9 Inflammatory Mediators and Intracellular Signalling

Richard Korbut and Tomasz J. Guzik

9.1 Introduction

Inflammation is a protective response of the macroorganism to injury caused by trauma, noxious chemicals or microbiological toxins. This response is intended to inactivate or destroy invading organisms, remove irritants and set the stage for tissue repair. The inflammatory response consists of immunological and non-immunological reactions. The latter are triggered by the release from the injured tissues and migrating cells of lipid-derived autacoids, such as eicosanoids or "platelet-activating factor" (PAF); large peptides, such as interleukin-1; small peptides, such as bradykinin; and amines, such as HISTAMINE or 5-hydroxytryptamine. These constitute the chemical network of the inflammatory response and result in clinical and pathological manifestations of inflammation (Table 9.1). The concept of the inflammatory response was introduced over 2000 years ago with its description by Cornelius Celsus as "*rubor et tumor cum calore et dolore*" (redness and swelling with heat and pain). Centuries later, in the nineteenth century, this definition was extended by Rudolph Virchow to include loss of function ("*functio laesa*"). It was Virchow and his pupils, including J. Cohnheim, who explained the scientific basis for Celsus' description of inflammation. They found that the redness and heat reflected increased blood flow and that the swelling was related to the exudation of fluid and

Table 9.1 Symptoms of inflammation induced by inflammatory mediators

Symptom	Mediators
Vascular permeability	Vasoactive amines Bradykinin Leukotrienes C_4, D_4, E_4 PAF Complement (C3a and C5a) Substance P Nitric oxide
Vasodilatation	Nitric oxide PGI_2, PGE_1, PGE_2, PGD_2 Hydrogen peroxide
Vasoconstriction	Thromboxane A_2, Leukotrienes C_4, D_4, E_4 Superoxide
Chemotaxis and leukocyte adhesion	Chemokines LTB_4, HETE, lipoxins Complement (C5a) Bacterial antigens
Pain	Bradykinin Prostaglandins
Fever	IL-1, TNF, IL-6 Prostaglandins
Tissue and endothelial damage	Reactive oxygen species Nitric oxide Lysosomal enzymes

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R. Korbut (✉)
Jagiellonian University School of Medicine,
Krakow, Poland
e-mail: mfkorbut@cyf-kr.edu.pl

T. J. Guzik
Department of Internal and Agricultural Medicine,
Jagiellonian University School of Medicine,
Krakow, Poland

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to the accumulation of cells, while pain follows [1]. The first understanding of the mechanism of inflammation was introduced by Elie Metchnikoff, who concluded in his book *Lectures on the Comparative Pathology of Inflammation* published in 1893 that "…inflammation is a local reaction, often beneficial, of living tissue against an irritant substance" [2]. This definition still stands today. For the first time, he observed that this reaction is mainly produced by phagocytic activity of the mesodermic cells and that it includes "the chemical action of the blood plasma and tissue fluids …", thus introducing the concept of mediators of inflammation [2]. Numerous further studies since then have identified the roles of individual mediators in inflammation, and we are beginning to understand the genetic and molecular aspects of the genesis of the inflammatory process. Inflammatory mediators include a plethora of cell-derived molecules (e.g. CHEMOKINES, CYTOKINES, antimicrobial peptides and reactive oxygen and nitrogen species) and of activated biochemical cascades originating in the vascular compartment (e.g. REACTIVE OXYGEN SPECIES, nitric oxide, COMPLEMENT, coagulation and fibrinolytic systems).

9.2 Eicosanoids

Arachidonic acid (AA) metabolites are formed rapidly from lipids of the cellular membrane, following activation of cells by numerous chemical and physical stimuli. They exert their effects locally (*autacoids*), affecting virtually every step of inflammation [3]. Eicosanoids encompass cyclic prostanoid structures, i.e. *prostaglandins* (PGs), *prostacyclin* (PGI_2) and *thromboxane* A_2 (TXA_2), and also straight-chain *leukotriene* structures (LTs), i.e. chemotactic LTB_4 and pro-inflammatory peptidolipids (LTC_4, LTD_4, LTE_4) [Fig. 9.1]. Subsequently, a new group of molecules was added to the family of eicosanoids,

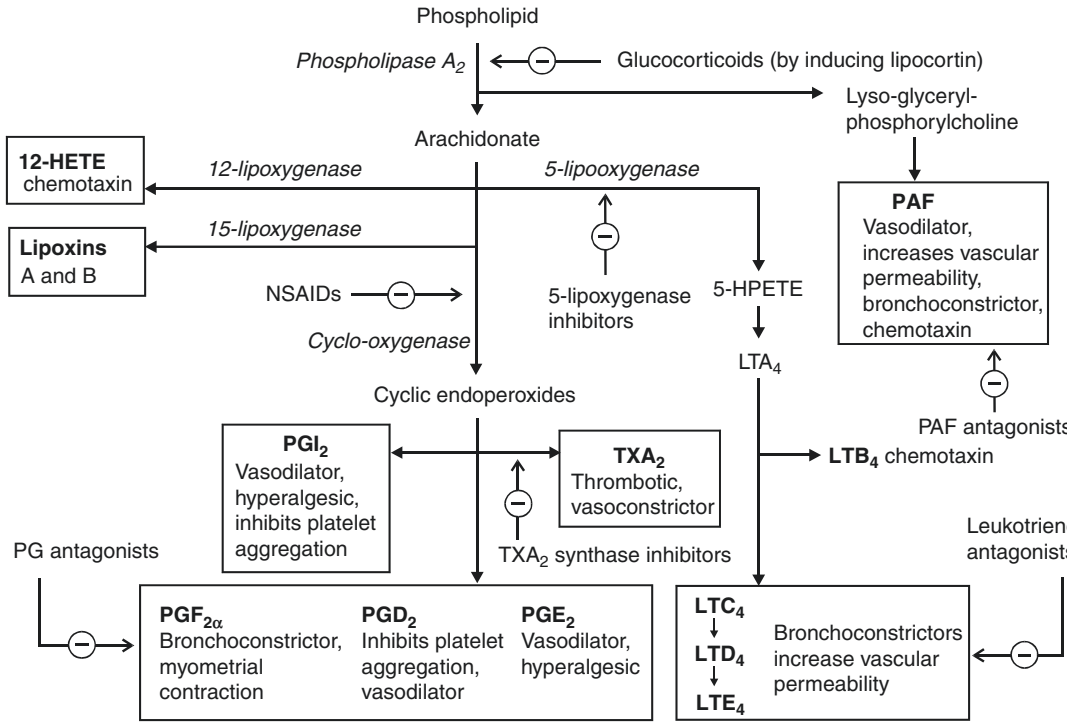

Fig. 9.1 Mediators derived from phospholipids and their actions, the sites of action of anti-inflammatory drugs

