrast to single-agent therapy, only the combination therapy leads to T-cell-dependent regression of established breast tumors (Muller et al. 2005). Zhang et al. similarly reported significantly higher levels of cytotoxicity of T cells against 1-MT treated IDO transfected BGC-823 gastric undifferentiated adenocarcinoma cells than the levels of cytotoxicity against untreated IDO transfected BGC-823 cells (Zhang et al. 2011).

Boasso et al. reported that in comparison to healthy individuals, IDO mRNA expression was higher in peripheral blood mononuclear cells (PBMCs) from HIV(+) patients and that the proliferation of $CD4^+$ T cells in PBMCs from HIV-infected patients was enhanced upon inhibition of IDO in vitro with 1-MT (Boasso et al. 2007). Baban et al. demonstrated that IDO acts a critical switch to modulate the generation of regulatory T cells (Tregs) and the development of IL-17-secreting T helper cells (Baban et al. 2009). The authors demonstrated that the inhibition of IDO by 1-D-MT in CpG oligonucleotide-stimulated $CD19^+$ plasmacytoid dendritic cells induced the conversion of Tregs into $T_{HELPER}17$-like cells. Similarly, Curti et al. reported that 40 out of 76 primary AML samples but none of the healthy bone marrow-derived mononuclear cells or $CD34^+$ hematopoietic stem cells expressed IDO both at the mRNA and protein level (Curti et al. 2007a). Moreover, the addition of both 1-D-MT and 1-L-MT in cocultures containing only IDO^+ AML cells in allogeneic mixed leukocyte reactions with $CD3^+$ T cells boosted allogeneic T-cell proliferation. Same group also demonstrated that IDO^+ AML blasts both in vitro and in vivo induced the conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ FoxP3-expressing Tregs, an effect which was abrogated by 1-MT (Curti et al. 2007b).

The inhibition of IDO activity by the use of 1-D-MT has been extensively used also in other in vivo murine models such as the lupus-prone mice, mice with HIV-1 encephalitis, toxoplasmosis, HSV-1 infection or leishmaniasis, α-galactosylceramide (α-GalCer)-induced mouse hepatitis model, murine renal ischemia-reperfusion injury model, cerebral ischemia-reperfusion model, murine $T_{HELPER}1$ cell-induced autoimmune encephalomyelitis (corresponding to human multiple sclerosis) model, OT-1 and OT-2 T-cell receptor transgenic mouse models, B16 melanoma model, MHC-deficient GM-CSF transfected B16 melanoma model, surgically induced 4T1 metastatic mammary carcinoma model, and phorbol myristate acetate-induced skin tumor model (Ravishankar et al. 2012; Potula et al. 2005; Divanovic et al. 2012; Ito et al. 2010; Mohib et al. 2008; Jackman et al. 2011; Mellor et al. 2004; Sharma et al. 2009; Munn et al. 2005; Gu et al. 2010; Muller et al. 2008; Kwidzinski et al. 2005).

El Kholy et al. investigated the effects of the inhibition of IDO on cultured leukemia blast cells and the consequences on T-cell proliferation (El Kholy et al. 2011). They reported that 52 % of the isolated peripheral blood mononuclear cells (MNCs) representing leukemia blasts from 25 patients but none from the 25 healthy controls expressed IDO and IDO expression was significantly correlated with IDO enzymatic activity. Moreover, they showed that culturing patient MNCs with 10 μg/ml Adriamycin significantly decreased the proliferation of MNCs and the addition of 1 mmol/ml 1-MT (1-methyl tryptophan) into cultures further reduced the proliferation of leukemia blasts, especially after 72 h, whereas incubation with 1-MT alone did not diminish blastic proliferation. Similarly, Miyazaki et al. reported that human glioma cell lines LN229 (glioblastoma), U251 (astrocytoma), T98G (glioblastoma multiforme), and U87 (glioblastoma) expressed IDO mRNA, which was enhanced upon IFN-γ stimulation, resulting in tryptophan depletion and kynurenine production in cultured cells (Miyazaki et al. 2009). The authors also demonstrated that the addition of 1-MT alone increased tryptophan and decreased kynurenine levels in culture supernatants. Moreover, the addition of temozolomide, bischloroethylnitrosourea (BCNU), etoposide, and cisplatin in IFN-γ-stimulated LN229 cell cultures significantly decreased the viability of cells that was further decreased by the addition of 1-MT, which suggests that the use of IDO inhibitors such as 1-MT may help improve therapeutic efficacy.

The expression of IDO was also shown in ovarian, endometrial, and cervical cancers (Ino 2011). In most of these studies, IDO expression was correlated with reduced number of tumor-infiltrating lymphocytes and poor clinical outcome (Okamoto et al. 2005; Ino et al. 2008; Inaba et al. 2010). Inaba et al. reported the expression of IDO in more than half of the ovarian cancer cases and demonstrated the increase of IDO expression with advanced (stages II–IV) disease (Inaba et al. 2009). The authors pointed out worse progression-free and overall survival (OS) outcomes in patients with increased IDO expression, suggesting that IDO is both a contributor to disease biology and a prognostic indicator in ovarian cancers. Moreover, they also demonstrated that while 1-MT did not show any effect, the combination of paclitaxel with 1-MT synergistically improved survival in a murine ovarian cancer peritoneal carcinomatosis model with transplanted IDO-overexpressing SKOV3 cells. Similarly, Yoshida et al. reported significantly reduced tumor outgrowth with 1-MT + paclitaxel combination in comparison to paclitaxel

alone in nude mice that received subcutaneous IDO-expressing human endometrial cancer cells (Yoshida et al. 2008).

Attempts to identify novel, second-generation IDO inhibitors have recently introduced new molecules with distinct properties for preclinical testing. Yue et al. reported a hydroxyamidine chemotype to be used as a backbone in novel potent competitive IDO inhibitors and showed dose-dependent efficacy in a murine B16 melanoma model (Yue et al. 2009). One year later, Liu et al. demonstrated that INCB024360, a novel orally active hydroxyamidine small molecule inhibitor, selectively inhibited IDO1 activity without interfering with IDO2 or TDO at an IC50 value of 10 nM, increased T-cell and DC viability in cocultures, decreased conversion to Treg cells, and inhibited tumor growth in murine PAN02 pancreatic adenocarcinoma model in a T-cell-dependent fashion (Liu et al. 2010). Koblish et al. also reported that both orally active IDO1-selective hydroxyamidine small molecule inhibitors INCB023843 and INCB024360 were well tolerated and exhibited durable suppression of IDO1 activity in mice and dogs (Koblish et al. 2010). Moreover, the authors demonstrated potent suppression of tryptophan metabolism and tumor growth in murine CT26 colon and PAN02 pancreatic adenocarcinoma models in a dose- and T-cell-dependent fashion. These promising preclinical results with the hydroxyamidine small molecule inhibitors, which exhibited good efficacy and toxicity profiles with desirable pharmaceutical properties, lead to the initiation of testing in a number of clinical studies (Table 13.2).

1-Alkyl tryptophan derivatives comprise another class of novel IDO inhibitors. Sun et al. reported that novel 1-alkyl tryptophan derivatives demonstrated functions similar to 1-MT in inhibiting IDO expression and enzymatic activity in IFN-γ-treated dendritic cells (Sun et al. 2010). However, they also showed a differential selectivity for those derivates, as 1-propyl-tryptophan (1-PT) and 1-isopropyl-tryptophan (1-isoPT) moderately inhibited IDO1 expression and enzymatic activity in dendritic cells, whereas 1-butyl-tryptophan (1-BT) and 1-ethyl-tryptophan (1-ET) mainly suppressed IDO2 expression. Fung et al. discovered that phenylhydrazine, a hydrazine derivative, potently inhibited IDO1 at nontoxic concentrations, after screening of a fragment library (Fung et al. 2013). Muller et al. suggested the use of another novel IDO inhibitor, the ethyl pyruvate, as they demonstrated that ethyl pyruvate suppressed the expression of IDO1 in vitro in IFNγ- and LPS-stimulated U937 human lymphoma cells and inhibited tumor growth in a T-cell-dependent manner in murine B16-F10 melanoma and $Bin1^{-/-}$ MR KEC (Myc + ras-transformed keratinocytes from Bin1 deficient mice) tumor models in vivo (Muller et al. 2010). Vasilyeva et al. later confirmed the findings by Muller et al. showing that ethyl pyruvate administration accelerated tumor regression and decreased tumor incidence in mice immunized with human H-29 colorectal adenocarcinoma cells (Vasilyeva et al. 2012). A tryptophan analog, methylthiohydantoin-DL-tryptophan, and(−)-epigallocatechin gallate (EGCG), the major catechin in green tea, were both reported to significantly block IDO expression and activity (Okamoto et al. 2007; Ogawa et al. 2012). The latter molecule was also shown to significantly reduce IDO mRNA expression in the colonic epithelium and stroma as well as to decrease the total number of aberrant crypt foci in a rat azoxymethane-induced colon carcinoma

Table 13.2 Current clinical trials that investigate the use of IDO inhibitors in cancer treatment as of 10.02.2014 are shown

Clinicaltrials.gov number	Intervention	Phase	Disease	No. of patients enrolled/estimated enrollment	Primary endpoint	Status
NCT00739609	1-Methyl-D-tryptophan	I	Relapsed/refractory solid tumors (breast, lung, melanoma, pancreas, etc.)	17	Safety and efficacy	Terminated due to low enrollment
NCT00567931	1-Methyl-D-tryptophan	I	Metastatic or refractory adult solid tumors	46	Safety and toxicity	Active but not recruiting
NCT01191216	1-Methyl-D-tryptophan and docetaxel	I	Metastatic adult solid tumors	30	Safety and toxicity (max. tolerated dose)	Active but not recruiting
NCT01792050	Indoximod (1-methyl-D-tryptophan) + docetaxel versus placebo + docetaxel	II	Metastatic breast cancer	154	Efficacy (progression-free survival)	Recruiting
NCT02052648	Indoximod (1-methyl-D-tryptophan) and temozolomide	I/II	Glioblastoma multiforme, glioma, gliosarcoma, malignant brain tumors	18	Safety and dosing	Not yet recruiting
NCT01042535	Adenovirus-p53 transduced dendritic cell vaccine and 1-methyl-D-tryptophan	I/II	Recurrent/metastatic breast cancer/advanced adult solid tumors with mutated p53	37	Max. tolerated dose and objective response rate by RECIST criteria	Recruiting
NCT01560923	Indoximod (1-methyl-D-tryptophan) sipuleucel-T versus placebo + sipuleucel-T	II	Metastatic castration resistant prostate cancer	50	Safety, efficacy, immune response to sipuleucel-T	Recruiting
NCT01604889	INCB024360 + ipilimumab versus placebo + ipilimumab	I/II	Unresectable or metastatic melanoma	136	Safety and tolerability; OS	Recruiting

NCT01685255	INCB024360 versus tamoxifen	II	Biochemical-recurrent-only epithelial ovarian cancer, primary peritoneal carcinoma, fallopian tube cancer	110	Progression-free survival using RECIST 1.1	Recruiting
NCT01822691	INCB024360	II	Myelodysplastic syndrome	40	Best ORR	Recruiting
NCT01195311	INCB024360	I	Advanced malignancies	50	Safety and tolerability as measured by adverse events and dose-limiting toxicities	Active, not recruiting
NCT01961115	INCB024360 and the multi-peptide melanoma vaccine MELITAC 12.1	II	Stage III/IV melanoma	12	Changes in the concentration and number of $CD8^+$ T cells infiltrating the tumor by IHC	Recruiting
NCT02042430	INCB024360 before therapeutic conventional surgery	II	Stage III/IV epithelial ovarian/fallopian tube or primary peritoneal cancer	12	Number of participants with an increase in $CD8^+$ T cells	Recruiting

The table is prepared by the data obtained from the clinicaltrials.gov website

model (Ogawa et al. 2012). Recently, arctigenin and trachelogenin molecules derived from *Mediterranean Carthamus tinctorius* (Safflower) lignans are reported to intensely interact with the binding site of the IDO enzyme and interfere with the enzymatic activity in PBMC cultures in vitro (Temml et al. 2013).

Alternatively, instead of targeting IDO1, Nakano et al. recently introduced the targeting of the metabolites of tryptophan catabolism that act on tumor cells (Nakano et al. 2012). It is known that the administration of exogenous kynurenine and quinolinic acid resulted in increased activation and nuclear translocation of β-catenin, enhanced proliferation of HCT 116 and HT-29 human colon cancer cells, and increased tumor growth in mice (Platten et al. 2012; Johnson and Munn 2012; Thaker et al. 2013). Accordingly, to interfere with kynurenine production and regulation in tumor cells and in the tumor microenvironment, the authors reported a novel kynurenine production-inhibiting benzenesulfonamide derivative, the *compound 1*, which suppressed IDO mRNA transcription, STAT1-dependent transcriptional activity, and DNA binding without inhibiting IDO enzymatic activity or STAT1 expression or phosphorylation.

In vivo inhibition of IDO activity was proposed to boost the action of antitumor vaccines, due to the actions observed both against the immune cells and tumor cells (Platten et al. 2012; Johnson and Munn 2012; Sharma et al. 2009; Gu et al. 2010). Li et al. investigated concomitant use of 1-MT and a tumor cell lysate-pulsed dendritic cell (DC) vaccine in a murine model of pancreatic adenocarcinoma (Li et al. 2010). The authors reported that IDO mRNA and protein expression as well as $FoxP3^+$ Treg counts were higher in spleens and tumor-draining lymph nodes of mice with tumors than the control healthy mice. Moreover, concomitant use of the tumor cell lysate-pulsed DC vaccine and 1-MT was shown to reduce tumor growth rate in tumor-bearing mice in comparison to mice treated with either DC vaccine or 1-MT alone, suggesting an enhancement of antitumor vaccine efficacy, possibly by reducing the number of Treg cells. However, due to a wide variety of immune-balancing functions of IDO, much effort has also been given to specifically target IDO inhibition locally at the tumor microenvironment or directly at the tumor intracellular compartment. Accordingly, Yi et al. conjugated 1-MT with fibroblast-activating protein-α (FAPα), a protein that is known to be expressed only on reactive stromal fibroblasts in 90 % of epithelial cancers but not on normal adult human tissues (Yi et al. 2011). They demonstrated that 1-MT could easily dissociate from the resulting FAPτ-MT tumor vaccine and inhibited IDO activity in LPS- or IFN-γ-stimulated RAW264.7 murine macrophages and CNE2 nasopharyngeal carcinoma cells (with no phagocytic activity) in vitro, as observed by the reduction in tryptophan depletion and kynurenine production. Moreover, in mice with FAPα-expressing 4T1 mammary carcinoma, FAPτ-MT administration resulted in significant and possibly $CD8^+$ T-cell-dependent reduction of tumor growth and increased survival in comparison to the mice receiving CpG or CpG+ FAPτ, achieving similar tumor volumes with mice receiving FAPτ + free 1-MT lavage. Importantly, the authors reported that FAPτ-MT vaccine did not lead to any pregnancy failures in pregnant BALB/C or C57BL/6 mice, proving that the vaccine did not cause any systemic immune activation and acted as a local IDO inhibitor, which might be a clinically applicable potential tumor vaccine.

The association of the expression of hepatic and neuronal enzyme tryptophan 2,3-dioxygenase (TDO) with cancer progression has recently been elucidated (Opitz et al. 2011). Opitz et al. clearly demonstrated that human glioma cell lines and glioma-initiating cells but not astrocytes constitutively express TDO, which catabolizes tryptophan into kynurenine that acts as an endogenous ligand of the human aryl hydrocarbon receptor (AHR) (El Kholy et al. 2011). The authors showed that the proliferation of both $CD4^+$ and $CD8^+$ T cells was impaired due to the production of kynurenine by active TDO in a concentration dependent manner, with enhanced motility and survival of tumor cells through the autocrine/paracrine activation of AHR. Moreover, the infiltration of TDO-expressing tumors by T cells was much lower than that of the tumors that did not express TDO and increased expression of TDO or AHR in glioma patients (WHO grades II–IV) correlated with decreased OS in comparison to the glioma patients with lower tumoral TDO and AHR expression. One very interesting finding of Opitz et al. was that TDO expression was not confined only to malignant gliomas, but was also seen in malignant melanoma, hepatocellular carcinomas, renal cell carcinomas, and non-small cell lung carcinomas as well as the cancers of the breast, colon, urinary bladder, and ovaries in high rates, suggesting that TDO expression is a common feature of various cancers. Correspondingly Pilotte et al. reported that selective inhibition of TDO but not IDO in a TDO-expressing murine P815 mastocytoma model by the selective TDO inhibitor, the compound LM10, rendered the mice the ability to reject TDO-expressing tumors upon systemic administration, with good oral bioavailability and well tolerability profiles (Pilotte et al. 2012). Furthermore, in a recent structure-activity study on a series of 3-(2-(pyridyl)ethenyl) indoles, more than 70 novel derivatives were evaluated for their ability to potently inhibit TDO, and among them, the *compound 58* was discovered to show highly selective potent TDO inhibition ($K(i) = 5.5\ \mu M$) with good oral bioavailability (Dolusic et al. 2011).