namely, *lipoxins* (LXA_4 and LXB_4), which are products of PLATELET 12-lipoxygenase metabolism of NEUTROPHIL LTA_4 (transcellular biosynthesis). Eicosanoids are synthesized by the cyclo-oxygenation (prostanoids) or lipoxygenation (leukotrienes) of a 20-carbon $\omega - 6$ polyunsaturated fatty acids (PUFAs)—5,8,11,14-eicosatetraenoic acid (AA, arachidonic acid) [Fig. 9.1]. AA is an important structural constituent of cellular phospholipids and first must be liberated by acylhydrolases—directly by phospholipase A_2 (PLA_2) or indirectly by PLC before it becomes the substrate for the synthesis of eicosanoids.

9.2.1 Prostanoids

Prostanoids are produced by the *cyclooxygenase* pathway. Prostaglandin H synthase (PGHS) is a dimeric complex which contains cyclooxygenase (COX) and peroxidase (Px). The COX cyclizes the AA to an unstable cyclic 15-hydroperoxy prostaglandin endoperoxide (PGG_2), while the Px converts the 15-hydroperoxy to a 15-hydroxy group, in this way yielding PGH_2. Eventually, the end product of PGHS (the complex which contains either constitutive COX-1, inducible COX-2 or the COX-3) is an unstable cyclic prostaglandin endoperoxide (PGH_2), which in various types of cells is converted by corresponding isomerases or synthases to the stable prostanoids: PGD_2, PGE_2, $PGF_{2\alpha}$ and unstable prostanoids, i.e. PGI_2 or TXA_2. Special biological significance has been ascribed to PGI_2 synthase in vascular endothelial cells and TXA_2 synthase in blood platelets. The transcellular metabolism providing PGH_2 from activated platelets to endothelial cells is the main source of vascular PGI_2 [4]. The biological activity of stable prostanoids is terminated by catabolic enzymes, such as prostaglandin 15-hydroxy dehydrogenase (15-PGDH), D^{13}-reductase or α and ω oxidases which are present in high concentration in the lungs. These enzymes also break down inactive TXB_2 and 6-keto-$PGF_{1\alpha}$.

The role of individual cyclooxygenase enzymes in the development of inflammation remains unclear. The discovery of the inducible form, COX-2, led to the hypothesis that COX-1 is a constitutive enzyme responsible for the physiological activities of prostaglandins, while COX-2, which is expressed during inflammation, produces "bad" prostaglandins, which generate pain and fever. This hypothesis quickly turned out to be simplistic, and both enzymes show their activities under both physiological and pathological conditions [5]. Moreover COX-2 inhibitory drugs possess less analgesic properties than non-selective inhibitors. The picture became even more complicated in 2002 with the discovery of COX-3. This isoenzyme is not a separate genetic isoform (like COX-2), but a splice variant of COX-1. In fact, COX-1 mRNA gives rise to four different isoforms including classical COX-1, COX-3 (splice variant including intron 1) and two partially truncated, inactive PCOX-1a and 1b. COX-3 due to the presence of intron 1, which changes its conformational structure, shows significantly diminished activity (25%) [6]. It is expressed mainly in the human brain and the heart. It has been suggested that COX-3 is an isoform particularly involved in the mechanisms of pain and fever during inflammation. Some suggestions exist that this isoform is inhibited by paracetamol, which could explain its analgesic actions.

The biosynthesis of prostanoids is initiated by transductional mechanisms in an immediate response to the activation of various cell membrane receptors or to various physical and chemical stimuli. These lead to an increase in the cytoplasmic levels of calcium ions $[Ca^{2+}]i$, and in this way, they activate acyl hydrolases, which thereby release free AA for metabolism by the PGHS. Alternatively, these enzymes can be induced by delayed transcriptional mechanisms which are usually activated by CYTOKINES or bacterial toxins. The spectrum of prostanoids produced by individual tissues depends on the local expression of individual enzymes. For example, vascular endothelium possesses prostacyclin synthase and COX-2, but lacks thromboxane synthase, present in turn in the platelets. Accordingly, the major prostanoid released by endothelium is PGI_2, while platelets produce TXA_2.

Prostanoids regulate vascular tone and permeability in the development of inflammation. They (TX) also induce PLATELET aggregation and

thrombus formation. Prostaglandins (in particular PGE_2) are also involved in the pathogenesis of the pain and fever which accompany inflammation.

Most actions of prostanoids appear to be brought about by activation of the cell surface receptors that are coupled by G proteins to either adenylate cyclase (changes in intracellular c-AMP levels) or PLC (changes in triphosphoinositol—IP_3 and diacylglycerol—DAG levels). The diversity of the effects of prostanoids is explained by the existence of a number of distinct receptors. The receptors have been divided into five main types, designated DP (PGD), FP (PGF), IP (PGI_2), TP (TXA_2) and EP (PGE). The EP receptors are subdivided further into EP_1 (smooth muscle contraction), EP_2 (smooth muscle relaxation), EP_3 and EP_4, on the basis of physiological and molecular cloning information. Subtype-selective receptor antagonists are under development. Only one gene for TP receptors has been identified, but multiple splice variants exist. PGI_2 binds to IP receptors and activates adenylate cyclase. PGD_2 interacts with a distinct DP receptor that also stimulates adenylate cyclase. PGE_1 acts through IP receptors, PGE_2 activates EP receptors, but it may also act on IP and DP receptors.

While most prostaglandins participate in the pathomechanism of inflammation, a more recently discovered member of this family—the 15-deoxy-Δ-12,14-prostaglandin J2 (15d-PGJ2)—is the dehydration end product of the PGD_2 and differs from other prostaglandins in several respects. 15d-PGJ2 has been shown to act via PGD_2 receptors (DP1 and DP2) and through interaction with intracellular targets. In particular, 15d-PGJ2 is recognized as the endogenous ligand for the intranuclear receptor PPARγ. This property is responsible for many of the anti-inflammatory functions of 15d-PGJ2.

9.2.2 Products of the Lipoxygenation of Arachidonic Acid

AA can be metabolized to straight-chain products by lipoxygenases (LOXs) which are a family of cytosolic enzymes that catalyse oxygenation of all polyenic fatty acids with two cis double bonds that are separated by the methylene group to corresponding lipid hydroperoxides [7] (Fig. 9.1). As in the case of AA, these hydroperoxides are called hydroperoxyeicosatetraenoic acids (HPETE's). Different LOX enzymes vary in their specificity for inserting the hydroperoxy group, and tissues differ in LOXs that they contain. Platelets contain only 12-LOX and synthesize 12-HPETE, whereas leukocytes contain both 5-LOX and 12-LOX producing both 5-HPETE and 12-HPETE. HPETE's are unstable intermediates, analogous to PGG_2 or PGH_2 and are further transformed by peroxidases or non-enzymatically to their corresponding hydroxy fatty acids (HETE's). 12-HPETE can also undergo catalysed molecular rearrangement to epoxy-hydroxyeicosatrienoic acids called *hepoxilins*. 15-HPETE may also be converted by lipoxygenation of LTA_4 to the trihydroxylated derivatives, the *lipoxins* (Fig. 9.1).

9.2.3 Leukotrienes

In activated leukocytes an increase in $[Ca^{2+}]i$ binds 5-LOX to five-lipoxygenase-activating protein (FLAP), and this complex converts AA to 5-HPETE, which in turn is the substrate for LTA_4 synthase. In the course of transcellular metabolism between leukocytes and blood cells or endothelial cells, unstable LTA_4 is converted by corresponding enzymes to stable chemotactic LTB_4 or to cytotoxic cysteinyl-containing leukotrienes—C_4, D_4, E_4 and F_4 (also referred to as sulphidopeptide leukotrienes or peptidolipids) (Fig. 9.1). Note that the transcellular metabolism of AA can bring about either "protection", as is the case during the platelet/endothelium transfer of PGH_2 to make cytoprotective PGI_2 [1], or "damage", as in the case of the leukocyte/endothelium transfer of LTA_4 to make cytotoxic LTC_4 [6].

Consecutive splicing of amino acids from the glutathione moiety of LTC_4 occurs in the lungs, kidney and liver. LTE_4 is already substantially deprived of most of the biological activities of LTC_4 and LTD_4. Also LTC_4 may be inactivated by oxidation of its cysteinyl sulphur atom to a sulphoxide group. The principal route of inactivation of LTB_4 is by ω-oxidation. LTC_4 and LTD_4 comprise an important endogenous bronchocon-

strictor, previously known as the "slow-reacting substance of anaphylaxis" (SRS-A) [8].

Three distinct receptors have been identified for LTs (LTB_4, LTC_4 and LTD_4/LTE_4). Stimulation of all of them appears to activate PLC. LTB_4, acting on specific receptors, causes adherence, chemotaxis and activation of polymorphonuclear leukocytes and monocytes, as well as promoting cytokine production in macrophages and lymphocytes. Its potency is comparable with that of various chemotactic peptides and PAF. In higher concentrations, LTB_4 stimulates the aggregation of PMN's and promotes DEGRANULATION and the generation of superoxide. It promotes adhesion of neutrophils to vascular endothelium and their transendothelial migration [9]. The cysteinyl-LTs are strongly cytotoxic and cause bronchoconstriction and vasodilatation in most vessels except the coronary vascular bed.

9.2.4 Lipoxins (Lipoxygenase Interaction Products)

Lipoxins are formed by a sequential transcellular metabolism of arachidonic acid by 15- and 5- or by 5- and 12-lipooxygenases [10]. The cellular context is critical for the synthesis of lipoxins (Fig. 9.2). Lipoxins have several anti-inflammatory properties as well as concomitant pro-inflammatory actions. Lipoxins inhibit the adhesion molecule expression on endothelium, cause vasodilatation and attenuate LTC_4-induced vasoconstriction by antagonism of $cysLT_1$ receptor. They also inhibit chemotaxis, adhesion and transmigration, IL-1β and superoxide production of polymorphonuclear leukocytes. On the other hand, lipoxins stimulate MONOCYTE adhesion and increase IL-4 formation [10, 11]. There is an inverse relationship between the amount of lipoxin and leukotriene production, which may indicate that lipoxins may be "endogenous regulators of leukotriene actions". High-affinity G-protein-coupled lipoxin receptors (ALXR) have been identified on numerous cells, including monocytes, PMNs fibroblasts and endothelial and epithelial cells. Receptor expression may be upregulated by interferon γ, IL-13 or even IL-1β. Activation of this receptor modulates phosphatidylinositol 3-kinase (PI3-kinase) activity. Lipoxins may also competitively bind and block the cys-LT_1 receptor. There are also suggestions that lipoxins may also bind within the cell, to ligand-activated transcription factors, therefore regulating gene expression in the nucleus.