Current clinical trials that are registered to clinicaltrials.gov investigating the use of IDO inhibitors in cancer treatment are listed in Table 13.2. As it is seen from the table, most of the studies are Phase I or II trials with small sample sizes. Moreover, still no outcome data has been reported from these ongoing clinical trials. It should also be kept in mind that due to the multifaceted roles played by tryptophan, tryptophan metabolism, and its catabolites in humans, sufficient preclinical effort should be given before the application of any novel IDO inhibitor or other therapeutic to human subjects. As more potent and specific IDO inhibitors are identified that are suitable for clinical use, patients will likely benefit from combination studies with conventional chemotherapeutics and/or tumor vaccines.

13.3.2 Tryptophan Catabolites as Disease Biomarkers

A number of studies previously demonstrated that IDO expression and enzymatic activity can be used as a prognostic marker for various human cancers, such as cancers of the breast, colon, ovaries, endometrium as well as for melanoma, lymphomas, and leukemias (Platten et al. 2012; Johnson and Munn 2012; Soliman et al. 2013; Ninomiya et al. 2011). In this section, however, the prognostic value of

tryptophan catabolites such as kynurenine will be discussed, though the number of studies evaluating the prognostic value of tryptophan catabolites is limited. Yet, the value of kynurenine or kynurenine/tryptophan levels in predicting patient outcome or as a prognostic factor has been tested in various studies. Creelan et al. reported that the baseline plasma kynurenine to tryptophan ratio as an indirect indicator of IDO activity was higher in patients with stage III non-small cell lung cancer (NSCLC) than that of healthy controls (Creelan et al. 2013). A total of 33 patients in this single-arm phase II study received gemcitabine and carboplatin for induction, followed by simultaneous paclitaxel, carboplatin, and 74 Gy thoracic irradiation. Interestingly, kynurenine/tryptophan levels increased both after the induction treatment and the following chemoradiation in these patients. Although the baseline kynurenine/tryptophan levels correlated neither with tumor-related parameters such as size, histological grade, and clinical stage or OS, the post-induction increase in kynurenine/tryptophan levels was shown to significantly associate with worse overall and progression-free survival. However, due to the small sample size and the lack of immunohistochemical data and data about other correlative immunological markers such as interferon levels, CD3 and FoxP3, the results from this study should be carefully deduced. Furthermore, Sagan et al. demonstrated that serum levels of kynurenic acid were significantly higher in patients with lung adenocarcinoma (71 patients with adenocarcinoma and 96 with squamous cell carcinoma, a total of 227 non-small cell lung cancer patients) than those in patients with squamous cell lung carcinoma (107.1 ± 62.8 pmol/ml; 95 % CI: 92.4–132.3 pmol/ml vs. 82.1 ± 47.6 pmol/ml; 95 % CI: 78.5–91.2 pmol/ml; $p = 0.027$) (Sagan et al. 2012). Yet, in a very recent prospective study, Chuang et al. investigated circulating levels of tryptophan and six of its metabolites as well as the kynurenine/tryptophan ratios in 893 incident lung cancer cases and 1,748 matched controls and demonstrated that tryptophan levels as well as kynurenine/tryptophan ratios were associated with increased overall lung cancer risk after adjustment for risk factors [the odds ratios comparing the 5th and the 1st quartiles were 0.52 (95 % CI: 0.37–0.74) for tryptophan and 1.74 (95 % CI: 1.24–2.45) for kynurenine/tryptophan ratio, respectively] (Chuang et al. 2013). Moreover, the authors claimed that according to their previous work, plasma methionine strongly correlated with tryptophan, and after adjustment for methionine, the associations of tryptophan and kynurenine/tryptophan ratios increased considerably. They also stated that kynurenine/tryptophan ratios positively correlated with squamous cell carcinoma, as the odds ratio comparing the 5th and the 1st quartiles was 2.83 (95 % CI: 1.62–4.94).

Yoshikawa et al. investigated the value of serum L-kynurenine concentration as a prognostic indicator in 73 patients with diffuse large B-cell lymphoma (Yoshikawa et al. 2010). Patients who were younger than 70 years recruited for this study received eight cycles of R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone), whereas those older than 70 years received six cycles of R-THP (tetrahydropyranyl Adriamycin, a less-cardiotoxic anthracycline derivative of doxorubicin)-COP therapy. The authors reported that the complete response rate and OS were better in patients with serum L-kynurenine levels lower than 1.5 μM than those with L-kynurenine levels $\geq$1.5 μM, which was the median serum

L-kynurenine level for all patients. Serum L-kynurenine levels were found to significantly correlate with poor performance status and clinical stage. Moreover, they indicated that according to the multivariate analyses, only serum L-kynurenine concentration was an independent prognostic factor for OS in these patients. Similarly, significantly elevated levels of tryptophan catabolites were also reported for patients with myelodysplastic syndromes, which correlated with cytopenia development (Berthon et al. 2013). Moreover, the authors demonstrated that elevated tryptophan catabolites inhibited the propagation of hematopoietic progenitor cells in vitro.

In a series of studies, Sakurai et al. investigated IDO enzymatic activity in Japanese breast cancer patients receiving trastuzumab, conventional chemotherapy, or hormonotherapy, by determining plasma tryptophan and kynurenine levels using high-performance liquid chromatography (HPLC). In recurrent breast cancer patients receiving chemotherapy, Sakurai et al. observed that tryptophan/kynurenine levels did not differ at the pre- and posttreatment periods in patients under weekly paclitaxel regimen, whereas in comparison to the re-chemotherapy patients with tri-weekly docetaxel, the tryptophan/kynurenine levels were significantly elevated in post-chemotherapy patients treated with weekly paclitaxel, indicating that weekly paclitaxel might be a better option than tri-weekly docetaxel for recurrent breast cancer patients (Sakurai et al. 2008). They also demonstrated that in comparison to the pre-chemotherapy period, tryptophan/kynurenine levels were significantly higher following chemotherapy in recurrent breast cancer patients who were older than 36 years but not in those younger than 35 years, suggesting that IDO-related immune alterations as well as the effects of chemotherapeutics on IDO enzymatic activity might directly associate with patients' ages (Sakurai et al. 2009). The same authors reported that compared to the breast cancer patients receiving hormonotherapy, tryptophan/kynurenine levels increased only in patients treated with conventional chemotherapy [either in the form of FEC (fluorouracil, epirubicin, and cyclophosphamide) or weekly paclitaxel] at the posttreatment period in comparison to the pre-treatment period (Sakurai et al. 2010). Moreover, tryptophan/kynurenine levels in patients treated with chemotherapy were significantly higher than those of the patients who received hormonotherapy alone at the posttreatment period. However, the long-term follow-up results of the same patient groups argued against their initial findings, showing that post-FEC and post-weekly paclitaxel kynurenine/tryptophan levels were higher than the levels before the initiation of chemotherapy and that kynurenine/tryptophan levels at the posttreatment period were significantly higher in patients receiving chemotherapy than those of the patients under hormone therapy (Sakurai et al. 2011). In 2012, Sakurai et al. reported that compared to the patients receiving trastuzumab therapy, tryptophan/kynurenine levels were significantly lower in patients treated with conventional chemotherapy (with either epirubicin and cyclophosphamide or weekly paclitaxel) at the posttreatment period (Sakurai et al. 2012). The latter two reports collectively point out to the fact that in comparison to conventional chemotherapy, more specific treatments such as hormone therapies or targeted therapies might be more effective in reducing the enzymatic activity of IDO, thereby attenuating the detrimental effects on antitumor immunity in breast cancer patients.

Serum levels of tryptophan, kynurenine, tyrosine, and kynurenine/tryptophan (to estimate IDO activity) in 33 stage I–III breast cancer patients and 24 controls (patients without any detectable breast cancer) were compared in a recent study by Lyon et al. (2011). Although not statistically significant, tryptophan levels were relatively lower and kynurenine as well as tyrosine levels were higher in patients with breast cancer before the initiation of chemotherapy in comparison to the controls. However, kynurenine to tryptophan ratios were significantly higher in breast cancer patients before the initiation of chemotherapy in comparison to the controls. Moreover, although not statistically significant, levels of tryptophan, kynurenine as well as the kynurenine to tryptophan ratios were lower and tyrosine levels were higher in breast cancer patients at the end of chemotherapy than those before the initiation of chemotherapy. Still, the sample size and results were inadequate to evaluate chemotherapy efficacy in this study, where the contents of the chemotherapy administered were not mentioned either.

13.3.3 Tryptophan Metabolism and Cancer-Related Fatigue

Evidence supports that tryptophan metabolism may directly be linked to cancer-related fatigue (CRF). CRF is described as the sense of persistent tiredness that is resistant to rest and sleep and that is related either to the cancer itself or the cancer treatment administered, which eventually leads to disability to perform daily activities (Ryan et al. 2007). Vagal afferent neural activation, dysregulation of the muscle and ATP metabolism as well as the cytokine network, the dysfunction of the hypothalamic-pituitary-adrenal axis, and circadian rhythm as well as the progression of cancer-related anemia were all shown to be involved in the development of CRF (Wang 2008). Apart from these, dysregulation of the 5-hydroxytryptamine (serotonin, 5-HT) neurotransmitter system was hypothesized to associate with CRF (Barsevick et al. 2010). Serotonin is known to be involved in various functions, such as sleep, memory, learning, appetite, body temperature, mood, and behavior control; the regulation of muscle contraction, endocrine, and cardiovascular functions; as well as the regulation of depression. It was proposed that elevated levels of 5-HT and/or the upregulation of some 5-HT receptor subtypes in the brains of cancer patients, resulting from either the cancer itself or the administered treatment, caused persistent tiredness and decreased somatomotor drive. Animal studies demonstrated that the accumulation of tryptophan, the precursor for 5-HT, in the hypothalamus and brain stem following prolonged exercise leads to increased production of 5-HT by some neurons, which results in physical and mental fatigue. Moreover, it was previously reported that patients with chronic fatigue syndrome had elevated levels of plasma-free tryptophan and several studies showed that the use of some selective serotonin reuptake inhibitors (SSRIs) decreased exercise performance in humans (Ryan et al. 2007). Yet, the mechanisms in humans are not well understood. However, considering the fact that the rate-limiting step for 5-HT synthesis in the brain is the transport of tryptophan from the circulation into the brain, two possible

pathways were suggested to associate with increased tryptophan and 5-HT accumulation. The first pathway involves the reduction in the circulating levels of branched-chain amino acids (BCAAs) due to capturing by the muscles following exercise, resulting in increased tryptophan transport. The second pathway is based on increased free fatty acids (FFAs) concentrations after exercise. Elevated plasma FFA level enhances the dissociation of tryptophan from albumin, resulting in more available unbound tryptophan in plasma to be transported into the brain.

It should be noted that CRF is a complex disease with multifactorial contributors. Therefore, it would not be rational to try to explain the developmental mechanism with only one single pathway. Accordingly, dysregulation of inflammatory signals and cytokine network may also be equally important. Evidence suggests that 5-HT metabolism is also affected by proinflammatory cytokines such as the tumor necrosis factor-α (TNF-α) (Zhu et al. 2006). Similarly, systemic cytokines can influence the activation of afferent peripheral nerves and the production of other neuromediators like prostaglandins at the blood-brain interface (Zhu et al. 2010). Moreover, it was previously reported that 5-HT metabolism may also be modified through the activation of IDO by the action of proinflammatory cytokines like TNF-α, interleukin-1β (IL-1β), interferon-α (IFN-α), and IFN-γ (Catena-Dell'Osso et al. 2013).

13.3.4 Melatonin and Cancer Chemotherapy

13.3.4.1 Melatonin

Melatonin, N-acetyl-5-methoxytryptamine, derived from an essential amino acid tryptophan, is a small lipophilic molecule, an indoleamine hormone which is mainly secreted by the pineal gland (Carlberg 2000). It is intricately involved in the regulation of human chronobiological and endocrine function (Carlberg 2000; Brzezinski 1997; Claustrat et al. 2005). In addition to the pineal gland, melatonin is also synthesized in the retina, lens, bile, gastrointestinal tract, bone marrow, skin, and the immune system cells (Brzezinski 1997). Its synthesis shows a circadian pattern: melatonin synthesis is very low during the daytime, ascends in the evening, and peaks between 02:00 and 04:00 h (4–8 h after the unset of darkness) (Claustrat et al. 2005). Nighttime levels of circulating melatonin are often 1–20 times higher than daytime concentrations (Arendt 2000). It then gradually declines. It is found in high concentrations in the bone marrow, intestines, and in the mitochondria and nucleus at subcellular levels (Reiter et al. 2009). Recently, its presence is detected in the gingival crevicular fluid and saliva (Cutando et al. 2007).

Peak blood levels of melatonin are different between individuals and depend on age. High and young secretors have plasma levels of melatonin ranging from 54 to 75 pg/ml, whereas levels in low and elderly secretors range from 18 to 40 pg/ml (Brzezinski 1997). Endogenous melatonin half-life in serum is 30–60 min and exogenous melatonin half-life is 12–48 min (Brzezinski 1997; Claustrat et al. 2005). Melatonin is not stored to any extent. Melatonin has various local and systemic

functions that play critical roles in controlling inflammatory reactions (Claustrat et al. 2005). It also has antioxidative and free radical scavenging activity and oncostatic and immunomodulatory functions (Brzezinski 1997; Cutando et al. 2007).

13.3.4.2 Anticancer Effects of Melatonin

Melatonin has multiple physiological antitumor activities: antiproliferative action, modulation of cell cycle, differentiation, and apoptosis; inhibition of telomerase activity, antioxidant, anti-angiogenic and anti-inflammatory effects, inhibition of metastases, stimulation of cytokine production, changing fatty acid transport and metabolism, epigenetic effects, hormonal effects (antiestrogenic effects); and prevention of circadian disruption (Regelson and Pierpaoli 1987; Jung and Ahmad 2006; Hill et al. 2009; Mediavilla et al. 2010; Sanchez-Barcelo et al. 2012; Lissoni 2002). Several clinical studies have demonstrated that cancer progression is associated with a progressive decline in the pineal function and in melatonin secretion, mainly during the dark period of the day (Lissoni 2002; Lissoni et al. 1986). Several studies have shown reduced levels of melatonin in patients with certain type of cancers compared with age-matched normal, healthy people (Brzezinski 1997; Lissoni et al. 1986; Mills et al. 2005; Lee 2006; Hu et al. 2009). Therefore, diminished nocturnal secretion of melatonin is a very common cancer progression-associated endocrine deficiency (Lissoni 2002). Some examples of the anticancer activities of melatonin and rationales for integrating melatonin with various chemotherapy regimens in cancer treatment are outlined in Tables 13.3 and 13.4 (Mediavilla et al. 2010; Sanchez-Barcelo et al. 2012; Lissoni 2002).

13.3.4.3 Melatonin and Cancer Treatment

Many cancer types (breast, NSCLC, metastatic renal cell carcinoma, hepatocellular carcinoma, brain metastases from solid tumors, lymphomas, melanoma, soft tissue sarcoma, gastric carcinoma, pancreatic carcinoma, liver cancer, colorectal cancer, testicular cancer, head and neck carcinoma) have shown to be responsive to melatonin in different settings (Sanchez-Barcelo et al. 2003; Cerea et al. 2003; Lissoni et al. 2001, 2002, 2003; Gonzalez et al. 1991; Italian Study Group for the Di Bella Multitherapy Trials 1999; Barni et al. 1990; Neri et al. 1994; Viviani et al. 1990). Interestingly, many of these studies were conducted by Lissoni et al. (1987, 1989, 1990, 1991, 1992a, b, 1993a, b, c, d, 1994a, b, c, d, 1995a, b, c, d, e, f, 1996a, b, c, 1997a, b, c, d, 1998, 1999a, b, 2002, 2003 and Aldeghi et al. 1994). In those studies, melatonin was combined with other types of cancer therapies such as radiation therapy (Lissoni et al. 1996b), hormonal therapy (tamoxifen and LHRH analogs) (Lissoni et al. 1995a, 1996a, 1997b; Yan et al. 2002), chemotherapy [cisplatin, etoposide, irinotecan, 5-fluorouracil (5-FU), anthracyclines, gemcitabine, oxaliplatin, paclitaxel, folates, mitoxantrone, transcatheter arterial chemoembolization] (Mills et al. 2005; Lee 2006; Hu et al. 2009; Sanchez-Barcelo et al. 2003; Cerea et al.