A separate group of lipoxins was termed aspirin-triggered lipoxins (ATLs), as their synthesis is the result of acetylation of cyclooxygenase-2,

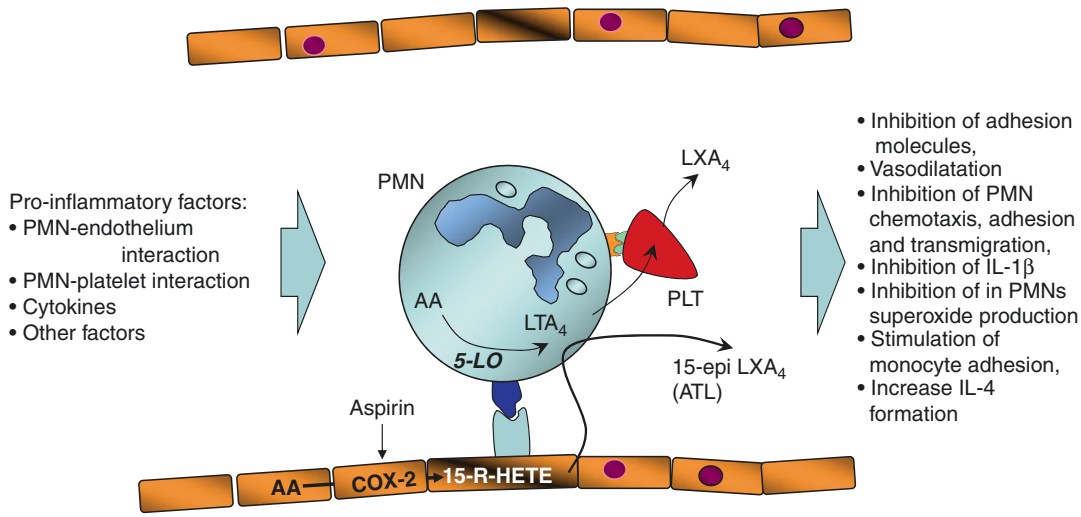

Fig. 9.2 Transcellular synthesis of lipoxins and their actions

which inhibits endothelial cell prostanoid formation and promotes synthesis of 15(R) HETE. These are then converted in PMNs to 15*R*-enantiomeres: 15-epi LXA_4 or 15-epi-LXB_4. ATLs share many actions of lipoxins, albeit with much greater potency [12]. Due to their anti-inflammatory properties, lipoxin analogues may find an important place in the treatment of inflammation [10, 11].

9.2.5 Other Pathways of Arachidonic Acid Metabolism

AA can be also metabolized by a NADPH-dependent cytochrome P-450-mediated monooxygenase pathway (MOX). The resulting 19-HETE, 20-HETE and a number of epoxyeicosatrienoic and dihydroxyeicosatrienoic acid isomers show vascular, endocrine, renal and ocular effects, the physiological importance of which remains to be elucidated [13].

Recently, a non-enzymatic, free radical-mediated oxidation of AA, while still embedded in phospholipids, has been discovered. Subsequently, acyl hydrolases give rise to a novel series of regioisomers of *isoprostanes*. Formed non-enzymatically, isoprostanes lack the stereospecificity of prostanoids. Highly toxic isoprostanes might contribute to the pathophysiology of inflammatory responses which are insensitive to currently available steroidal and non-steroidal anti-inflammatory drugs. The most thoroughly investigated regioisomer of isoprostanes is 8-epi-$PGF_{2\alpha}$. It has a potent vasoconstrictor action which is mediated by vascular TXA_2/PGH_2 receptors.

9.2.6 Actions and Clinical Uses of Eicosanoids

Eicosanoids produce a vast array of biological effects. TXA_2, $PGF_{2\alpha}$ and LTs represent cytotoxic, pro-inflammatory mediators. TXA_2 is strongly thrombogenic through aggregation of blood platelets. LTC_4 injures blood vessels and bronchi subsequent to activation of leukocytes. On a molecular level, their cytotoxicity is frequently mediated by stimulation of PLC or inactivation of adenylate cyclase. Cytoprotective, but not necessarily anti-inflammatory, actions are mediated by PGE_2 and PGI_2. They are both naturally occurring vasodilators. PGI_2 is the most comprehensive anti-platelet agent which is responsible for the thromboresistance of the vascular wall. PGE_2 through a similar adenylate cyclase-dependent mechanism inhibits the activation of leukocytes. PGE_2 is also responsible for protection of the gastric mucosa. PGE_2 and $PGF_{2\alpha}$ may play a physiological role in labour and are sometimes used clinically as abortifacients. Locally generated PGE_2 and PGI_2 modulate vascular tone, and the importance of their vascular actions is emphasized by the participation of PGE_2 and PGI_2 in the hypotension associated with septic shock. These prostaglandins also have been implicated in the maintenance of patency of the ductus arteriosus. Various prostaglandins and leukotrienes are prominent components released when sensitized lung tissue is challenged by the appropriate antigen. While both bronchodilator (PGE_2) and bronchoconstrictor ($PGF_{2\alpha}$, TXA_2, LTC_4) substances are released, responses to the peptidoleukotrienes probably dominate during allergic constriction of the airway. The relatively slow metabolism of the leukotrienes in lung tissue contributes to the long-lasting bronchoconstriction that follows challenge with antigen and may be a factor in the high bronchial tone that is observed in asthmatics in periods between attacks. Prostaglandins and leukotrienes contribute importantly to the genesis of the signs and symptoms of inflammation. The peptidoleukotrienes have effects on vascular permeability, while LTB_4 is a powerful chemoattractant for polymorphonuclear leukocytes and can promote exudation of plasma by mobilizing the source of additional inflammatory mediators. Prostaglandins do not appear to have direct effects on vascular permeability. However, PGE_2 and PGI_2 markedly enhance oedema formation and leukocyte infiltration by promoting blood flow in the inflamed region. PGEs inhibit the participation of lymphocytes in delayed hypersensitivity reactions. Bradykinin and the CYTOKINES (such as TNF-α, IL-1, IL-8) appear to liberate prostaglandins and prob-

ably other mediators that promote hyperalgesia (decreased pain threshold) and the pain of inflammation. Large doses of PGE_2 or $PGF_{2\alpha}$ given to women by intramuscular or subcutaneous injection to induce abortion cause intense local pain. Prostaglandins also can cause headache and vascular pain when infused intravenously. The capacity of prostaglandins to sensitize pain receptors to mechanical and chemical stimulation appears to result from a lowering of the threshold of the polymodal nociceptors of C fibres. Hyperalgesia also is produced by LTB_4. PGE_2 when infused into the cerebral ventricles or when injected into the hypothalamus produces fever. The mechanism of fever involves the enhanced formation of CYTOKINES that increase the synthesis of PGE_2 in circumventricular organs in and near to the preoptic hypothalamic area, and PGE_2, via increases in c-AMP, triggers the hypothalamus to elevate body temperature by promoting increases in heat generation and decreases in heat loss.

Synthetic PGE1, acting through IP and EP receptors, is given by infusion to maintain the patency of the ductus arteriosus in infants with transposition of large vessels until surgical correction can be undertaken. PGI_2 (epoprostenol) is occasionally used to prevent PLATELET aggregation in dialysis machines through inhibition of the thrombocytopenic action of heparin [14]. PGI_2 is also used for the treatment of primary and secondary pulmonary hypertension [15]. Stable analogues of PGI_2 (e.g. iloprost) as well as of PGE_1 are used in selected patients with peripheral vascular disease [14]. The PGE_1 analogue, misoprostol, is approved in the USA for the prevention of peptic ulcers, especially in patients who are required to take high doses of non-steroidal anti-inflammatory drugs (NSAID) for treatment of their arthritis.

9.2.7 Pharmacological Interference with Eicosanoid Synthesis and Actions

PLA_2 and COX are inhibited by drugs which are the mainstays in the treatment of inflammation. We discovered that GLUCOCORTICOSTEROIDS (hydrocortisone, dexamethasone) inhibit the generation of prostanoids in vivo through prevention of the release of AA from phospholipids [16]. This effect is mediated by intracellular steroid receptors which, when activated, increase expression of lipocortins which inhibit phospholipases. Many other actions of glucocorticosteroids on AA metabolism are known, one of them being inhibition of COX-2 transcription. These problems are further discussed in Chap. 32.

Aspirin selectively inhibits COX-1 explaining its inhibitory effect on the biosynthesis of TXA_2 in platelets (causing reduced thrombotic tending), of PGI_2 in endothelial cells and of PGE_2 in gastric mucosa (leading to gastric damage). This action of aspirin is more pronounced than that on the biosynthesis of prostanoids at the site of inflammation, where inducible COX-2 is most active. Consequently, aspirin at low doses seems to be a better antithrombotic than anti-inflammatory drug. Aspirin irreversibly acetylates the active centre of COX-1. Unlike endothelial cells, platelets lack the machinery required for de novo synthesis of COX-1, and, accordingly, aspirin-induced inhibition of TXA_2 synthesis in platelets is essentially permanent (until new platelets are formed), in contrast to the easily reversible inhibition of PGI_2 synthesis in vascular endothelium. The net effect of aspirin is, therefore, a long-lasting antithrombotic action. Unfortunately, most NSAIDs are more effective inhibitors of COX-1 than of COX-2. Meloxicam was the first clinically available drug which is claimed to be a selective COX-2 inhibitor—an anti-inflammatory drug with few side effects on the gastrointestinal tract, which causes no bleeding. However, population studies have verified that while protective for gastric mucosa, high doses of COX-2 selective inhibitors may induce cardiovascular (due to inhibition of endothelial COX-2) or renal side effects [17]. NSAID's usually are classified as mild analgesics, and they are particularly effective in settings in which inflammation has caused sensitization of pain receptors to normally painless mechanical or chemical stimuli. NSAID's do not inhibit fever caused by direct administration of prostaglandins, but they do inhibit fever caused by agents that enhance the synthesis of IL-1 and other CYTOKINES, which presumably cause fever at least in part by inducing the endogenous synthesis of prostaglandins.

9.3 Platelet-Activating Factor (PAF)

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a specialized phospholipid with an alkyl group (12–18C) attached by an ether bond at position 1 of glycerol and acetylated at position 2. PAF is not stored in cells, but it is synthesized from 1-*O*-alkyl-2-acyl-glycerophosphocholine as required (Fig. 9.3) [18]. Initially, PLA_2 converts the precursor to the inactive 1-*O*-alkyl-2-lysoglycerophosphocholine (lyso-PAF) with concomitant release of AA. Incidentally, in GRANULOCYTES, AA produced in this way represents a major source for the synthesis of PGs and LTA_4. In a second step, lyso-PAF is acetylated by acetyl coenzyme A in a reaction catalysed by lyso-PAF acetyltransferase. This is the rate-limiting step. The synthesis of PAF in different cells is stimulated during antigen-antibody reactions or by chemotactic peptides (e.g. f-MLP), CYTOKINES, thrombin, collagen and autacoids. PAF can also stimulate its own formation. Both PLA_2 and lyso-PAF acetyltransferase are calcium-dependent enzymes, and PAF synthesis is regulated by the availability of Ca^{2+}. The anti-inflammatory action of glucocorticosteroids is at least partially dependent on inhibition of the synthesis of PAF by virtue of the inhibitory effect of lipocortin on the activity of PLA_2.

Inactivation of PAF also occurs in two steps. Initially, the acetyl group of PAF is removed by PAF acetylhydrolase to form lyso-PAF; this enzyme is present in both cells and plasma. Lyso-PAF is then converted to a 1-*O*-alkyl-2-acyl-glycerophosphocholine by an acyltransferase. This latter step is inhibited by Ca^{2+}.