Table 13.3 Anticancer activities of melatonin that are related to cancer treatment are summarized

Direct cytotoxic or cytostatic effects by the modulation of cell cycle, differentiation, and apoptosis (see Sect. 13.2.1)
Increases the duration of cycle in different cell lines (MCF-7, HepG2, HL-60, etc.), expanding the G1 phase
Decreases DNA synthesis (MCF-7 cells)
Upregulates the expression of p53 and p21 in MCF-7 cells
Downregulates the expression of cyclin D1
Induces cell differentiation in different types of normal and tumor cells by modulating oncogene and endocrine receptor expression
Induces apoptosis by different mechanism involving caspases 7 and 9 and TGF-β1[a]
Anti-inflammatory effects
Inhibits the secretion of inflammatory cytokines, such as IL-6
Inhibition of telomerase
Inhibits the basal expression of hTERT[a] in MCF-7 cells
Inhibits hTERT expression induced by natural estrogens and xenoestrogens (cadmium)
Antiangiogenesis
Reduces serum levels of VEGF[a] in advanced cancer patients
At pharmacologic doses, inhibits VEGF expression induced by hypoxia
At pharmacologic doses, inhibits the expression of HIF[a]-1α
Inhibition of metastases
Reduces the invasiveness of MCF-7 cells
Increases the expression of E-cadherin and β1-integrin in MCF-7 cells
Immunomodulatory effects
Stimulates the production of NK[a] cells, monocytes, and leukocytes
Stimulates the production of cytokines, including IL-2, IL-6, IL-12, and TNF[a]-α
Fatty acid transport and metabolism
Blocks tumor linoleic acid uptake and its conversion to 13-HODE[a], which normally activates EGFR/MAPK[a] mitogenic signaling
Epigenetic effects
Inhibits p300 histone acetyl transferase in macrophages, thus inhibiting p52 acetylation and binding to the DNA and silencing iNOS and COX[a]-2 genes
Increases histone H3 acetylation in C17.2 neural stem cells
Prevention of circadian disruption
Modulates the expression of the clock genes: *mPER1, mClock,* and *mBmal1* in neuronal cultures of mouse striatum
In different prostate cancer cell lines, upregulates clock and Per2 proteins, whereas downregulates Bmal1 protein
Antiestrogenic effects
Shares properties of the selective estrogen receptor modulators (SERM)
Shares properties selective estrogen enzyme modulators (SEEM)

Modified and adapted from Mediavilla et al. 2010; Sanchez-Barcelo et al. 2012; Lissoni 2002
[a]*TGF* transforming growth factor, *hTERT* human telomerase reverse transcriptase, *VEGF* vascular endothelial growth factor, *HIF-1α* hypoxia inducible factor 1α, *NK* natural killer, 13-HODE:13(S)-hydroxyoctadecadienoic acid, *COX-2* cyclooxygenase 2

Table 13.4 The rationale for the use of melatonin alone or in combination with chemotherapy in cancer treatment is summarized

Rationale for melatonin administration to cancer therapy
Endocrine replacement therapy of cancer progression-related decline in pineal functions
Anticancer activities of melatonin (see Table 13.3)
Palliative therapy:
Thrombocytopenia
Neoplastic cachexia
Depression and asthenia
Rationale for melatonin-chemotherapy combination in cancer treatment
Increased efficacy:
Prevention of chemotherapy-induced lymphocyte damage with potentially increased survival
Antioxidant-induced increase in cytotoxic activity of chemotherapy and enhancement of tumor response rate
Prevention of chemotherapy toxicity:
Myelosuppression, especially thrombocytopenia
Neurotoxicity
Cardiotoxicity
Asthenia
Immunosuppression-related symptoms

Modified and adapted from Lissoni (Mediavilla et al. 2010; Sanchez-Barcelo et al. 2012; Lissoni 2002)

2003; Lissoni et al. 1987, 1989, 1991, 1996a, b, c, 1997a, 1998, 1999a, 2002, 2003; Gonzalez et al. 1991; Barni et al. 1990; Todisco et al. 2001), immune therapies (interferon, interleukin-2) (Lissoni et al. 1993a, b, c, d, 1994c, d, 1995d, e, f, 1997c, d; Aldeghi et al. 1994; Barni et al. 1992, 1995; Brivio et al. 1995; Bregani et al. 1995; Brackowski et al. 1994), and supportive care (Lissoni et al. 1992a, 1994b).

In 2012, two meta-analyses of randomized control trials (RCTs) were published. In the first meta-analysis, the efficacy and safety of melatonin in concurrent chemotherapy and radiotherapy for solid tumors were analyzed (Wang et al. 2012). Eight eligible RCTs were included ($n=761$). In all trials, 20 mg melatonin was administered orally once a day. Melatonin significantly improved complete and partial remissions (16.5 % vs. 32.6 %, $p<0.00001$) as well as the 1-year survival rate (28.4 % vs. 52.2 %, $p=0.001$). However, the same origin of the six of the eight included RCTs (six studies were from the same center), the lack of any information about the methods of randomization used, the lack of any placebo controls, the unblinded nature of those RCTs, and the unknown loss to follow-up rates in six of the eight included RCTs comprise the main limitations of this meta-analysis. In the second meta-analysis, 21 RCTs (melatonin with or without chemotherapy in solid tumors) were included (Seely et al. 2012). Melatonin dose was 10–40 mg p.o. once daily. The pooled relative risk (RR) for 1-year mortality was 0.63 ($p<.001$), and the rates for complete response (RR 2.33), partial response (RR 1.90), and stable disease (RR 1.51) were significantly higher in the melatonin arm. In trials combining melatonin with chemotherapy, adjuvant melatonin decreased 1-year mortality rate

(RR 0.60) and improved complete response (RR 2.53), partial response (RR 1.70), and stable disease rates (RR 1.15). However, same limitations were also present in the latter study.

13.3.4.4 Melatonin and Chemotherapy Side Effects

Cancer chemotherapy has multiple side effects. Common side effects are myelosuppression, nausea-vomiting, hair loss, fatigue, and specific organ toxicities related to the class of chemotherapeutic agent used, such as the cardiotoxicity of anthracyclines, stomatitis-mucositis of antimetabolites, and neurotoxicity of taxanes (Casciato 2009; Skeel 2011; Freter and Perry 2008). In a meta-analysis by Wang et al., radiochemotherapy-related side effects were significantly lower in the melatonin arm than those in the control arm (thrombocytopenia 19.7 % vs. 2.2 %, $p<0.00001$; neurotoxicity 15.2 % vs. 2.5 %, $p<0.0001$; fatigue 49.1 % vs. 17.2 %, $p<0.00001$) (Wang et al. 2012). In the second meta-analysis, melatonin also significantly reduced asthenia, leukopenia, nausea-vomiting, hypotension, and thrombocytopenia according to the non-melatonin arm (Seely et al. 2012). Myeloprotective effects of melatonin, especially on thrombocytopenia, were observed in some trials (Lissoni et al. 1999a, 2001; Viviani et al. 1990). These effects of melatonin might be due to a direct action of the pineal hormone on hematopoiesis (Maestroni et al. 1994). The myeloprotective effect of melatonin in combination with carboplatin and etoposide in 20 advanced lung cancer patients was investigated in a randomized double-blinded study (Ghielmini et al. 1999). Melatonin was given orally at a dose of 40 mg/day for 21 days in the evenings. At the end of the study, there was no significant difference between the melatonin and non-melatonin arms.

Cardiotoxicity is an important and dose-limiting side effect of anthracyclines (see Table 13.1). They increase the production of free radicals, thus altering cellular levels of antioxidant enzymes and causing molecular damage (Casciato 2009; Skeel 2011). Melatonin has protective effects for multiple organs (Wang et al. 2005; Topal et al. 2004). In one study by Zhang et al., the protective effect of melatonin against Adriamycin-induced cardiotoxicity was investigated in rat breast cancer models (Zhang et al. 2013). In that study, 116 rats were randomly divided into the Adriamycin (A) group and the melatonin + Adriamycin (M + A) group. Using conventional light and electron microscopy, it was observed that injury to rat myocardial tissues within the M + A group was significantly alleviated compared to those within the A group. The 1-month survival rate was also higher in the M + A group than that of the A group. In conclusion, the authors suggested that melatonin might have a protective effect in the myocardium by reducing Adriamycin-induced oxidative damage.

Mucositis and stomatitis are important side effects of chemotherapy. They are also the main and dose-limiting toxicities of antimetabolites (see Table 13.1) (Casciato 2009; Skeel 2011; Freter and Perry 2008). Melatonin is lipophilic in nature, which makes it possible to easily cross cell membranes and access to almost every cell. In addition, melatonin is mainly a cell protector rather than a hormone. Recently its presence was detected in saliva as well as gingival and crevicular fluids.

Moreover, melatonin has a significant role in protecting the oral cavity through its antioxidant, anti-inflammatory, immunomodulatory, antiviral, antimycotic, and anticancer actions (Cutando et al. 2007, 2011; Chava and Sirisha 2012). Melatonin protects the oral cavity and gastrointestinal tract from conditions such as stomatitis, esophagitis, and peptic ulcer (Czesnikiewicz-Guzik et al. 2007). In one randomized study, cancer patients were randomized to chemotherapy-only and chemotherapy + melatonin (20 mg/day p.o. in the evenings) arms. Grade I–III stomatitis was less frequently observed in the melatonin arm than that in the chemotherapy-only arm; however, the difference was not statistically significant (2 vs. 9 patients, respectively) (Lissoni et al. 1997e). Hence, melatonin can be used both as a coadjuvant in oral hygiene aids and as an antimicrobial agent in local therapy to benefit from its natural anti-inflammatory actions. The same properties of melatonin may also be beneficial in the treatment of cancer patients; however, further studies are needed (Czesnikiewicz-Guzik et al. 2007).

Neurotoxicity is the dose-limiting toxicity of taxanes (see Table 13.1) (Casciato 2009; Skeel 2011; Freter and Perry 2008). Types of neuropathy that are mainly caused by taxanes include peripheral neuropathy, myalgia, arthralgia, and motor weakness. Mild to moderate neuropathy develops nearly 60–90 % of the patients receiving chemotherapy with taxanes. Among others, docetaxel has less neurotoxic effects than paclitaxel. As melatonin was shown to exhibit neuroprotective properties in preclinical animal studies, the use of melatonin in taxane-containing chemotherapy protocols is rational (Rogerio et al. 2005; Chang et al. 2002). Accordingly, the neuroprotective effect of melatonin was investigated in one pilot clinical study (Nahleh et al. 2010). Twenty-two breast cancer patients treated either with paclitaxel or docetaxel were recruited for the study. Patients received melatonin 21 mg daily at bedtime. Grade I (23 %) and grade II (22 %) neuropathy was seen in 45 % of the patients. None of the patients had grade III neuropathy. The number of patients who developed neuropathy during the study was lower than the historical controls. Similarly, in a meta-analysis by Wang et al., neuropathy was significantly lower in the melatonin arm than that in the non-melatonin arm (2.5 % vs. 15.2 %, respectively, $p < 0.0001$) (Lissoni et al. 1995d; Starr 1969).

In various clinical trials, chemotherapy-induced vomiting and alopecia could not be prevented by melatonin (Regelson and Pierpaoli 1987; Sanchez-Barcelo et al. 2003). However, psychogenic vomiting was reduced by melatonin (Lissoni et al. 1997e).

Cancer- and chemotherapy-related anorexia and cachexia syndrome is one of the most common causes of death among patients with cancer (Mantovani et al. 2001). In a controlled trial in 100 patients with metastatic cancer, melatonin was found to significantly reduce bodyweight loss (Lissoni et al. 1996d). Melatonin could also decrease the circulating levels of TNF (Braczkowski et al. 1995; Lissoni et al. 1994e).

Melatonin dose for insomnia and jet lag is 1.5–5 mg/day. In all studies that were outlined in this chapter, melatonin dose was between 10 mg and 40 mg/day (Wang et al. 2012; Seely et al. 2012). 20 mg/day was the most common dose used in clinical trials (Lissoni et al. 1995d). This raises the question of toxicity and whether there are any significant side effects at these dose levels. However, none of the trials reported any significant side effects due to melatonin use. The most common side

effects of melatonin were sedation and sleepiness in some patients. It is accepted that to avoid the sedative action, melatonin should be administered in the evenings (Wang et al. 2012).

13.3.4.5 Chronochemotherapy

Chronomodulated chemotherapy (circadian chronochemotherapy) is the administration of chemotherapeutic drugs according to the biological clock (Kelleher et al. 2014; Levi et al. 2007, 2010). Melatonin is the main hormone for the circadian rhythm. Mechanisms of control over time operate for both normal healthy cells and cancer cells. Cells divide and rest at different times in a day. These actions are tightly regulated by nine specific clock-related genes in the brainstem. However, rest and division hours of cancer cells are different from healthy cells. Therefore, a common hypothesis suggests that if a chemotherapeutic is applied right at a specific time point when cancer cells divide but healthy cells rest, the efficacy of that chemotherapeutic can be increased and toxicity can be decreased. Proportion of cells in the S phase of the cell cycle in the skin, bone marrow, intestines, and oral mucosa varies by 50 % in each 24-h period (Kelleher et al. 2014; Levi et al. 2007, 2010; Mormont and Levi 1997; Huang et al. 2011; Levi 1997). Dihydropyrimidine dehydrogenase (DHD) is the catabolizing enzyme for 5-FU. Enzyme activity was shown to increase almost up to 40 % between 22:00 and 00:00 h (Levi 1997). According to various studies taxanes, platinum-based drugs, and antimetabolites are suitable for chronochemotherapy (Table 13.1) (Levi et al. 2007, 2010; Mormont and Levi 1997). One trial compared the toxicity of anthracyclines and cisplatin given at one of two dosing times to 30 patients with advanced ovarian cancer. Adriamycin at 06:00 h and cisplatin between 16:00 and 20:00 h produced significantly fewer side effects, such as myelosuppression and renal toxicity, than treatment given 12 h apart (Levi et al. 1990). Platinum drugs given in the afternoons and 5-FU continuously infused at nights significantly reduced side effects in comparison to classical chemotherapeutic agents for patients with metastatic colorectal cancer and NSCLC (Levi et al. 1997; Focan et al. 1995). Levi et al. investigated the use of conventional chemotherapy with chronomodulated chemotherapy in metastatic colorectal cancer patients (Levi et al. 1997). They reported that continuous infusion of oxaliplatin in the afternoons and 5-FU at nights significantly increased tumor overall response rate (ORR) (51 % vs. 27 %, respectively; $p=0.003$) and delayed median time to treatment failure (6.4 months on chronochemotherapy vs. 4.9 months on conventional therapy arm, $p=0.006$). Moreover, severe stomatitis was observed in 76 % of the patients in the conventional therapy arm but only in 14 % of the patients in the experimental arm ($p<0.0001$). The rates for peripheral sensory neuropathy were 31 % and 16 %, respectively ($p<0.01$). However, median survival (15.9 vs. 16.9 months, respectively) and 3-year survival rates (22 % vs. 21 %, respectively) were similar in both groups. Similarly, continuous infusion of carboplatin and 5-FU at nights was compared to conventional chemotherapy in NSCLC patients. Adverse events were lower and the quality of life was better in the chronochemotherapy arm

than that of the conventional treatment arm (Focan et al. 1995). In another study, conventional (during official working hours) chemotherapy versus chronomodulated paclitaxel (continuous IV infusion), carboplatin (infusion between 4 and 8 pm), and 5-FU (infusion between 10 pm and 7 am) combination chemotherapy were compared in patients with recurrent or metastatic head and neck squamous cell carcinoma (Chen et al. 2013). The ORR was higher (71.43 % vs. 42.86 %, $p<0.05$) and the OS was longer (15.3 months vs. 10.6 months, $p<0.05$) in the chronomodulated therapy arm than those of the conventional therapy arm. In addition, adverse events were significantly lower (46.43 % vs. 76.19 %, $p<0.05$) and grade III–IV adverse events were significantly lower (7.14 % vs. 33.33 %, $p<0.05$) in the experimental arm than those of the conventional therapy arm. Liao C et al. also reported a meta-analysis comparing chronomodulated chemotherapy and conventional chemotherapy for patients with advanced colorectal cancer (Liao et al. 2010). The authors analyzed 5 randomized studies from 79 articles. A total of 958 patients were included in the study. There was a significant OS benefit (hazard ratio: 0.82; $p=0.023$) in favor of the chronomodulated therapy. However, the ORR was not significantly different between the two arms (relative risk: 1.27, $p=0.196$). Grade III–IV mucositis [odds ratio (OR) = 2.26] and asthenia (OR = 2.15) were higher, whereas grade III–IV neutropenia (OR = 0.26) was lower in the chronomodulated chemotherapy arm than those in the conventional therapy arm, without reaching statistical significance. The incidences for nausea/vomiting, peripheral neuropathy, and grade III–IV diarrhea were similar in both treatment arms.

In summary, chronomodulated chemotherapy was shown to have some advantages (more effective, less toxic) over conventional chemotherapy. Yet, more high-quality randomized controlled trials are needed for the confirmation of those benefits.

13.4 Conclusion

Despite much has recently been revealed about the biology of tryptophan and of tryptophan catabolites in cancer, the relative significance of the expression of IDO1, IDO2, or TDO in various cancer settings is yet unknown. Increased catabolism of tryptophan results in tryptophan depletion and the accumulation of various catabolites such as kynurenine. In cells of the immune system, especially in T cells, tryptophan depletion activates the GCN2 stress kinase pathway resulting in T-cell anergy and apoptosis, whereas kynurenine production activates signals through AHR leading to the suppression of T-cell differentiation and functions. On the other hand, in tumor cells, kynurenine and other catabolites exhibit a protumorigenic role, increasing the motility and survival of tumor cells. Taken altogether, tryptophan catabolism creates an immunosuppressive microenvironment both around the tumor and tumor-draining lymph nodes. Studies indicate that tryptophan catabolites are worth testing as potential biomarkers predicting disease activity and patient outcome. Moreover, constituents of the metabolic and catabolic pathways related to tryptophan are potential targets to develop certain therapeuticals. The use of IDO/

TDO inhibitors together with conventional chemotherapeutics and tumor vaccines is also a promising research field. Yet, randomized prospective multicenter trials with large sample sizes are warranted to validate the initial promising results obtained with preclinical and early clinical studies. Due to its possible effects in protecting cancer patients from the side effects of cancer chemotherapeutics and in potentiating the oncostatic efficacy, melatonin may also be beneficial in the treatment of cancer patients, especially in adjuvant settings. However, similar to IDO/TDO inhibitors, randomized, double-blinded, multicenter, and international trials with larger sample sizes are required to verify the efficacy and safety of melatonin use. Chronochemotherapy is as well a remarkably promising area that increasingly attracts scientific interest and attention.