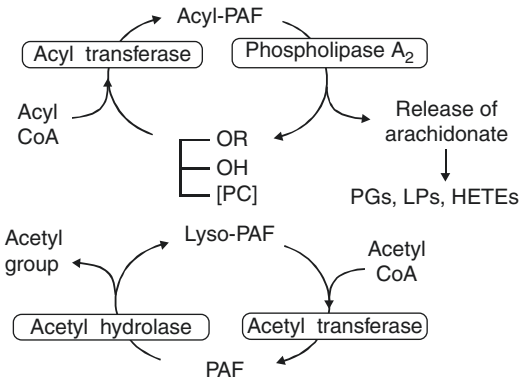

Fig. 9.3 The synthesis and metabolism of platelet-activating factor (PAF)

PAF is synthesized by PLATELETS, NEUTROPHILS, MONOCYTES, BASOPHILS and MAST CELLS, EOSINOPHILS, renal mesangial cells, renal medullary cells and vascular endothelial cells. In most instances, stimulation of the synthesis of PAF results in the release of PAF and lyso-PAF from the cell. However, in some cells (e.g. endothelial cells), PAF is not released and appears to exert its effects intracellularly.

PAF exerts its actions by stimulating a single G protein-coupled, cell-surface receptor [19]. High-affinity binding sites have been detected in the plasma membranes of a number of cell types. Stimulation of these receptors triggers activation of phospholipases C, D, and A_2 and mobilization of $[Ca^{2+}]i$. Massive direct and indirect release of AA occurs with its subsequent conversion to PGs, TXA_2 or LTs. Eicosanoids seem to function as extracellular representatives of the PAF message. As its name suggests, PAF unmasks fibrinogen receptors on platelets, leading directly to platelet aggregation. In endothelial cells, the synthesis of PAF may be stimulated by a variety of factors, but here PAF is not released extracellularly. Accumulation of PAF intracellularly is associated with the adhesion of neutrophils to the surface of the endothelial cells and their diapedesis, apparently because it promotes the expression or exposure of surface proteins that recognize and bind neutrophils. Activated endothelial cells play a key role in "targeting" circulating cells to inflammatory sites. Expression of the various *adhesion molecules* varies among different cell types involved in the inflammatory response. For example, expression of E-selectin is restricted primarily to endothelial cells and is enhanced at sites of inflammation. P-selectin is expressed predominantly on platelets and on endothelial cells. L-selectin is expressed on leukocytes and is shed when these cells are activated. Cell adhesion appears to occur by recognition of cell surface glycoprotein and carbohydrates on circulating cells by the adhesion molecules whose expres-

sion has been enhanced on resident cells. Endothelial activation results in adhesion of leukocytes by their interaction with newly expressed L-selectin and P-selectin, whereas endothelial-expressed E-selectin interacts with glycoproteins on the leukocyte surface, and endothelial ICAM-1 interacts with leucocyte INTEGRINS.

PAF also very strongly increases vascular permeability. As with substances such as histamine and bradykinin, the increase in permeability is due to contraction of venular endothelial cells, but PAF is 1000–10,000 times more potent than histamine or bradykinin.

Intradermal injection of PAF duplicates many of the signs and symptoms of inflammation, including vasodilatation, increased vascular permeability, hyperalgesia, oedema, and infiltration of neutrophils. Inhaled PAF induces bronchoconstriction, promotes local oedema, accumulates EOSINOPHILS and stimulates secretion of mucus. In anaphylactic shock, the plasma concentration of PAF is high, and the administration of PAF reproduces many of the signs and symptoms of experimental anaphylactic shock. PAF receptor antagonists prevent the development of pulmonary hypertension in experimental septic shock. Despite the broad implications of these experimental observations, the clinical effects of PAF antagonists in the treatment of bronchial ASTHMA, septic shock and other inflammatory responses have been rather modest.

PAF receptor antagonists include PAF structural analogues, natural products (e.g. ginkgolides from *Ginkgo biloba*) and, interestingly, triazolobenzodiazepines (e.g. triazolam). The development of PAF receptor antagonists is currently at an early stage of clinical development, still leaving the hope that such antagonists may find future therapeutic application in inflammation and sepsis.

9.4 Innate Immune Signalling Receptors

Several families of innate immune signalling receptors are currently known. Their functionality and subcellular location vary. These receptors include the transmembrane Toll-like receptors (TLRs) and C-type lectin receptors, while other receptors are located in the cytosol, including the retinoic acid-inducible gene-I-like helicases (RLRs) and the nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs). The common property and function of these receptors is to detect a broad variety of molecular entities including lipids, nucleic acids, proteins and their combinations. It is most likely that these innate immune receptors evolved to recognize specific molecules associated with microbial invasion and thus are designed to orchestrate antimicrobial defence—virtually all of these receptors can also detect molecular changes that occur during tissue damage during virtually every kind of inflammation. Moreover, their importance may be emphasized by the fact that they are located at the beginning of the inflammatory cascade.

9.4.1 Toll-Like Receptors

The Toll-like receptors (TLR1-10) are a part of the innate immune defence, recognizing conserved pathogen-associated molecular patterns (PAMPs) on microorganisms [20]. TLRs and their signalling pathways are present in mammals, fruit flies and plants. Ten members of the TLR family have been identified in humans, and several of them appear to recognize specific microbial products, including lipopolysaccharide, bacterial lipoproteins, peptidoglycan, bacterial DNA and viral RNA. Signals initiated by the interaction of TLRs with specific microbial patterns direct the subsequent inflammatory response including mononuclear phagocytic cell cytokine production. Thus, TLR signalling represents a key component of the innate immune response to microbial infection [20]. Interestingly, recent data indicates that TLRs play an important role not only in the modulation of innate immunity but also in the initiation of specific responses of adaptive immunity (Table 9.2). Moreover, T cells express certain types of TLRs during development and activation stages, and they participate in the direct regulation of adaptive immune

Table 9.2 Toll-like receptors in the regulation of adaptive immunity

Toll-like receptor	Recognized molecular pattern of microorganisms	Effect on T-cell function	Classical co-stimulatory effects
TLR1/2	Triacyl lipopeptides	Inhibition or reversal of regulatory T cells suppressive function	
TLR2	Peptidoglycan	Increase of regulatory T cell suppressive function	x
TLR3	ssRNA (viral), dsRNA, respiratory syncytial virus	Expressed; unclear function	x
TLR4	Lipopolysaccharide	Increase of regulatory T cell suppressive function	x
TLR5	Flagellin		x
TLR6	Lipoteichoic acid Zymosan, Diacyl lipopeptides		
TLR7	ssRNA (viral; inc influenza)	Inhibition or reversal of regulatory T cells suppressive function	
TLR8	ssRNA (viral)		
TLR9	Ds DNA (HSV); CpG dinucleotide motifs, haemozoin		x

response, possibly as co-stimulatory molecules. Co-stimulation of CD4+ effector cells with anti-CD3 mAb and TLR-5 ligand flagellin enhances T-cell proliferation and production of IL-2 levels equivalent to those achieved by co-stimulation with classical APC involving CD28. Moreover, CpG-containing oligodeoxynucleotides (CpG-ODN) can co-stimulate primary T cells in the absence of APCs. Finally, TLR activation on APCs may direct the development of immune responses into the regulatory T cells or Th17 pathway. These mechanisms are further discussed in Chaps. 2, 3 and 5.