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Chapter 14
Indoleamine 2,3-Dioxygenase-Competent Regulatory Dendritic Cells and Their Role in Alloimmune Regulation and Transplant Immune Tolerance

Atilla Engin and Ayse Basak Engin

Abstract Underlying mechanisms of immune tolerance, including the characterization of antigen-presenting cells (APCs) and regulatory T (Treg) cells and the function of indoleamine 2,3-dioxygenase (IDO) activity that may play key roles in promoting operational tolerance, have been discussed in this chapter. Donor dendritic cells (DCs) migrate from graft and present the donor major histocompatibility complex (MHC) molecules to allospecific T cells. Allorecognition occurs when donor MHC molecules are processed by host APCs. Treg cells have an indispensable role in the creation of peripheral allograft tolerance; therefore, Treg cell differentiation is important to create a new T-cell repertoire. Immune tolerance primarily occurs in the thymus that is referred to as central tolerance. Elimination of self-reactive T cells and clonal anergy are two principal mechanisms of peripheral tolerance (Fig. 14.1). Allorecognition can occur by two distinct pathways; direct recognition is achieved by foreign MHC class II molecules on the surface of donor bone marrow-derived cells, and indirect recognition is executed by internalized donor class II histocompatibility molecules. Allogeneic DCs are required for both rejection and tolerance of allografts. While CD28, CD5, and CD43 contribute to negative selection of the tolerance-susceptible population, costimulatory molecule, cytotoxic T lymphocyte antigen (CTLA)-4 signaling in thymocytes may diminish the efficacy of clonal deletion. The programmed death 1 (PD-1) receptor and its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) play an important role in tolerance. Tolerogenic DCs present antigen to antigen-specific T cells but fail to deliver

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adequate endogenous costimulatory signals for T-cell activation and proliferation. Pathways in the B7-CD28, family of costimulatory molecules, regulate T-cell activation and tolerance. Acute rejection of vascularized solid organ allotransplants is essentially mediated through direct allorecognition, while indirect allorecognition is commonly associated with chronic rejection of transplants. The subpopulation of CD4+ T lymphocytes that coexpress the forkhead family transcription factor-3 (Foxp3) plays a unique role as Tregs. Foxp3+ Treg cells are potent mediators of dominant self-tolerance in the periphery. Interleukin (IL)-2 secretion from conventional T cells is critical for development of suppressive activity of Treg. Increased inherent IDO activity, which is the rate-limiting enzyme for the tryptophan (Trp) catabolism in transplanted cells, plays an important role in the induction of immune tolerance. IDO expression by DCs in response to interferon (IFN)-gamma stimulation may suppress T-cell responses and promote tolerance either through direct effects of Trp depletion and Trp metabolites on T cells or through the effects of IDO on DCs. Overexpression of IDO in transplanted organs can prolong allograft survival due to peripheral tolerogenic effect. Treg activation by IDO+ plasmacytoid (p)DCs requires an intact amino acid-responsive general amino acid control nonderepressible (GCN)2 pathway in the Tregs. GCN2 and mammalian target of rapamycin (mTOR) react to amino acid deprivation. Following liver transplantation, the incidence of chronic rejection is lower than the other posttransplant organ grafts, and immunosuppression withdrawal is also successful in a high proportion of liver recipients. Liver graft acceptance results from donor antigen-specific tolerance which is demonstrated by the extension of tolerance to other grafts of donor origin. However, both underlying mechanisms of spontaneous liver transplant tolerance and transfer of tolerance from the liver to the second graft are not known precisely.

Keywords Allorecognition • Dendritic cells • Regulatory T cells • Spontaneous liver transplant tolerance • Immunosuppression • General amino acid control nonderepressible (GCN)2 • Mammalian target of rapamycin (mTOR) • Interleukin-2 • Cytotoxic T lymphocyte antigen (CTLA)-4 • Clonal deletion

14.1 Introduction

Successful transplantation requires immune tolerance development against allogeneic antigens. Efficacious immune tolerance may prevent a host versus graft reaction, which leads to graft rejection. Induction of immune tolerance decreases the risk of acute and chronic graft rejection after transplantation and can improve transplanted organ survival (Alpdogan and Van Den Brink 2012). In this regard considerable interest focused on various mechanisms of clinical transplant tolerance such as clonal deletion, clonal diversion, receptor editing, anergy, induction of regulatory T (Treg) cells, expression of immunomodulatory molecules, and production of immunosuppressive components against the potential threat of high-affinity T-cell receptor (TCR) for self-peptide–major histocompatibility complex (MHC) complexes

(Steinman et al. 2003; Ezzelarab and Thomson 2011). Many recent studies intend to clarify the role of dendritic cells (DCs), Treg cells, and myeloid-derived suppressor cells in transplant tolerance. Donor DCs migrate from graft and present donor MHC molecules to allospecific T cells. Allorecognition occurs when donor MHC molecules are internalized, processed, and presented as peptides by host antigen-presenting cells (APCs). Alloantigen recognition on specialized APCs may have a tolerazing effect (Game and Lechler 2002). Positively selected T cells have TCR with low affinity for self-peptide–MHC complexes (Xing and Hogquist 2012). In this case Treg cell differentiation is important to create a new T-cell repertoire. Therefore, Treg cells have an indispensable role in maintaining immunological unresponsiveness to self-antigens and creation of peripheral allograft tolerance (Sakaguchi et al. 2008). However, DCs are absolutely necessary for the maintenance of both central and peripheral tolerance against self-antigens (Ohnmacht et al. 2009).

14.2 T-cell Tolerance

T cells recognize the pathogen fragments in the context of surface MHC molecules on host cells. The tolerance of T cells begins as soon as a TCR is formed and expressed on the cell surface of a T-cell progenitor in the thymus. Immune tolerance that primarily occurs in the thymus before the maturation and circulation of T cells is referred to as "central tolerance" (Xing and Hogquist 2012). Central tolerance is efficient, but it is also incomplete. In the presence of the deleting ligand, as many as 25–40 % of T cells reactive to a self-peptide can escape clonal deletion in the thymus and in the periphery. Persistence of low-affinity self-specific T cells in the periphery and the presence of a deleting ligand can specifically decrease the structural diversity of the TCR repertoire (Bouneaud et al. 2000). The two principal mechanisms of peripheral tolerance are activation-induced cell death and anergy. In CD4+ T lymphocytes, activation-induced cell death is induced by repeated stimulation, with high levels of interleukin (IL)-2 productions. Under these conditions, the T cells coexpress Fas (CD95) and Fas ligand (FasL), and engagement of Fas triggers apoptotic death of the T cells (Van Parijs et al. 1998). IL-2 is responsible for rapid amplification of the number of Treg cells in peripheral lymph nodes to insure suppression of self-reactive T cells that escape negative selection, thereby maintaining tolerance (Malek et al. 2008). Peripheral tolerance to self-antigens occur through the elimination of self-reactive T cells and by the clonal anergy (Rocha and von Boehmer 1991; Jones et al. 1990). DCs have a critical role in the pathogenesis of allograft tolerance. While DCs generate tolerance by deleting self-reactive T cells in the thymus, they also induce tolerance to low doses of soluble antigens which are captured by receptors in peripheral lymphoid organs (Steinman et al. 2003). In humans, the two major distinct subpopulations of DC are the "conventional" myeloid DC (mDC) and plasmacytoid DC (pDC). mDC and pDC are distinguished by both cell surface markers and function (Liu 2005; Shortman and Naik 2007). In particular pDCs are involved in the regulation of the induction and maintenance

of tolerance (Arpinati et al. 2003). Since mDCs may have inflammatory functions and pDCs may have tolerogenic potential, it was shown that the elevated mDC/pDC ratio is associated with allograft rejection (Gupta et al. 2009, 2010). There is a transfer of antigens from tissues to the MHC class I and II products of APCs during the rejection of transplants. DCs, known for strong costimulatory functions, are also efficient at forming MHC–peptide complexes from phagocytosed cells. Although DCs stimulate T cells directly, inactivation of self-reactive T cells occurs via DCs in the thymus and in the periphery by the efficient transfer from peripheral to lymph node DCs (Inaba et al. 1998). DCs are professional APCs. CD4+CD25+ Treg cells are recognized as professional regulatory cells. DCs not only initiate T-cell immunity by uptake, processing, and presentation of specific antigens, but also induce immune tolerance by deletion of T cells and/or induction of Treg cells. CD4+CD25+ Treg cells maintain immune tolerance by suppressing the function of CD4+ and CD8+ T cells, B cells, macrophages, DCs, and NK cells (Chen 2006). Treg cells were first defined as CD4+CD25+ double-positive cells with suppressive functions on immunological response (Sakaguchi et al. 1995). Thymus-derived naturally occurring CD25+CD4+ Tregs suppress immune responses in transplantation. In this case the alloantigen-specific CD25+ CD4+ Tregs are much more effective suppressors of transplantation reactions than polyclonal populations (Yamazaki et al. 2006). The trigger for allograft rejection is allorecognition which occurs when the host immune system detects same-species, nonself-antigens. Allorecognition can occur by two distinct pathways. The direct pathway results from the recognition of foreign MHC class II molecules on the surface of donor bone marrow-derived cells. Indirect allorecognition occurs when donor class II histocompatibility molecules are internalized, processed, and presented as peptides by recipient APCs. In addition to antigen recognition, T-cell activation requires the provision of costimulatory signals (Game and Lechler 2002; Benichou and Tocco 2013), whereas a block in DC maturation reduces the initial sensitization to the transplant and may induce antigen-specific tolerance, by the direct pathway whereby allo-MHC and minor histocompatibility antigens are presented by DCs from the allograft at the levels of the transplant donor and by the indirect pathway whereby recipient DCs present peptides from allo-MHC and minor histocompatibility antigens from the graft at the levels of the recipient (Steinman et al. 2003).

A large number of thymocytes escape negative selection by using an endogenous TCR-alpha chain which is created by secondary rearrangement maintaining normal thymocyte development. Thus, secondary rearrangements of the TCR-alpha chain gene play an important role in the formation of the T-cell repertoire (Wang et al. 1998). Sequential rearrangement of TCR-alpha chain genes facilitates enhanced production of useful thymocytes by increasing the frequency of production of both in-frame rearrangements and positively selectable TCR-alpha/beta heterodimers (Petrie et al. 1993). TCR internalization and increased gene rearrangement at the endogenous TCR-alpha locus change the specificity of the TCR, and this event is known as “receptor editing” (McGargill et al. 2000). Finally, a state of unresponsiveness can be induced in self-reactive thymocytes. It is called as anergy. Thymocytes that recognize self-peptide–MHC with low affinity induce positive

selection, whereas those with high affinity undergo negative selection. TCR can discriminate between low- and high-affinity ligands in clonal deletion (Xing and Hogquist 2012). Thymic DCs make a significant contribution to Treg cell induction as well as to negative selection. Peripheral DCs can migrate into the thymus, where they induce the development of Treg cells and the deletion of self-reactive CD4 thymocytes. It may be interpreted that central tolerance to peripherally expressed antigens is induced by migrating DCs (Proietto et al. 2008).

DCs can contribute to the expansion and differentiation of T cells that regulate or suppress other immune T cells. Distinct developmental stages and subsets of DCs and T cells can account for the different pathways to peripheral tolerance, such as T-cell deletion, induction of T-cell anergy and induction of Treg, expression of immunomodulatory molecules, and production of immunosuppressive factors, IL-10; transforming growth factor-beta (TGF-beta); and indoleamine 2,3-dioxygenase (IDO). In particular APC expressing IDO plays a critical role in maintaining peripheral tolerance (Steinman et al. 2003; Ezzelarab and Thomson 2011). Following transplantation, donor DCs migrate from the graft and present donor MHC molecules to allospecific T cells via the direct pathway. Recipient DCs that have processed donor alloantigen present allopeptides on self (recipient) MHC molecules to donor-reactive T cells through the indirect pathway (Herrera et al. 2004). Therefore, allogeneic DCs are required for both rejection and tolerance of allografts. Engagement of the TCR on CD4+CD8+ thymocytes initiates either a program of survival and differentiation (positive selection) or death (clonal deletion), which are dictated in large part by the affinity of the TCR for self-peptide–MHC complexes. Three genes, bim, nur77, and ian1, are consistently upregulated in cells which are subjected to clonal deletion (Baldwin and Hogquist 2007). TCR and the costimulatory molecule CD28 potently induce apoptosis of CD4+CD8+ thymocytes (Punt et al. 1994). Three cell surface molecules, namely, CD28, CD5, and CD43, contribute to negative selection of the tolerance-susceptible population. The costimulatory function of these three molecules can be blocked by IL-4 and IL-7 (Kishimoto and Sprent 1999).

On the other hand, costimulatory molecule, cytotoxic T lymphocyte antigen (CTLA)-4 signaling in thymocytes may diminish the efficacy of clonal deletion (Buhlmann et al. 2003; Takahashi et al. 2005). Thus, clonal deletion eliminates self-reactive clones from the repertoire, whereas clonal diversion imprints self-reactive clones with suppressive or regulatory function. Both clonal deletion and clonal diversion require TCR interaction with self-MHC ligands in the thymus (Xing and Hogquist 2012). Peripheral-tolerance mechanisms are crucial to control tolerance of lymphocytes that first encounter their cognate self-antigens outside of the thymus. Both anergy and deletion of self-reactive T cells can occur in the periphery (Xing and Hogquist 2012).

The programmed death 1 (PD-1) receptor and its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) play important roles in tolerance. Engagement of PD-1 receptor by second ligand, PD-L2, dramatically inhibits TCR-mediated proliferation and cytokine production by CD4+ T cells (Latchman et al. 2001). PD-1 is induced on activated peripheral T cells, B cells, and myeloid cells. B7-H1 is widely expressed on resting cells and upregulated on activated T cells, B cells, DCs, and many tissue

cells of nonlymphoid organs, including the liver, whereas B7-DC is expressed exclusively on DCs and monocytes (Morita et al. 2010). PD-L1 also has an affinity for the costimulatory molecule B7.1 (CD80) but not for B7.2 (CD86). T-cell activation requires two different signals which consist of TCR signal and costimulatory signal. CD28 is the main costimulatory receptor and has two ligands, B7.1 (CD80) and B7.2 (CD86) that are expressed on APCs (Alpdogan and Van Den Brink 2012). In particular CD28 signals are critical for T-cell activation, proliferation, and survival after T-cell interaction with APCs (Jenkins et al. 1991). T cells become activated in the presence of a TCR signal and a costimulatory signal mediated by CD28 ligation and will then secret cytokines such as IL-2. Subsequent signaling through the IL-2R complex can fully activate the phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway (Fig. 14.1). However, T-cell activation in the absence of a second signal induces a

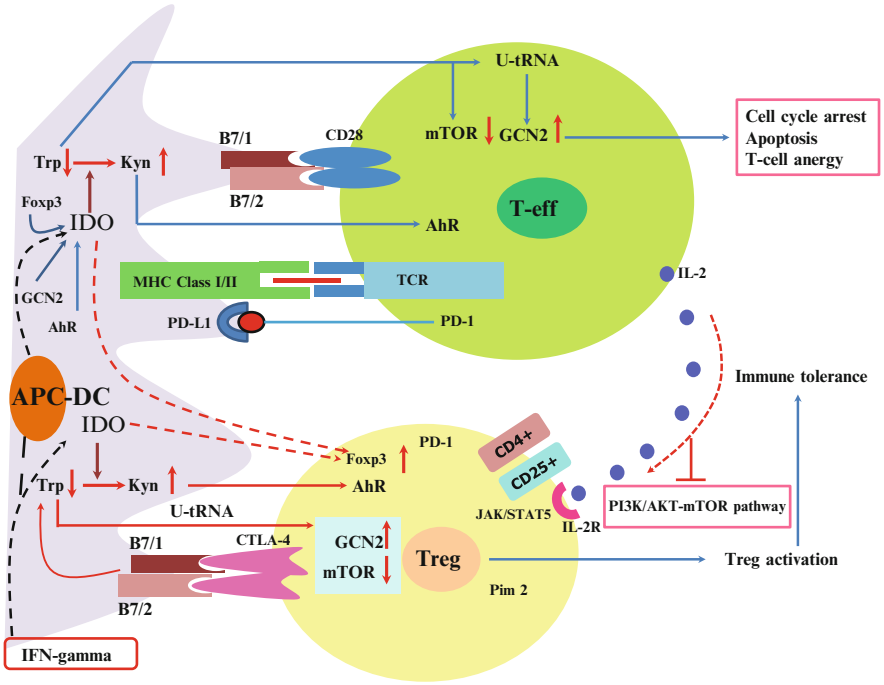

Fig. 14.1 Induction of immunogenic and tolerogenic molecules in dendritic cell. *APCs* antigen-presenting cells, *AhR* aryl hydrocarbon receptor, *CTLA* cytotoxic T lymphocyte antigen-4, *DCs* dendritic cells, *Foxp3* forkhead family transcription factor-3, *GCN* general amino acid control non-derepressible 2, *IDO* indoleamine 2,3-dioxygenase, *IFN-gamma* interferon gamma, *IL* interleukin-2, *IL-2R* IL-2 receptor, *Kyn* kynurenine, *MHC* major histocompatibility complex, *mTOR* mammalian target of rapamycin, *PI3K* phosphatidylinositol 3-kinase, *PD-1* programmed death receptor 1, *B7-1 and B7-2 PD-1* PD-L2 and PD-L1 ligands, *AKT* protein kinase B, *JAK* janus kinase 2, *Treg* regulatory T cell, *STAT* signal transducers and activators of transcription, *TCR* T-cell receptor, *Teff* T effector cell, *Trp* tryptophan, *U-tRNA* uncharged (tryptophanyl)-tRNA

state of long-term hyporesponsiveness in T cells, termed "anergy," which is characterized by an active repression of TCR signaling and IL-2 expression (Xing and Hogquist 2012). Tolerogenic DCs present antigen to antigen-specific T cells but fail to deliver adequate endogenous costimulatory signals for T-cell activation and proliferation (Gallucci et al. 1999). Normally, thymus produces immunoregulatory CD4+CD25+ T cells that are anergic to TCR stimulation and are able to suppress proliferation of other T cells (Itoh et al. 1999).