9.4.1.1 Inflammasomes

Inflammasomes are cytoplasmic protein complexes critical in the regulation of inflammation. The term was first introduced in 2002 to describe a caspase-1-activating multimolecular complex consisting of caspase-1, caspase-5, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and NLRP1 (a NLR family member).

NLRs are a family of 20 intracellular immune receptors characterized by the presence of leucine-rich repeats (LRRs) near the C terminus and a central nucleotide-binding and oligomerization (NACHT) nucleotide-binding domain (NBD). The LRR domains of this family are thought to play a role in autoregulation, the recognition of PAMPs and/or protein-protein interactions. The NBDs can bind ribonucleotides, possibly regulating self-oligomerization. In spite of these similarities, different NLRs differ in their N-terminal domains. Most of these have an N-terminal pyrine domain (PYD) and are therefore called NLRP (NALP). Other NLRs have an N-terminal caspase recruitment domain (CARD) and include nucleotide-binding oligomerization domain-containing-1 (NOD1, also known as NLRC1), NOD2 (NLRC2). CARD domain-containing-4 NLRs are also vital (NLRC4, also known as CARD12 or IPAF). Other NLR family members have an acidic transactivation domain or a baculoviral inhibitory repeat-like domain, such as the member of NLR family, apoptosis inhibitory protein 5 (NAIP5).

The most important property of some NLR as discussed above is their ability to create inflammasomes. This involves particularly NLRP1, NLRP3 and NLRC4 which assemble multimolecular complexes in response to various activators, leading to the activation of inflammatory caspases. Through caspase-1 activation, the INFLAMMASOME controls the maturation of the cytokines of the IL-1 family.

For instance, an example is the NLRP3 inflammasome which can be activated by a myriad of microbial factors including Sendai virus, influenza A virus, adenoviruses, *Staphylococcus aureus*, *Listeria monocytogenes*, *E. coli*, *Mycobacterium marinum* and *Neisseria gonorrhoeae* as well as the fungal *Candida albicans*. NLRP3 is activated by products of the above

microbes, including MDP, bacterial RNA, LPS, Pam2CysK4, poly(I:C) as well as bacterial toxins such as nigericin or listeriolysin O (from *L. monocytogenes*), or α-toxin and β- and γ-hemolysins (from *S. aureus*). In summary, activation of cytokine receptors or pattern recognition receptors such as TLRs leads to the induction of pro–IL-1β and NLRP3. In the next step, NLRP3 inflammasome assembly is triggered by low intracellular potassium levels (e.g. through formation of pores by bacterial toxins) which influences lysosomal stability and the binding of a putative ligand that is generated by proteolytic activity after lysosomal damage or by the action of ROS. The assembled NLRP3 inflammasome results in activation of caspase-1, which proteolytically activates IL-1β family cytokines. The produced pro-inflammatory IL-1β family cytokines can act on other cell types or act in a feed-forward loop. These mechanisms are excellently discussed in a recent review by Stutz et al. [21]

9.5 Cytokines

Cytokines are peptides produced by immune cells, which play key roles in regulating virtually all mechanisms of inflammation including innate immunity, antigen presentation, cellular differentiation, activation and recruitment as well as in repair processes (see Chap. 6). They are produced primarily by macrophages and lymphocytes, but also by other leukocytes, endothelial cells and fibroblasts. Substances considered to be cytokines include interleukins 1–35, interferons, tumour necrosis factors (TNF), platelet-derived growth factor (PDGF), transforming growth factor-β, CHEMOKINES (which will be discussed separately) and the COLONY-STIMULATING FACTORS. Major cytokine superfamilies are listed in Table 9.3, and the most important ones are discussed below. Further details are also given in Chap. A5. The cytokine production profile in response to immune insult determines the nature of the immune response (cell-mediated, humoral, cytotoxic or allergic) [22, 23].

Interleukin-1 is the term given to a family of four cytokines consisting of two active, *agonists*, IL-1α and IL-1β, an endogenous IL-1-receptor antagonist (IL-1ra), and the recently cloned cytokine IL-18, which is structurally related to IL-1. Both IL-1α and IL-1β as well as a related protein IL-18 are synthesized as a less active precursor. Their secretion in response to various stimuli (antigens, endotoxin, cytokines or microorganisms) depends on the cleavage of the pro-cytokines to their active forms by IL-1-converting enzyme (ICE or caspase 1). IL-1α remains cell-associated and is active mainly during cell-to-cell contact, while the soluble IL-1β is a form predominant in biological fluids. IL-1 is an important inflammatory mediator, and it is believed to be implicated in several acute (e.g. systemic inflammatory response syndrome—SIRS in sepsis) or chronic (e.g. rheumatoid arthritis) inflammatory diseases. IL-1 is also important in immune responses, facilitating interaction of both B and T cells with antigen.