Induction and maintenance of T-cell tolerance require PD-1, and its ligand PD-L1 can limit effector T-cell responses and protect tissues from immune-mediated tissue damage (Keir et al. 2008). Pathways in the B7-CD28, family of costimulatory molecules, regulate T-cell activation and tolerance. B7-1 and PD-L1 interact with affinity intermediate to that of B7-1-CD28 and B7-1-CTLA-4 (Butte et al. 2007). CTLA-4 plays a critical role in peripheral tolerance. The activating receptor CD28 engages the same B7-1 and B7-2 molecules as the inhibitory receptor CTLA-4; CTLA-4–immunoglobulin (CTLA-4–Ig) can bind to B7 molecules expressed on DCs and activate a pathway of Trp catabolism that can lead to indirect inhibition of lymphocyte activation and T-cell death (Alegre and Fallarino 2006).

14.3 Mechanism of Transplant Tolerance

Actually tolerization of T cells responding to alloantigens through the indirect allorecognition pathway has become a major challenge in the field of transplantation (Benichou and Tocco 2013). In contrast to the fetus that is able to subvert the immune system and establish a state of immune tolerance, solid allografts require artificial immunosuppression of the host to avoid destruction. Although fetus shares a very similar early history of implantation with allograft, it is initiated at the single-cell stage in the absence of any vascularization. By contrast, allografts represent an enormous bulk of cells comprising a preexisting vascular tree that is connected immediately and without delay to the systemic circulation of the host (Dürr and Kindler 2013). Inducing organ-specific transplant tolerance is an elusive goal and major challenge in clinical transplants. A better understanding of the mechanisms of transplant tolerance is important and timely and may improve the immunosuppressive therapy (Liu et al. 2014). While elimination of donor passenger leukocytes brings about some prolongation of graft survival by reducing direct alloreactivity, it neither achieves tolerance nor suppresses indirect alloresponses (Lechler and Batchelor 1982). Early acute rejection of vascularized solid organ allotransplants is essentially mediated through direct allorecognition, while indirect allorecognition is commonly associated with chronic rejection of transplants. It is a slow process characterized by graft vasculopathy and tissue fibrosis (Baker et al. 2001). Therefore, in the absence of ongoing immunosuppression, abrogation of both types of inflammatory alloresponses will be required to achieve tolerance to allogeneic transplants which is defined as indefinite graft survival without chronic dysfunction (Benichou and Tocco 2013).

T lymphocytes have a critical role in allograft rejection, graft failure, and graft versus host disease (GVHD). T-cell tolerance occurs by two different mechanisms. The first is the depletion of self-reactive T cells during their maturation in the thymus (Sykes 2007; Griesemer et al. 2010). The second mechanism is the suppression or elimination of self-reactive mature T cells in the periphery, which involves either immunologically active suppressive cells such as Tregs or the inactivation of autoreactive T-cell clones by inhibitory molecules (Mueller 2010). If T cells are unable to proliferate and produce cytokines such as IL-2 or IL-4 in response to antigens, they may become anergic (nonresponsive) to those antigens. In some instances suppressor/regulatory cells cause anergy or clonal deletion of T cells by secreting inhibitory cytokines or inducing T-cell apoptosis in the periphery. Peripheral immune tolerance mechanisms are critical for controlling mature T cells with low-/moderate-affinity TCRs to self-MHC–peptide complexes (Mueller 2010). A small amount of antigenic stimulation can induce T-cell tolerance by partial downregulation of TCR on self-reactive CD8+ cells (Ferber et al. 1994). However, T-cell apoptosis is generally required for the induction of peripheral transplant tolerance (Wells et al. 1999).

The subpopulation of CD4+ T lymphocytes that coexpress the forkhead family transcription factor-3 (Foxp3) plays a unique role as Tregs that modulate many aspects of the immune response through numerous mechanisms. Stimulation of the TCR via recognizing self-peptide–MHC is required for their expression of Foxp3. TCR stimulation alters Foxp3-dependent transcriptional regulation, protein–protein interaction, and Foxp3 recruitment to the specific genomic loci. Antigen-specific Tregs inhibit dendritic cell functions including the expression of costimulatory molecules and the presentation of antigen during the early generation of the immune response (Shevach 2011; Ohkura and Sakaguchi 2010). Foxp3+ Tregs are primarily generated in the thymus (tTreg) but also may be generated extrathymically at peripheral sites (pTreg). One prominent mechanism of action of polyclonal tTregs is to inhibit T effector cell trafficking to the target organ, while antigen-specific inducible Tregs primarily prevent T-cell priming by acting on antigen-presenting DCs (Shevach and Thornton 2014). Naturally occurring Treg cells specifically express Foxp3, which is the main inducer, regulator, and survival factor in Treg development and function. Foxp3 expression in mature Treg cells is necessary for maintenance of Treg cell functions (Hori et al. 2003; Fontenot et al. 2003). Most Foxp3+ cells differentiate in the thymus from immature CD4+CD8+ precursors, as an alternative to conventional CD4+ T cells (Benoist and Mathis 2012). The Foxp3+-induced Treg cell repertoire is also drawn from peripheral naive conventional CD4+ T cells (Curotto de Lafaille and Lafaille 2009). CD4+CD25+ T cells suppress the proliferation of CD4+ as well as CD8+ T cells, which requires direct cell contact with Treg (Shevach 2002). Foxp3+ Treg cells are distributed throughout the body in a wide array of nonlymphoid tissues, even in the absence of any overt inflammatory responses. This suggests that Foxp3+ Treg cells constitutively function to help to maintain immune tolerance and prevent autoimmunity at these sites (Sather et al. 2007). Foxp3+ Treg cells are potent mediators of dominant self-tolerance in the periphery (Sakaguchi et al. 2010).

Thymic Treg cell development follows a two-step process based on the requirement for TCR-derived signals, through a Foxp3-negative CD25high (CD25hi) intermediate that secondarily converts to Foxp3high (Foxp3hi) under the influence of IL-2. The TCR costimulator CD28 acts in the TCR-dependent phase of Treg cell development (Lio and Hsieh 2008). Furthermore survival and function of Treg cells are dependent on the presence of IL-2 (Sadlack et al. 1993, 1995). Expression of IL-2 receptor (IL-2R, CD25) on their surface and signaling through IL-2R are required for optimum T regulatory function (Furtado et al. 2002). In fact Treg cells are anergic after stimulation, and therefore, IL-2 secretion from conventional T cells is critical for development of suppressive activity of Treg (Thornton et al. 2004; Malek and Bayer 2004). The ultimate immunosuppressive function of Treg cells may occur by cell–cell contact, mediated by the costimulatory molecule CTLA (Li et al. 2008), and programmed death ligand PD-L1, and by soluble immunosuppressive factors such as IL-10 and TGF-beta (Bestard et al. 2007; Zuber et al. 2009).

In four of five patients who received combined transplantation from haploidentical donors, long-term organ allograft survival without ongoing immunosuppression was achieved (Fudaba et al. 2006). Long-term tolerance in combined transplantation recipients is due to deletion or anergy of donor-reactive T cells. The Treg cells can directly suppress the effector CD4+ and CD8+ T cells by reducing IL-2 production and inducing activated T-cell apoptosis (Andreola et al. 2011). In the thymus, developing T lymphocytes (thymocytes) bearing a T-cell receptor (TCR)–CD3 complex that engages self-antigens are induced to undergo apoptosis. Thymocytes lacking the proapoptotic B-cell lymphoma-2 (Bcl-2) family member, Bim, are refractory to apoptosis induced by TCR–CD3 stimulation. Bim is an essential initiator of apoptosis in thymocyte-negative selection (Bouillet et al. 2002). Eventually apoptosis is involved in many aspects of the control of peripheral T-cell numbers at the termination of an immune response and maintenance of peripheral tolerance to tissue antigens (Newton and Strasser 2000). Bim is required for peripheral deletion mediated by cross-tolerance. The absence of Bim prevents their deletion, leading to a dramatic accumulation of autoreactive T cells (Davey et al. 2002).

14.4 Contribution of IDO to Metabolic Immune Regulation

IDO catalyzes the initial and rate-limiting step of L-Trp catabolism in the kynurenine (Kyn) pathway following interferon-gamma (IFN-gamma) stimulation (Taylor and Feng 1991). This step involves the oxidative cleavage of the 2,3 double bond in the indole moiety of L-Trp, resulting in the production of N-formyl Kyn. IDO-dependent T-cell suppression and tolerance induction by DCs indicate that L-Trp catabolism has significant impacts on T-cell proliferation and differentiation (Sugimoto et al. 2006).

Actually solid organ allografts, which most often exhibit MHC antigen mismatches with respect to the recipient, require immune tolerance to avoid rejection. Chemical immunosuppressors have to be administered to the recipient to achieve

graft survival (Dürr and Kindler 2013). However, increased inherent IDO activity in transplanted cells has been demonstrated to have antirejection properties. IDO-competent DCs induce Treg cells through its high levels of IDO activity. This event constitutes peripheral tolerogenic pathway for prolonged graft survival (Mulley and Nikolic-Paterson 2008). In this respect transfer of recombinant IDO genes into tissue allografts creates de novo systemic tolerance to the transplanted organ, without receiving any other immunosuppression (Swanson et al. 2004; Liu et al. 2006). The immunologic effects of IDO are not confined only to the cells expressing IDO. Thus, professional APCs expressing IDO can affect both the APC itself and also neighboring T cells that interact with APCs (Opitz et al. 2011; Pilotte et al. 2012; Munn and Mellor 2013).

Substantially IDO contributes to "metabolic immune regulation" by catalyzing oxidative degradation of the essential amino acid Trp along the Kyn pathway. Actually IDO is intracellular and its metabolic effects begin with inherently local signals. Immunologic effects of IDO subsequently spread to adjacent cells which may respond to Kyn metabolites and to the reduced access to Trp (Pallotta et al. 2011). State of immune activation includes the secretion of IFN-gamma by APCs or activated T cells. IDO expression by DCs in response to IFN-gamma stimulation may suppress T-cell responses and promote tolerance either through direct effects of Trp depletion and Trp metabolites on T cells or through effects of IDO on the DCs (Hainz et al. 2007). If so, IDO activity should be correlated with induction of guanosine triphosphate cyclohydrolase (GTPCH), the key enzyme in pteridine biosynthesis (Taylor and Feng 1991). Kyn to Trp ratio increases with neopterin in non-rejecting allograft recipients. Thus, significant positive correlation between Kyn to Trp ratio and neopterin indicates that increase in IDO activity depends on IFN-gamma stimulation (Brandacher et al. 2007a). Virtually IDO is involved in intracellular signaling events responsible for the self-amplification and maintenance of a stably regulatory phenotype in pDCs (Pallotta et al. 2011). Activation of DCs induces the production of functional IDO, which causes depletion of Trp and subsequent inhibition of T-cell proliferation (Hwu et al. 2000).

IDO-related peripheral tolerance can be summarized in four consecutive mechanisms: (1) Trp conversion to Kyns is activated in DCs by CTLA-4; (2) an increased IDO-dependent tolerogenesis correlates with the inhibition of DNAX activation protein of 12 kDa (DAP12), an adapter molecule associated with activating receptors; (3) a tolerogenic phenotype can be acquired by DCs lacking functional IDO through the paracrine production of Kyns by IDO-competent DCs; and (4) the suppressive effect of Treg cells generated in a microenvironment subsequent to Trp deprivation (Belladonna et al. 2007). In this context, IDO may attenuate the ability of DCs to stimulate effective T-cell responses in a number of ways. T cells activated by IDO-expressing DCs recognize antigen and enter the cell cycle, but Trp degradation due to IDO activity blocks cell cycle progression at a mid-G1 arrest point. Unfortunately restoration of Trp cannot allow further cell cycle progression and enhance T-cell apoptosis (Munn et al. 1999). Thus, IDO-induced tryptophan metabolites suppress the T-cell response. T cells, once stopped in their proliferation, cannot be restimulated. IDO-catalyzed Trp metabolites exert a cytotoxic action on

CD3+ cells and are responsible for suppression of allogeneic T-cell proliferation which is likely due to T-cell death (Terness et al. 2002). Nevertheless, in the absence of free Trp, T cells enter the cell cycle, but cell cycle progression halts in mid-G1 phase, and T cells become susceptible to death via apoptosis, in part through Fas-mediated signaling (Lee et al. 2002). As mentioned above, IDO-dependent Trp catabolism has profound effects on T-cell proliferation, differentiation, effector functions, and viability (Mellor and Munn 2004). To achieve all these, IDO modifies immune responses in two ways: by producing Kyn, a natural ligand for the aryl hydrocarbon receptor (AhR), and by depleting Trp, a trigger for the amino acid-sensing signal transduction pathways (Pallotta et al. 2011). Later Kyn binds to the AhR in T cells (Fig. 14.1). This activation leads to AhR-dependent CD25+Foxp3+ Treg cell generation (Mezrich et al. 2010). The AhR might act as a sensor to the outside environment, leading to alteration of the acquired immune system that might have relevance in transplantation (Van Voorhis et al. 2013).

Since TCR has a determinant role for guidance of developing T cells to the Foxp3+CD4+CD25+ Treg cell lineage and differentiation, TCR signaling is essential for suppressive function of Treg cells (Ohkura and Sakaguchi 2010). Virtually the CD4+ CD25+ Treg cells are generated in the thymus and the periphery (Roncador et al. 2005; Liang et al. 2005). Both thymus-derived T CD4+ CD25+ Foxp3+ natural cells and peripherally induced T CD4+ CD25+ Foxp3+ cells prevent migration of effector cells to target organs and inhibit their cooperation with APCs. The suppressive function of CD4+ CD25+ Foxp3+ Tregs depends on interactions between stimulatory (IL-2, CTLA-4) and inhibitory (glucocorticoid-induced tumor-necrosis-factor-receptor-related protein (GITR, CD28) signals, on stimulation of IDO activity in dendritic cells via CD80/CD86 (B7/1-B7/2) molecules, and finally on cell–cell inhibition of effector cells by membrane-bound TGF-beta (Wilczynski et al. 2008). GITR is a surface receptor molecule that has been shown to be involved in inhibiting the suppressive activity of Treg cells and extending the survival of T effector cells (Shimizu et al. 2002). IDO induction follows reverse signaling of CTLA-4 to its ligands CD80/86 and acts as a counter-regulatory mechanism to T-cell stimulation. Moreover IDO is required in tolerance induction to vascularized organ allografts or in effecting costimulation blockade (Hainz et al. 2007). IDO appears to contribute to the network of tolerogenic signals in many instances. Indeed Treg cells modulate Trp catabolism by inducing IDO activity; this is done by a mechanism that is dependent on CTLA-4 and that suppresses downstream T-cell activation (Brandacher et al. 2007b). Eventually overexpression of IDO in transplanted organs can prolong allograft survival due to peripheral tolerogenic effect (Jia et al. 2009). Serial second, third, and fourth adoptive transfers of allograft from CD40Ig-treated recipients into secondary recipients led to indefinite donor-specific allograft acceptance. Accepted grafts displayed increased IDO expression. Donor alloantigen-specific CD8+ Tregs may promote local graft immune privilege through IDO expression (Guillonneau et al. 2007). Treg activation by IDO+ pDCs requires an intact amino acid-responsive general amino acid control non-derepressible (GCN)2 pathway in the Tregs. GCN2 is a 2alpha serine–threonine protein kinase that becomes aware of amino acid deficiency. CD80/86 (B7) and toll-like receptor 9 (TLR9) ligands stimu-

late IFN-alpha expression in CD19+ DCs (Fig. 14.1). They acquire potent T-cell regulatory functions due to induced expression of the intracellular enzyme IDO. It seems likely that the GCN2 pathway enhances Treg cells and inhibits T effector cells (Manlapat et al. 2007). IDO-induced Treg cell activation markedly upregulates PD-L1 and PD-L2 expression on target DCs. The ability of Tregs to suppress target T-cell proliferation is abolished by antibodies against the PD-1/PD-L pathway. Treg cell activation is also prevented by CTLA-4 blockade (Sharma et al. 2007). In this case upon Trp depletion, amino acid deprivation causes a rise in intracellular concentration of uncharged (tryptophanyl)-tRNA (U-tRNA), which activates the GCN2 kinase and initiates downstream signaling (Dong et al. 2000). U-tRNA triggers a signal transduction pathway and activates GCN2 kinase that phosphorylates the translation initiation factor, eukaryotic initiation factor (eIF) 2alpha kinase (Fig. 14.1). While IDO-mediated Trp depletion can act as a potent regulatory signal via GCN2 kinase, mTOR responds to amino acid withdrawal by different mechanisms (Gallinetti et al. 2013). mTOR has evolved to sense and integrate diverse signals from the environment and plays a critical role in regulating metabolism, which is essential for T-cell activation. Stimulation of naive CD4+ mTOR-deficient T cells lead to the generation of Foxp3+ T cells even under normally activating conditions (Powell et al. 2012). Full T-cell activation with TCR engagement and costimulation in the presence of rapamycin results in profound T-cell anergy, despite the fact that these cells produce abundant amounts of IL-2 (Powell et al. 1999). Rapamycin selectively expands the naturally occurring CD4+CD25+Foxp3+ Treg cells which retain their suppressive effect (Battaglia et al. 2005). The rapamycin-induced anergic cells have a more profound block in the production of IFN-gamma. It is claimed that T-cell activation in the presence of rapamycin can lead to graft tolerance (Powell et al. 1999). Thus, mTOR inhibitors induce anergy and promote the selective expansion of Tregs. Decreased activation of the PI3K/Akt/mTOR pathway in Tregs helps ensure expression of sufficient levels of Foxp3 to allow their proliferation and survival. After treatment with rapamycin, Tregs are relatively resistant to apoptosis. A serine–threonine kinase, Pim-2, is activated in response to cytokine stimulation through the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Fig. 14.1) (McMahon et al. 2011). Pim-2 is constitutively expressed by resting CD4+CD25+Foxp3+ Treg cells but not CD4+CD25- T effector cells. However, in the presence of rapamycin, Foxp3 can regulate Pim-2 expression in T effector cells, and there is a positive correlation between Treg expansion and Foxp3 expression. Pim-2 offers rapamycin resistance (Basu et al. 2008). IL-2 stimulation of CD4+CD25+ Tregs initiates the transcription of STAT5-dependent genes and increases cellular survival. IL-2R signaling through mTOR is required for differentiation of T effector cells, whereas Treg cells use JAK/STAT5 pathway (Bensinger et al. 2004). Treg activation by alloantigen and IL-2 leads to an activity of the STAT5 pathway and only very little mTOR activity. Expression of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a negative regulator of the PI3K/Akt/mTOR pathway, remains high in Tregs during stimulation (Zeiser et al. 2008).

IDO-induced activation of GCN2 leads to cell cycle arrest and functional anergy in CD8+ T cells (Munn et al. 2005). Both Trp starvation and Trp catabolites contribute to regulatory environment affecting CD8+ as well as CD4+ T-cell function. Not only Trp catabolism is an effector mechanism of tolerance, but it also results in GCN2-dependent generation of autoimmune-preventive regulatory T cells. Consequently GCN2 enhances Treg activity and inhibits T effector cells. The IDO pathway contributes to regulation of Foxp3+ Treg lineage commitment and function (Fallarino et al. 2006). mTOR activity is inhibited in inflammatory settings in which amino acids are catabolized by IDO, arginase, tryptophan hydroxylase, and other enzymes (Dalton and Noelle 2012; Cobbold et al. 2009). GCN2 seems to be an important mechanism implicated in IDO-based immunosuppression. The IDO pathway contributes to regulation of Foxp3+ Treg lineage commitment and function. In vitro, Trp deprivation acts synergistically with Kyn metabolites to drive de novo differentiation of Foxp3+ Tregs from uncommitted CD4+ T cells (Fallarino et al. 2006; Chen et al. 2008; Manches et al. 2008). DC-expanded Tregs maintain stable expression of Foxp3, survived longer, and effectively contained the differentiation of alloreactive T cells into IFN-gamma-producing effector cells both in vitro and in vivo. DC-expanded Tregs provide a clinically advantageous means of preventing unwanted immune reactions to allografts (Zeng et al. 2009). The CD4+CD25+ subpopulation of T cells has been shown to be crucial in self-tolerance and also to prevent allograft rejection (Sakaguchi et al. 2001; Wood and Sakaguchi 2003; Sakaguchi 2004). Alloantigen-specific and highly suppressive regulatory CD4+CD25+ T cells have been shown to preferentially accumulate within the graft, where they hold effector cells in check by exertion of regulatory mechanisms (Graca et al. 2002; Cobbold et al. 2004). As CD25 is not uniquely expressed by Tregs, it can also be expressed by activated effector cells. However, the identification of Foxp3 provides an opportunity to specifically identify Tregs and to track them in vivo so as to better define the mechanisms involved in their regulation of immune responses (Fontenot et al. 2005; Uhlig et al. 2006). The transcription factor Foxp3 is a specific marker of Tregs. The Foxp3 gene, a member of the forkhead winged-helix protein family of transcription factors, is a specific molecular marker for CD4+ CD25+ Treg cells that controls Treg cell development and function (Hori et al. 2003). CD4+CD25+ Treg cells, which specifically express the Foxp3, are essential for the maintenance of immunological self-tolerance and immune homeostasis. Stimulation of the TCR via recognizing self-peptide–MHC is required for their expression of Foxp3 in the course of their development in the thymus (Ohkura and Sakaguchi 2010). The induction of Foxp3+ Treg cells by DCs depends on the IDO pathway (Manches et al. 2008). During acute rejection of kidney allografts, an augmented Foxp3 gene expression and increased CD4+CD25+Foxp3+ and other cell populations are observed in graft biopsies. Immunosuppressive drugs modulate the number and function of circulating Tregs and Foxp3 expression (Dummer et al. 2012). Foxp3 CD4+ T cells are frequently associated with rejection (Zhang et al. 2009). Donor-specific Treg cells that have no previous exposure have immunosuppressive properties that are stimulated in the peripheral blood of tolerant patients, and the frequency of these cells decreases in intolerant patients. Therefore, Treg

cells may promote transplant tolerance (Nafady-Hego et al. 2010). Following transfer of regulatory CD4+ T cells from tolerant recipients to a secondary irradiated recipient, regulatory CD25+ Foxp3 + and CD25+ Foxp3−CD4+ T cells accumulate in the graft and induce the expression of IDO by graft endothelial cells (EC) by an IFN-gamma-dependent mechanism. This induction is strictly dependent on IFN-gamma, a molecule that plays a crucial role in allograft tolerance (Thebault et al. 2007). Plasma IDO activity is much higher in acute GCHD (aGVHD) patients than in those without aGVHD. Although IDO activity decreases after alleviation of aGVHD, fluctuation of plasma IDO indicates the recurrence of aGVHD. IDO activity may represent a potential biomarker for the diagnosis and evaluation of aGVHD (Xu et al. 2013). IDO gene expression correlates with the severity of acute rejection, and IFN-gamma-induced IDO-positive DCs may attenuate acute rejection and catalyze local Trp metabolism via IDO enzyme expression (Sun et al. 2012). IDO activity can be interpreted as a negative feedback pathway that limits uncontrolled immune responses. The newly emerging concepts of manipulating IDO competence may be planned as a therapy for downregulating immune responses in transplantation (Heitger 2011).

14.5 Mechanisms of Spontaneous Liver Transplant Tolerance

With the introduction of new immunosuppressants to the clinical application, overall posttransplant survival currently exceeds 85 % in the first year and is approaching 75 % at 5 years in orthotopic liver transplant (Roberts et al. 2004). Moreover the 5-year liver graft and patient survival of alcoholic hepatitis and alcoholic cirrhosis patients are 75 % and 73 % and 80 % and 78 %, respectively, according to United Network for Organ Sharing database (UNOS) between 2004 and 2010 (Singal et al. 2012). Following liver transplantation, the incidence of chronic rejection is lower than the other posttransplant organ grafts, and immunosuppression withdrawal is also successful in a high proportion of liver recipients (Benítez et al. 2013). The liver is an immunologically privileged organ that may be tolerated with less immunosuppression after transplantation, and immunosuppressants can sometimes even be completely withdrawn (Sánchez-Fueyo and Strom 2004). Sixty percent of pediatric recipients of parental living-donor liver transplants remained off immunosuppression therapy for at least 1 year with normal graft function and stable allograft histology (Feng et al. 2012). Because of the inherent tolerogenic property, liver transplantation is a unique clinical event. Approximately 20 % of liver transplantation recipients can spontaneously withdraw immunosuppressive therapy without rejecting their grafts. This condition is referred to as operational or functional tolerance in which destructive immune response to graft is not detected in the absence of immunosuppression (Liu et al. 2013; Sánchez-Fueyo 2010).

Operational tolerance in liver transplant patients occurs much more frequently than that of the other transplanted organs; however, the rate of operational tolerance

varies considerably with the center and patient population (Alex Bishop et al. 2012). Both prospective and retrospective reports suggest that the probability of achieving operational tolerance is between 17 and 23 % for adult recipients and up to 40 % for pediatric patients (Trotter and O'Grady 2010). As mentioned above, the main challenge considering liver transplantation is minimization of immunosuppression with the goal of donor-specific tolerance (Fung 1999). Liver graft acceptance results from donor antigen-specific tolerance which is demonstrated by the extension of tolerance to other grafts of donor origin (Cunningham et al. 2013). In other words co-transplantation of a liver allograft can prevent rejection of other organ grafts from the same donor (Rasmussen et al. 1995; Kamada and Wight 1984). Liver transplants primarily may induce the peripheral tolerance itself. However, the mechanism of transfer of tolerance from the liver to the second graft is unknown (Liu et al. 2014). Actually the underlying mechanisms of spontaneous liver transplant tolerance are not known precisely. Several factors have been proposed for the ability of the transplanted liver to be spontaneously accepted by the recipient (Cunningham et al. 2013).

An increase in the frequency of CD4+CD25hi cells occurs when the immunosuppression is withdrawn in operationally tolerant liver transplant recipients. These patients exhibited a 3.5-fold increase in mRNA Foxp3 expression before the complete immunosuppression withdrawal, and this continued when immunosuppression therapy is stopped, whereas, in patients who suffer rejection, there is no increase in the CD4+CD25hi cells or Foxp3 expression (Pons et al. 2008). Thus, Foxp3+ CD4+CD25+ Treg cells induce functional tolerance and apoptosis of activated alloreactive T cells and eventually promote liver transplant tolerance. The Foxp3+ Treg cell-mediated immunosuppressive cascade is initiated primarily by liver DCs and PD-L1. The ratio of Foxp3+ Treg to T effector cells appears to determine liver transplant outcome (Li et al. 2008). Although the mechanisms of suppression of T effector cells by Tregs remain unclear, these cells are able to suppress antigen-specific responses via direct cell-to-cell contact, secrete anti-inflammatory cytokines, and inhibit the generation of memory T cells (Dummer et al. 2012). Actually Foxp3+ CD4+CD25+ Treg cells appear to support spontaneous acceptance of MHC-mismatched liver allografts (Li et al. 2006a). Indeed Foxp3+CD25+CD4+ T cells are markedly increased in the graft and recipient spleen following allogeneic orthotopic liver transplantation. These Foxp3+CD25+CD4+ cells can also express high levels of CTLA-4 in liver grafts. Increase in Foxp3+CD25+CD4+ Treg cells post-liver transplantation suggests that they play a key role in inducing or maintaining spontaneous liver transplant tolerance (Li et al. 2008). Liver graft rejection is associated with reduced Treg to effector T-cell ratio, diminished activated cell apoptosis, decreased CTLA-4 and IDO, and increased IL-2 gene expression in liver grafts and host spleens (Li et al. 2008, 2006b). However, overexpression of IDO within the liver allograft or APCs causes suppression of activated CD4 or CD8 T cells and promotes T-cell apoptosis (Brandacher et al. 2007b). It has been suggested that activation of CD8+ T cells predominantly within the liver would favor a response leading to tolerance. Actually primary intrahepatic activation of CD8+ T cells results in a defective CTL response. In fact competition for primary activation of

CD8+ T cells between the liver and secondary lymphoid tissues establishes the balance between immunity and tolerance (Bowen et al. 2004). The liver comprises various cell types including bone marrow-derived DCs and Kupffer cells and non-bone marrow-derived liver sinusoidal ECs, hepatic stellate cells, and sessile Kupffer cells. All major subpopulations of liver APCs respond to exposure to Treg by downregulation of their antigen-presenting function, upregulation of immunosuppressive molecules, and secretion of immunosuppressive cytokines (Hugues et al. 2006; Mahnke et al. 2007). In the periphery, cross-presentation of endogenous antigens by DCs, especially CD8+DCs, may induce CD8+ T-cell tolerance against self. APCs contribute to the induction, maintenance, and regulation of peripheral CD8+ T-cell tolerance by several mechanisms (Reynoso and Turley 2009). More recently it has been thought that liver DCs and Kupffer cells, costimulatory pathways, and activated T-cell apoptosis may participate in the induction of liver tolerance. DCs may modulate the amount of alloreactive T cells in liver graft recipients by expressing the co-inhibitory molecules such as PD-L1, CTLA-4, and IDO and by contributing to the induction of Foxp3+ Treg cells (Liu et al. 2014). DCs, especially pDCs, express high quantities of the co-inhibitory molecule PD-L1, which correlates with the elevated frequency of Foxp3+ Treg cells in operational liver transplant tolerance (Tokita et al. 2008). CTLA-4 signaling is also critical for liver transplant tolerance induction. CTLA-4 blockade promotes donor-specific T-cell activation, cytotoxicity, and T helper1 (Th1) polarization. In this instance liver allograft acute rejection is induced subsequent to protection of alloreactive T cells from apoptotic death (Li et al. 2005). It is evident that IDO mRNA and protein expression of Kupffer cells significantly increases with immune tolerance induction (Luan et al. 2012). Moreover Kupffer cells could promote immune tolerance by acting as incompetent APC and actively suppressing T-cell activation induced by other potent APCs. On the other hand, compared with DCs, Kupffer cells express significantly lower levels of MHC II, B7-1, B7-2, and CD40 (You et al. 2008). B7-H1 upregulation of liver allografts plays an important role in the apoptosis of infiltrating CD8+ cells and the induction of the spontaneous tolerance. Blockade of the PD-1/B7-H1 pathway leads to severe cell infiltration as well as transplant necrosis (Morita et al. 2010).

IDO signals are stronger in Kupffer cells or DCs of hepatectomized rats subjected to allogeneic or syngeneic hepatocyte transplantation. Transplanted hepatocytes may be protected from rejection by inhibiting indirect or direct recognition of donor antigen and further T-cell activation (Lin et al. 2008a). IDO mRNA is expressed in both the rejection phase and the induction phase of tolerance, but the signal is gradually lowered during the maintenance phase of tolerance. IDO may act as a local immunosuppressive molecule to protect transplanted cells from immune attack (Lin et al. 2008b). Liver allografts have significant increase in IDO gene expression and enzyme activity. IFN-gamma-induced IDO+ DCs may attenuate acute rejection and improve allograft survival rate following liver transplantation (Sun et al. 2012). Treg cells can suppress CD4+ T-cell stimulation by liver cells. Thus, liver cells may facilitate the transition from hepatic immune tolerance to hepatic inflammation by controlling Treg suppressor activity (Wiegard et al. 2005).

Activation of CD8+ T cells predominantly occurs within the liver but not within lymphoid tissues and favors leading to tolerance. The liver is a preferential disposal site for activated CD8+ T cells which are subjected to apoptosis. Either apoptotic cells migrate to the liver to die, or activated T cells receive an apoptotic signal within the liver tissue itself (Bertolino et al. 2001). Actually peripheral deletion is a major mechanism of self-reactive T-cell silencing. Moreover 80–90 % of autoreactive CD8+ T cells undergoing primary activation within the liver are rapidly deleted by a non-apoptotic mechanism as a result of their invading hepatocytes. Invasion of a cell into another cell is known as emperipolesis. In this event T cells are not passively engulfed by phagocytosis but needed to be metabolically active to enter hepatocytes. Consequently, T cells are destroyed by hepatocytes with the process of suicidal emperipolesis (Benseler et al. 2011). Donor leucocytes transferred with the liver appear to be responsible for both liver acceptance and the abortive activation of the recipient's T cells. T cells are activated to express IL-2 and IFN-gamma mRNA in the recipient lymphoid tissues, but not reach to the adequate levels within the graft. Subsequently the activated T cells die leading to specific clonal deletion of liver donor-reactive T cells (Bishop et al. 2002). Therefore, apoptosis may be important in blocking host alloactivity and inducing tolerance. Apoptosis of infiltrating leukocytes in liver allografts may represent an important process in the induction of spontaneous liver transplant tolerance and may underlie the abortive nature of the effector response observed within tolerated livers (Sharland et al. 1998). The extensive initial activation and differentiation of donor-reactive CD8+ T cells that occurs following liver transplantation lead to clonal depletion or deletion of the alloreactive CD8+ T-cell repertoire resulting in spontaneous tolerance induction (Steger et al. 2008). Eventually T-cell deletion may be responsible for spontaneous liver allograft acceptance. High levels of T lymphocyte apoptosis within the graft-infiltrating cell population are correlated with the liver allograft survival in non-immunosuppressed recipients (Qian et al. 1997).

14.6 Conclusion

In recent years to minimize dependence on immunosuppressive drug therapy, the induction of donor-specific tolerance in transplantation is the major objective. Therefore, comprehensive studies have been continuing to clarify the cellular and molecular basis of central and peripheral transplant tolerance. DCs and costimulatory molecules are important to initiate the Foxp3+ Treg cell-mediated immunosuppressive cascade. In this respect expression of the Trp metabolizing enzyme IDO by DCs is a major mechanism to elicit immune tolerance. In the light of this new insight, strategic integration of correct tools into transplantation protocols such as increasing IDO activity and mTOR inhibitors is expected to improve allograft survival.

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Chapter 15
Wine Flavor and Tryptophan

Atilla Engin

Abstract The intrinsic flavor properties of wine are dependent upon the flavor compounds' composition and how these influence sensory perception. Aromatic amino acids are catabolized by the transamination of the amino group and the formation of alpha-keto acids, which are decarboxylated to the aldehydes. Furthermore, they create highly desired wine aroma by transforming into aromatic alcohol. While only traces of tryptophan metabolites could be determined in grapes and grape musts, their amounts increase significantly during fermentation. Even under optimal fermentation conditions, the most efficient thiol-releasing *Saccharomyces cerevisiae* wine strain realizes less than 5 % of the thiol-related flavor potential of grape juice. Tryptophanase is able to convert an odorless substrate, L-tryptophan, into the odorous products methyl mercaptan and indole. Tryptophanase-encoded cysteine-beta-lyase releases up to 25 times more 4-mercapto-4-methylpentan-2-one (4MMP) and 3-mercaptohexan-1-ol (3MH) and transforms these compounds into free thiols. In order to produce wines with more pronounced aromatic profiles, low-temperature alcoholic fermentations are utilized frequently. Tryptophan metabolism of yeast influences fermentation performance during low-temperature wine fermentation. Actually, tryptophan uptake by yeast cells is sensitive to decreases in membrane fluidity caused by either high pressure or low temperature. Thus, tryptophan permease-expressing yeast cells can grow up under low-temperature conditions. Moreover, nitrogen deficiency in grape musts is one of the main causes of ineffective wine fermentations. In this respect, ammonium is the preferred nitrogen source for biomass production. Indeed, ammonium supplementation has a greater impact on wine aroma and color intensity. Additionally, tryptophanase activity and the rate of tryptophan synthesis increase with ammonium. Tryptophan and its metabolites are considered to be potential precursors of an aroma compound, 2-aminoacetophenone (AAP). If the amount of AAP increases significantly during fermentation, "untypical aging off-flavor" (UTA) may occur. Hence, a significant correlation is found between the level of AAP concentrations and poor quality of wine. On the contrary, indol-3-ethanol

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which is mainly formed during alcoholic fermentation from tryptophan does not reveal unpleasant odor. Pressure treatments impart aged-like characteristics to the wines and can influence the red wine physicochemical and sensorial characteristics. L-tryptophan is of primary importance for cell growth under high-pressure conditions. Indeed, the availability of tryptophan is the key factor for the continuation of growth at high pressure. Considering the flavor profile of wine, the ethanol stress-tolerant yeast strains are highly desired by winemakers. The enhancement of expression of genes related to tryptophan biosynthesis increases the ethanol stress tolerance of yeast cells. The fruity odors of wine are largely derived from the synthesis of esters and higher alcohols during yeast fermentation. The adaptation of yeast to metabolic conditions throughout the fermentation process by secreting aromatic alcohols is known as quorum sensing. Quorum-sensing molecules, tryptophol and phenylethanol, regulate the morphogenesis of *Saccharomyces cerevisiae* during nitrogen starvation. The production of aromatic alcohols is autostimulated by tryptophol. Melatonin is detected in grape extract within the range of 120–160 ng/g, while its isomer is found in musts and finished wines. Melatonin levels are higher in red wine than in white wine. Although melatonin in wine, thus, is believed to be derived from grapes, yeast also produces up to 100 ng/mg protein melatonin. However, the contribution of melatonin to the flavor of wine is not well understood.

Keywords Wine flavor • L-tryptophan • Tryptophanase • Tryptophol • Melatonin • *Saccharomyces cerevisiae* • Ethanol stress yeast • Volatile thiols • Quorum sensing • Yeast assimilable nitrogen

15.1 Introduction

The aroma of wine consists of 600–800 aroma compounds from which especially those typical for the variety are already present in grapes (Rapp 1998). Although hundreds of chemical compounds have been identified in grapes and wines, only a few compounds actually contribute to the sensory perception of wine flavor (Polásková et al. 2008). Hence, a small set of sensory attributes are of greatest importance to consumer preference. However, quality concepts of winemakers do not closely align with those of the consumers (Lattey et al. 2010). The perception of wine flavor and aroma is the result of interactions between a large number of chemical compounds and sensory receptors. The chemical profile of a wine depends on the type of grape, the fermentation microflora, storage conditions, and the aging of wine. In particular, the yeast contributes to wine aroma by biotransforming grape juice constituents into flavor-impacting components or neutral grape compounds into flavor-active compounds and by the de novo synthesis of many flavor-active metabolites (Styger et al. 2011). However, the addition of the precursor fraction brings about a significant increase in the floral properties of wine, irrespective of the yeast used. Nevertheless, the levels of 51 wine aroma chemicals have been found to

depend on the precursor fraction and, in most cases, also on the yeast strains (Loscos et al. 2007). The chemical composition of grapes mainly consists of sugars, organic acids, phenolic substances, amino acids, and volatile aroma compounds. The taste quality and taste intensity of amino acids depend on their molecular configuration and threshold values, respectively. L-Tryptophan is one of the most bitter amino acids with a threshold value of ct bitter = 4–6 mmol/l, whereas D-tryptophan, with ct sweet = 0.2–0.4 mmol/l, is the sweetest amino acid. A comparison of these threshold values with those of caffeine (ctbi = 1–1.2 mmol/l) and sucrose (ctsw = 10–12 mmol/l) shows that caffeine is about five times as bitter as L-tryptophan and that D-tryptophan is about 37 times as sweet as sucrose (Belitz et al. 2009).

15.2 Volatile Thiols and Wine Flavor

Volatile thiols, 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH), and 3-mercaptohexyl acetate (3MHA), are among the most potent aroma compounds of wine and can have a significant effect on wine quality. The enzymatic release of these aromatic thiols from grapes during fermentation enhances the varietal characters of wines (Swiegers et al. 2007). Optimal concentrations of these compounds in wine improve the wine aroma (Swiegers et al. 2007). Furthermore, when a wine is consumed, several oral physiological variables could affect aroma perception (Muñoz-González et al. 2011). For instance, the 4MMP concentration is correlated to the blackcurrant aroma in red wines, and 3MHA and 3MH present at high concentrations act as enhancers of the perception of this aroma (Rigou et al. 2014). The carbon-sulfur (CS) lyase activity is necessary for the transformation of cysteine-S-conjugated forms of 3MH and 4MMP into free thiols (Tominaga et al. 1998). Although great effort has been invested to identify their precursors in recent years, the origin of the majority of 3MH and 3MHA generated during wine fermentation still cannot be explained (Harsch et al. 2013). In fact, wine yeast strains have limited capacities to produce aroma-enhancing thiols. Even under optimal fermentation conditions, the most efficient thiol-releasing *Saccharomyces cerevisiae* wine strain realizes less than 5 % of the thiol-related flavor potential of grape juice. The *Escherichia coli* tnaA gene encodes a tryptophanase with strong cysteine-beta-lyase activity, is cloned, and is overexpressed in a commercial wine yeast strain CSL1 gene cassette. This modified strain expresses carbon-sulfur lyase activity and releases up to 25 times more 4MMP and 3MH (Swiegers et al. 2007). Actually, the metabolic enzyme tryptophanase is able to convert an odorless substrate like S-methyl-L-cysteine or L-tryptophan into the odorous products methyl mercaptan and indole (Xu et al. 2014). Indole is generated by reductive deamination from tryptophan via the intermediate molecule indolepyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine (-NH2) group of the tryptophan molecule is removed (Shimada et al. 2009). Recently, it was shown that L-tryptophan and L-tyrosine supplementation have negligible effects on the volatile profile of lychee wines, whereas L-phenylalanine results in the formation of

significantly higher amounts of 2-phenylethyl alcohol, 2-phenylethyl acetate, 2-phenylethyl isobutyrate, and 2-phenylethyl hexanoate. These findings suggest that selectively added amino acids may be used as a tool to modulate the volatile profile and flavor of lychee wines (Chinese dessert wine made of 100 % lychee fruit) (Chen et al. 2014).

Actually, sulfur compounds in wine can be a "double-edged sword." On the one hand, certain sulfur-containing volatile compounds such as hydrogen sulfide, imparting a rotten egg-like aroma, can have a negative impact on the perceived quality of the wine. On the other hand, some sulfur compounds such as 3MH, imparting fruitiness, can have a positive impact on wine flavor and aroma. Furthermore, these compounds can become less or more attractive or repulsive, depending on their absolute and relative concentrations (Swiegers and Pretorius 2007). The sensory analysis of the Sauvignon Blanc wine made with CSL1 indicates the increase in the characteristic volatile thiol aromas of passion fruit, grape fruit, and box hedge. At high concentrations, volatile thiols can elicit sweaty or cat's urine aromas (Swiegers et al. 2007). If the aromatic thiols, 3MH, 3MHA, and 4MMP, formed from nonvolatile cysteinylated precursors during fermentation are in small amounts, *Vitis vinifera* L. cv. Sauvignon Blanc wines have tropical fruit characters (Holt et al. 2011). The chemical profile of a wine partly depends on the type of grape (Cabernet Sauvignon, Chardonnay, Merlot, and Riesling wines). Approximately 90 % of cultivated grapes in the world are *Vitis vinifera*. *Vitis vinifera* (common grape vine) is a species of *Vitis*, native to the Mediterranean region, Central Europe, and Southwestern Asia, from Morocco and northern Portugal to southern Germany. There are currently between 5,000 and 10,000 varieties of *Vitis vinifera* grapes (Wine and Spirits Education Trust 2012). The qualitative and quantitative analyses of Trp and Trp metabolites in 39 grapes, 22 grape musts, and 16 wines demonstrated only traces of Trp metabolites that could be determined in the examined grapes and grape musts. But their amounts increase significantly during fermentation, whereas the amount of Trp decreases. The time of grape harvest shows no significant influences on the amount of Trp and Trp metabolites (Hoenicke et al. 1999). Thus, aroma characters of wine appear to be largely dependent on the yeast strain used during fermentation and winemaking techniques. Wines made from pressed juices (taken at 0.25 and 1.0 bar) contain less than half the concentration of 3MH and 3MHA, compared to wines made from free run juices (Patel et al. 2010). On the other hand, wines made from handpicked grapes have lower 3MH and 3MHA levels compared to the wines made from machine-harvested grapes (Allen et al. 2011).

15.3 Fermentation Temperature

The fermentation temperature influences the amount of volatile thiols irrespective of the yeast strain used. The final levels of 4MMP and 3MH in wines are higher when the alcoholic fermentation is conducted at 20 °C than at 13 °C. The 3MHA is

also correlated with the amount of 3MH in wines (Masneuf-Pomarède et al. 2006). In order to produce wines with more pronounced aromatic profiles, low-temperature alcoholic fermentations are utilized more frequently; however, the biggest drawback is the risk of ineffective fermentations. Low temperatures (13 and 25 °C as reference) restrict the yeast growth and lengthen the fermentations (Torija et al. 2003). Low-temperature fermentations produce wines with greater aromatic complexity and improve aromatic profile, but the success of these fermentations greatly depends on the adaptation of yeast cells to cold (López-Malo et al. 2014). Tryptophan metabolism of yeast influences fermentation performance during low-temperature (13 °C) wine fermentation. It has been previously reported that tryptophan is a limiting amino acid during *Saccharomyces cerevisiae* growth at low temperature. However, Lopez-Malo et al. recently demonstrated that deletion of TRP1 impairs tryptophan uptake and the growth rate of *Saccharomyces cerevisiae* at low temperature, whereas deletion of Tat2 (tryptophan permease or tryptophan amino acid transporter) provides the greatest fermentation activity and nitrogen consumption capacity at low temperature (López-Malo et al. 2014). Preadaptation of yeast to low temperatures is strain dependent and could improve the fermentation performance by reducing detrimental wine attributes such as acetic acid and fusel alcohol production (Llauradó et al. 2005). Tryptophan uptake by yeast cells is sensitive to decreases in membrane fluidity caused by either high pressure or low temperature (Abe 2011). However, Tat2 protein-expressing yeast cells can grow up under low-temperature conditions at 10 or 15 °C (Abe and Horikoshi 2000).

15.4 Yeast Assimilable Nitrogen and Wine

The total nitrogen content of grapes must range from 60 to 2,400 mg of nitrogen per liter; however, not all of this nitrogen will be assimilable (Zoecklein et al. 1999). The exact amount of free amino nitrogen will vary and can range 22–1,242 mg of nitrogen/l of yeast assimilable nitrogen (YAN) being derived from free amino acids (Spayd and Andersen-Bagge 1996). Nitrogen deficiencies in grape musts are one of the main causes of ineffective wine fermentations. The timing of the nitrogen additions influences the fermentation performance, the patterns of ammonium and amino acid consumption, and the production of secondary metabolites (Beltran et al. 2005). Primary or alpha amino acids, ammonium ion, and small peptides are referred to as YAN. Maximum yeast biomass yield and fermentation rate result when YAN exceeds 400 mg/l. Below 150 mg/l, YAN is the risk of ineffective fermentation (Blateyron et al. 2003).

Ammonium is the preferred nitrogen source for biomass production. The higher ammonium consumption correlates with a larger amount of glycerol, acetate, and acetaldehyde synthesis but with lower quantities of higher alcohols (Beltran et al. 2005). Several authors have reported that ammonium supplementation can improve wine sensory quality by lowering higher alcohol production (Rapp and Versini 1991).

Diammonium phosphate (DAP) [chemical formula (NH4)2HPO4, International Union of Pure and Applied Chemistry (IUPAC) name diammonium hydrogen phosphate] contains 21 % nitrogen. The standard addition of DAP to the juice or must (100–300 mg/l) without measuring the nitrogen concentration is a common practice among winemakers to prevent nitrogen-related fermentation problems. This application brings some disadvantages: acidification, accumulation of excessive wine phosphate, overproduction of acetate esters, the perception of volatile acidity, and suppression of varietal character. The nitrogen content of a must significantly affects the wine flavor and the yeast growth. Therefore, measuring the initial nitrogen concentration provides the opportunity to adjust DAP addition not only to achieve an adequate fermentation rate but also to more reliably guide the flavor profile and style of wine required (Ugliano et al. 2007; Carrau et al. 2008; Vilanova et al. 2007). Tryptophanase synthesizes L-tryptophan from D-serine in the presence of DAP. Furthermore, the activity of tryptophanase and the rate of tryptophan synthesis increase with DAP and reach to the maximum at 20 % concentration (Shimada et al. 2009). Five commercial *Saccharomyces cerevisiae* wine yeast strains are used to ferment a Chardonnay juice containing 110 mg/l of YAN, supplemented with DAP to increase YAN concentration to moderate (260 mg/l) and high (410 mg/l) levels (Ugliano et al. 2011).

DAP supplementation is likely to have a greater impact on wine aroma and color intensity. DAP-supplemented treatments give rise to higher concentrations of acetates, fatty acids, and fatty acid ethyl esters but lower concentrations of branched-chain fatty acids and their ethyl esters (Ugliano et al. 2008). However, there are many unanswered questions in the field of aroma ester synthesis (Saerens et al. 2010). DAP influences the expression of 350 genes in a commercial wine yeast strain. In this case, 185 genes involve in small molecule transporters and nitrogen catabolic enzymes are downregulated; 165 genes involve in amino acid metabolism, assimilation of sulfate, de novo purine biosynthesis, tetrahydrofolate one-carbon metabolism, and protein synthesis are upregulated (Marks et al. 2003). However, the impact of DAP addition on the production of fruity thiols, such as 4MMP, 3MH, and 3MHA, still needs to be determined.

Glutamine and tryptophan are the main amino acids consumed in all fermentation processes (Beltran et al. 2005). The yeasts may be able to use L-threonine, L-tryptophan, L-cysteine, and L-methionine not only as nitrogen sources but also as redox-active agents to balance the oxidation-reduction potential under conditions of restricted oxygen (Mauricio et al. 2001). However, aeration may increase the aroma compounds, thereby producing wines which have improved sensory properties. The consumption of YAN is not increased during the short aeration of young wine, whereas the consumption of L-proline, L-tryptophan, L-glutamic acid, ammonium ion, L-lysine, and L-arginine is accelerated (Mauricio and Ortega 1997). Aeration is found to increase the adenylate energy charge, growth, and viability of the yeast cells. Acetaldehyde reaches its highest level after the second aeration, which coincides with the exhaustion of the nitrogen source in the medium (Berlanga et al. 2001).

15.5 Untypical Aging Off-Flavor and Tryptophan

Tryptophan (Trp) and its metabolites, especially kynurenine and indole-3-acetic acid (IAA), are considered to be potential precursors of an aroma compound, 2-aminoacetophenone (AAP). More than 95 % of the total IAA is bound either as ester conjugate or as amide conjugate. Free IAA and other Trp metabolites are below the detection limit. If their amounts increase significantly during fermentation, "untypical aging off-flavor" (UTA) may occur. It is thought that the amounts of Trp and Trp metabolites in grape musts or wines largely depend on viticultural measures and climatic conditions of a vineyard (Hoenicke et al. 2001, 2002). The aroma-impact compounds of *Vitis vinifera* wines kynurenine and IAA are considered as potential precursors of AAP, which is triggered by an oxidative degradation of IAA (Hoenicke et al. 2002). Consequently, the amount of 2-AAP in wine can be referred to an oxidative degradation of IAA by superoxide and hydroxyl radicals, which can be formed in wine after the sulfuration by co-oxidation of sulfite to sulfate. Because the polyphenolic compounds of red wines have a scavenger effect on the radical oxidation of sulfite, UTA of wine due to AAP is completely blocked in red wines. In order to avoid the UTA in white wines, radical scavengers should be added to a medium (Christoph et al. 1999). IAA is known as a very instable compound and easily breaks down by enzymatic or nonenzymatic reactions. Formylaminoacetophenone (FAP) is the main degradation product of IAA. It is followed by 3-methyl-indole and AAP. Indole, esters of anthranilic acid, and AAP are intensive and unpleasant odor compounds. Conversely, indol-3-ethanol which is mainly formed during alcoholic fermentation from Trp does not reveal unpleasant odor. Therefore, a significant correlation is found between the level of AAP concentrations and poor quality of wine (Christoph et al. 1999). More frequently, the phenomenon of UTA is associated with higher concentrations of 2-AAP in *Vitis vinifera* white wines. A floor polish-like flavor may develop in white wines within a few months of storage. Oxidative degradation of IAA is triggered by sulfuration after fermentation. Therefore, actual differences in L-Trp and IAA concentrations of musts and wines are attributed more upon the soil type than yeast strain (Maslov et al. 2011).

15.6 Oxidative and Osmotic Stress

As mentioned above, oxidative degradation of tryptophan metabolites is very important for the taste of wine. Indeed, induction of the oxidative stress response has been described in *Saccharomyces cerevisiae* during industrial fermentation for wine yeast biomass production. However, a genetically engineered wine yeast strain overexpresses the TRX2 gene that codifies thioredoxin 2, especially under aeration conditions. The yeast cytoplasmic thioredoxin 2 is one of the most important redox controls together with glutathione/glutaredoxin system (Perez-Torrado et al. 2009; Grant 2001). Overexpression of the TRX2 gene in a wine yeast strain (TTRX2)

produces an increase in the fermentative capacity in the biomass obtained at the end of the process (Perez-Torrado et al. 2009). Enhanced fermentative capacity produced by the overexpression of thioredoxin 2 in a wine yeast strain correlates to an increased induction of several oxidative response genes and also to an increased activity of several ROS-scavenging enzymes (Gómez-Pastor et al. 2010).

Besides oxidative stress, osmotic stress is also the major cause of the stress response throughout the process of wine yeast biomass production (Pérez-Torrado et al. 2005). Elevated hydrostatic pressure inhibits cell division; nutrient uptake; biosynthesis of DNA, RNA, and proteins; and the association of multimeric proteins (Abe et al. 1999). L-tryptophan is of primary importance for cell growth under high-pressure conditions. Since the Tat2 protein level is downregulated by high pressure, tryptophan uptake is significantly inhibited into the cells. *Saccharomyces cerevisiae* expresses the Tat2 protein at high levels and becomes endowed with the ability to grow at high pressure as well as under low-temperature conditions at 10 or 15 °C. Tat2 gene encodes a high-affinity tryptophan permease which is required for tryptophan uptake. Consequently, the most critical target impaired by increasing hydrostatic pressure in growing cells is the tryptophan permease. The availability of tryptophan is the key factor for the continuation of growth at high pressure (Abe and Horikoshi 2000). Pressure treatments impart aged-like characteristics to the wines. The wine deposits of pressurized wines had higher total phenolic content, namely, proanthocyanidins (three- to tenfold). The results demonstrate that high hydrostatic pressure can influence long-term red wine physicochemical and sensorial characteristics (Santos et al. 2013). On the other hand, high-pressure treatments modify the alpha-helical and beta-sheet structures of wine proteins. Throughout the 60-day storage period, the alpha-helical structures in high-hydrostatic-pressure (HHP) samples are decreased. Structural changes by HHP (450 MPa for 3 and 5 min) improve the thermal stability of wine proteins and thus delay the haze formation in wine during storage (Tabilo-Munizaga et al. 2014).

15.7 Ethanol Stress and Quorum Sensing

Alcoholic fermentation is an essential step in wine production that is usually conducted by yeasts belonging to the species *Saccharomyces cerevisiae* (Carrasco et al. 2001). The increased ethanol concentration in a medium inhibits cell growth, damages cell viability, and reduces ethanol yield (Bai et al. 2004; Pina et al. 2004). Consequently, yeast cells are exposed to ethanol stress that affects cell growth and productivity. The ethanol-adapted strain shows active growth under the ethanol stress condition (Dinh et al. 2009).

Under high-gravity or very-high-gravity fermentation conditions, cells are exposed to ethanol stress due to the accumulation of ethanol, which affects the cell growth activity and productivity of target products. Therefore, the ethanol stress-tolerant yeast strains are highly desired by winemakers. The enhancement of expression of genes related to tryptophan biosynthesis increases the ethanol stress tolerance

of yeast cells. Moreover, the addition of tryptophan to the culture medium and overexpression of tryptophan permease gene confer ethanol stress tolerance to yeast cells (Hirasawa et al. 2007). The genes related to ribosomal proteins are highly upregulated in the ethanol-adapted strain. Furthermore, genes related to ATP synthesis in mitochondria are important for growth under ethanol stress (Dinh et al. 2009). The parental strain shows repressed expressions for many genes and is unable to withstand the ethanol stress and establishes a viable culture and fermentation. At least 82 genes have been identified as candidates and key genes for ethanol tolerance and subsequent fermentation under the stress (Ma and Liu 2010). The fruity odors of wine are largely derived from the synthesis of esters and higher alcohols during yeast fermentation. The ATF1- and ATF2-encoded alcohol acetyltransferases of *Saccharomyces cerevisiae* are responsible for the synthesis of ethyl acetate and isoamyl acetate esters, while the EHT1-encoded ethanol hexanoyl transferase is responsible for synthesizing ethyl caproate (Lilly et al. 2006). ATF deletion and overexpression strains clearly indicate that the expression level of ATF genes is an important factor controlling the volatile acetate ester production. While overexpression of ATF1 causes 10- to 80-fold increase in the production of acetate esters, overexpression of ATF2 causes smaller increases in ester formation (Verstrepen et al. 2003). Acetic acid is the main component of the volatile acidity of grape musts and wines. However, it has a negative impact on yeast fermentative performance and affects the quality of certain types of wine (Vilela-Moura et al. 2011). Weak organic acid stress inhibits the uptake of aromatic amino acids including tryptophol from the medium. *Saccharomyces cerevisiae* is often inhibited by the presence of high acetate levels in wine fermentations (Pretorius 2000). While the trp5D mutant is extremely acetate sensitive, this hypersensitivity is almost totally suppressed by a high level of tryptophan. Such tryptophan supplementation could restore the growth of the acetate-stressed trp5D mutant cells when compared to acetate-stressed wild-type (BY4741) cells lacking such supplementation (Bauer et al. 2003). The acetaldehyde concentration of the deacidified wine is 2.3 times higher and may have a detrimental effect on the wine aroma (Vilela-Moura et al. 2010).

Wine displays complex interactions between fungi, yeasts, and bacteria that begin in the vineyard and continue throughout the fermentation process until packaging. These interactions encompass yeast-yeast, yeast-filamentous fungi, and yeast-bacteria responses. Alcoholic fermentation is characterized by the successive growth of various yeast species and strains (Fleet 2003). At high cell density or under low-nutrient conditions, yeasts collectively adapt their metabolism by secreting aromatic alcohols in what is known as quorum sensing (Zupan et al. 2013). Quorum sensing is a process of intercellular communication. It allows individual cells to assess the population density and to coordinate behavior by secreting and sensing communication molecules. In *Saccharomyces cerevisiae*, quorum sensing regulates the transition between the solitary yeast form and the filamentous form. In these species, communication molecules are the aromatic alcohols tryptophol and phenylethanol (Wuster and Babu 2010). However, the mechanisms and role of quorum sensing in yeast are poorly understood. Furthermore, the separation, detection, and quantification of the putative quorum-sensing molecules 2-phenylethanol,

tryptophol, and tyrosol have been recently optimized (Zupan et al. 2013). Prefermentative cold maceration wines have particularly high concentrations of yeast metabolism-derived compounds such as tyrosol and tryptophol (Favre et al. 2014). It is suggested that quorum-sensing molecules, tryptophol and phenylethanol, regulate morphogenesis during nitrogen starvation conditions in *Saccharomyces cerevisiae* (Albuquerque and Casadevall 2012). *Saccharomyces cerevisiae* produces much more alcohol-based quorum-sensing molecules than the other yeast. Amounts of these alcohol-based compounds vary with growth conditions such as the availability of aromatic amino acids, ammonium sulfate, and NaCl in addition to pH and temperature. Tryptophol is only produced in the presence of tryptophan (Gori et al. 2011). When the individual yeast cells grow up to form a colony, the cell density increases and the nutrient availability decreases. On nitrogen-poor media, these conditions lead to the induction of genes involved in the synthesis of tryptophol. Tryptophol exhibits positive feedback regulation of the transcription of the genes for aromatic amino acid catabolism (ARO genes), which are necessary for the production of aromatic alcohols. The presence of tryptophol stimulates pseudohyphal growth by a Tpk2-dependent pathway (Hogan 2006). Tpk1, Tpk2, and Tpk3 encode the catalytic subunits of the cAMP-dependent protein kinase in *Saccharomyces cerevisiae*. At least one of them is required for normal cell growth (Toda et al. 1987). *Saccharomyces* mutants defective in producing aromatic alcohols show reduced filamentous growth, which can be suppressed by the addition of aromatic alcohols. Moreover, the production of aromatic alcohols is autostimulated by tryptophol. The autostimulatory effects of aromatic alcohols on morphogenesis require Tpk2p, a key component of the PKA signal transduction pathway. Furthermore, the production of tryptophol is under more strict nitrogen control. The positive feedback regulation by tryptophol could explain the cell density-dependent production of aromatic alcohols. Contrarily, high ammonia restricts the synthesis of aromatic alcohols by repressing the expression of their biosynthetic pathway (Chen and Fink 2006).

15.8 Carnitine: Long-Chain Fatty Acids and Flavor Compounds

L-carnitine is required for the transfer of activated acyl groups across intracellular membranes in eukaryotic organisms. In *Saccharomyces cerevisiae*, peroxisomal membranes are impermeable to acetyl-CoA, which is produced in the peroxisome. In a reversible reaction catalyzed by carnitine acetyltransferases (CATs), activated acetyl groups are transferred to carnitine to form acetylcarnitine which can be shuttled across membranes (Swiegers et al. 2001). Metabolic and physiological roles for carnitine in *Saccharomyces cerevisiae* are related to the activity of the carnitine shuttle. In yeast, the shuttle transfers peroxisomal activated acetyl residues to the mitochondria. However, acetyl-CoA can also be metabolized by the glyoxylate cycle to form succinate. The two pathways, therefore, provide a metabolic bypass for each other, and carnitine-dependent phenotypes have only been described in

strains with nonfunctional peroxisomal citrate synthase (Franken et al. 2008). The wine yeast *Saccharomyces cerevisiae* is central in the production of aroma compounds during fermentation. Some of the most important yeast-derived aroma compounds are esters. The esters ethyl acetate and isoamyl acetate are formed from alcohols and acetyl-CoA in a reaction, which is catalyzed by alcohol acetyltransferases. The pool of acetyl-CoA available in yeast cells could play a key role in the development of ester aromas. CAT catalyzes the reversible reaction between carnitine and acetyl-CoA to form acetylcarnitine and free CoA. This reaction is important in transferring activated acetyl groups to the mitochondria and in regulating the acetyl-CoA/CoA pools within the cell. CAT encodes the major mitochondrial and peroxisomal carnitine acetyltransferase, on the formation of esters and other flavor compounds during fermentation (Cordente et al. 2007). Carnitine causes a decrease in the level of conjugated dienes, lipid hydroperoxide, malondialdehyde, and dityrosine by about 20–30 % and a significant increase (by about 50 %) in the content of tryptophan (Augustyniak et al. 2008). In fact, carnitine protects cells from oxidative and organic acid stresses (Franken et al. 2008). Lipid components, including long-chain unsaturated fatty acids, derived from grapes could greatly influence wine quality. Sixteen fatty acids with carbon chain lengths from 12 to 26 were detected in 11 musts, with palmitic, linoleic, and linolenic acids generally predominating. Some polyunsaturated fatty acids abundantly expressed in grapes are immediately incorporated into yeast cells and accumulated as ethyl esters (Yunoki et al. 2005, 2007).

Carnitine palmitoyl transferase I (CPTI) is the rate-limiting enzyme in the mitochondrial oxidation of long-chain fatty acids (LCFAs). Although LCFA-CoAs themselves cannot enter the mitochondria, CPTI catalyzes the conversion of LCFA-CoA to LCFA-carnitine at the cytoplasmic face of the mitochondrial outer membrane. LCFA-carnitines are then transported into the mitochondria via additional proteins. The tryptophan residues at positions 391 and 452 are essential for the activity of the C-terminal 89 residue L-CPTI (Hostetler et al. 2011). Long-chain saturated fatty acids (SFA) are the most frequent membrane fatty acids throughout the fermentations. Lipid composition changes with the growth temperature. The optimal membrane fluidity at low temperatures is modulated by changes in the unsaturation degree in *Saccharomyces cerevisiae* strains (Torija et al. 2003).

15.9 Melatonin and Wine

Dietary indoleamines, melatonin, and serotonin, in different plant foods, including grapes, supporting the hypothesis that health benefits, are associated with Mediterranean dietary style (Iriti and Faoro 2006). Thus, wine grapes at different stages of development from the lag phase through veraison have been harvested from eight different vineyards to determine whether different patterns in melatonin and serotonin can be found in wine grapes during seed development and berry maturation. Melatonin is detected in 45 % of the fully developed purple, postveraison grapes but only found in 23 % of prelag phase samples. In fact, the actual

concentration of melatonin is highest in wine grapes harvested at the early stage of veraison when the seed is developing. Serotonin has been consistently detected at levels of about 8–10 mug/g in 30–35 % of grapes harvested during the veraison stage (Murch et al. 2010).

Melatonin levels fluctuate during the day/night cycle in plants grown under field conditions in a fruit organ of the species *Vitis vinifera*. When grapes are in the shade of leaves and branches, melatonin concentrations in these grapes are tenfold higher than those that are directly exposed to sunlight (Boccalandro et al. 2011). Melatonin and its possible synergistic action with the great variety of polyphenols may contribute to reducing the incidence of chronic illnesses associated with regular grape product consumption (Iriti and Faoro 2009). Several groups have reported widely diverse melatonin levels in wines. The ranges are from several picograms per milliliter to many nanograms per milliliter (Mercolini et al. 2008; Stege et al. 2010). Based on the published reports, melatonin levels are higher in red wine than in white wine (Stege et al. 2010). Melatonin is detected in grape extract within the range 120–160 ng/g, while its isomer is found in musts and finished wines. Gomez et al. demonstrated that *Saccharomyces cerevisiae* plays a decisive role in contributing to the content of melatonin and its isomer in wine (Gomez et al. 2012). Melatonin in wine, thus, is believed to be derived from grapes. However, yeast also produces up to 100 ng/mg protein and releases melatonin (Sprenger et al. 1999). *Saccharomyces cerevisiae* produces melatonin during fermentation in the winemaking process. Thus, Rodriguez-Naranjo et al. monitored 11 biogenic amines (agmatine, cadaverine, histamine, methylamine, 2-phenylethylamine, putrescine, spermidine, spermine, tyramine, tryptamine, and melatonin) during the making of five monovarietal wines (Merlot, Palomino Fino, Syrah, Tempranillo, and Tintilla de Rota). Safe levels of bioactive melatonin are formed by malolactic fermentation in all wines (Rodriguez-Naranjo et al. 2013). During this stage, the increase in these amines is accompanied by a significant decline in their amino acid precursors such as Trp (Marcobal et al. 2006). Mass spectrometry analyses revealed the presence of melatonin in dessert wines as well as the occurrence of three different melatonin isomers in grape products (Vitalini et al. 2013). The highest melatonin content is detected in skin, during the grape formation. During ripening, melatonin decreases in the skin of grapes while increasing in both seed and flesh (Vitalini et al. 2011). There are many external factors that can influence the levels of melatonin in grapes and wines.

15.10 Conclusion

Based on the available data, although it is suggested that tryptophan and its metabolites have pronounced effect on sensorial characteristics of wine by influencing the yeast growth under the conditions of low temperature or high pressure and ethanol stress, their functions have not been fully clarified in winemaking. Further research regarding the tryptophan metabolism in grapes and its continuation throughout the

fermentation process until packaging would lead to a broader insight into the aromatic profile of wine varieties.

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