One of the principal actions of IL-1 is activation of T lymphocytes and B cells by enhancing the production of IL-2 and expression of IL-2 receptors. In IL-1 knockout animals, diminished immune responses or state of tolerance is

Table 9.3 Main cytokine families

Cytokine family	Cytokines
IL-1 superfamily	IL-1, IL-18, IL-33
IL-6 like cytokines	IL-6, IL-11, IL-27, IL-30, IL-31 (oncostatins, cardiotrophin, etc.)
IL-10 family	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26
Interferon type III (lambda)	IL-28, IL-29
Common gamma chain family	IL-2/15; IL-3; IL-4; IL-7; IL-9; IL-13; IL-21
IL-12 family	IL-12, IL-23, IL-27, IL-35
Other	IL-5, IL-8; IL14; IL-16; IL-17/25(A); IL-32; IL-34
IL-17 family	IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F.
TNF ligand superfamily	TNF-alpha, 4-1BB ligand, B-cell-activating factor, FAS ligand, TNF-beta (lymphotoxin), OX40L, RANKL, TRAIL
Interferons	I (alpha) II (gamma) III (IL-28; IL-29)

observed. In vascular endothelial cells, IL-1 increases the synthesis of leukocyte adhesion molecules (VCAM-1, ICAM-1 and E-selectin), stimulates NO production, releases "platelet-derived growth factor" (PDGF) and activates PLA_2, thus inducing the synthesis of prostanoids and PAF. It stimulates fibroblasts to proliferate, to synthesize collagen and to generate collagenase. It regulates the systemic inflammatory response by stimulating synthesis of acute phase proteins (C-REACTIVE PROTEIN, amyloid and COMPLEMENT), producing neutrophilia and causing fever by altering a set point of temperature in the hypothalamus. IL-1 also induces the generation of other cytokines such as the interferons, IL-3 and IL-6, and, in bone marrow, the COLONY-STIMULATING FACTORS. It synergises with tumour necrosis factor-α (TNF-α) in many of its actions, and its synthesis is stimulated by TNF-α. The therapeutic effects of glucocorticoids in rheumatoid arthritis and other chronic inflammatory and autoimmune diseases may well involve inhibition of both IL-1 production and IL-1 activity. Production of IL-1ra alleviates potentially deleterious effects of IL-1 in the natural course of the disease.

IL-18 although structurally close to IL-1 family exerts actions more related to IL-12. It was originally derived from liver but is produced by numerous cell types (including lung, kidney and smooth muscle cells) apart from lymphocytes. In contrast to other cytokines, IL-18 pro-cytokine is constitutively expressed, and therefore its activity is regulated primarily by caspase-1. It plays a critical role in cellular adhesion being the final common pathway leading to ICAM-1 expression in response to IL-1, TNF-α and other cytokines. It also synergises with IL-12 in stimulating IFN-gamma production. Soluble IL-18 receptor may be particularly interesting from an immunopharmacological point of view as it has lost its signalling domain and may therefore serve as a potent anti-inflammatory molecule.

Interleukin 17 family (IL-17A-F) includes cytokines that share a similar protein structure, with four highly conserved cysteine residues critical to their 3-dimensional shape which is unique for this cytokine. Production of IL-17A, which is characteristic for a specific subset of T helper CD4+ lymphocytes called Th17 cells, places this cytokine as one of the most important regulators of autoimmune processes. IL-17 is particularly important as it regulates expression and function of numerous other immune pro-inflammatory signalling molecules. The role of IL-17 is also commonly associated with allergic responses. IL-17A, the best characterized member of this family, induces the production of many other cytokines (such as IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, TNF-α), CHEMOKINES (including IL-8, GRO-α and MCP-1) and prostaglandins (PGE_2) discussed in this chapter. IL-17 receptors, binding particularly IL-17A, are expressed on fibroblasts, endothelial cells, epithelial cells, keratinocytes and macrophages. As a result of these effects, the IL-17 family has been linked to many immune-/autoimmune-related diseases including rheumatoid ARTHRITIS, ASTHMA, SLE, allograft rejection and antitumour immunity.

9.5.1 Tumour Necrosis Factor-α and Tumour Necrosis Factor-β (TNF-α and TNF-β)

These cytokines are produced primarily in mononuclear phagocytes (TNF-α) and in lymphocytes (TNF-β) but also by numerous other cells. Activation of Toll-like receptors (TLR2 and TLR4) by LPS is the most commonly recognised intracellular pathway leading to production of TNF. TNF-α and TNF-β bind with similar affinity to the same cell surface receptors—TNFR 1 (p55) and TNFR 2 (p75). Therefore their activities are very similar. The generic name of these cytokines is based on tumour cytotoxic effects, but their pharmacological use in the treatment of tumours is limited by severe side effects. TNF is responsible for severe cachexia during chronic infections and cancer.

In endothelial cells these cytokines induce expression of adhesion molecules (ICAM-1 and VCAM-1) and synthesis of prostacyclin and of cytokines. TNFs act as chemoattractants, as well as potent activators for neutrophils and macrophages. TNF-α causes fever and releases acute

phase proteins. TNF and IL-1 produce many of the same pro-inflammatory responses which include induction of cyclooxygenase and lipoxygenase enzymes as well as the activation of B cells and T cells. It is finally important to point out that TNF is the primary mediator of haemodynamic changes during septic shock through its negative inotropic effects as well as an increase in vascular permeability.

TGF-α (transforming growth factor-α) is a trophic regulator of cell proliferation and differentiation which is important in repair processes, it is involved in angiogenesis and in the organization of extracellular matrix, and it is chemotactic for monocytes.

PDGF (platelet-derived growth factors) cause proliferation of fibroblasts, vascular endothelial cells and smooth muscle. They are implicated in angiogenesis, atherosclerosis and possibly in chronic ASTHMA.

Interferons constitute a group of inducible cytokines which are synthesized in response to viral and other stimuli. There are three classes of interferons (IFN), termed type I (IFN γ), type II (IFNsα and β) and type III (IFN λ). IFN-α is not a single substance but a family of 15 proteins with similar activities. The interferons have antiviral activity, and interferon-γ has significant immunoregulatory function and only modest antiviral activity. Interferon-λ is also an antiviral cytokine, but it signals through a distinct receptor complex, composed of the IFN-λR1 and interleukin-10R2 (IL-10R2) receptor chains. Thus this interferon (type III) is functionally an interferon but structurally is related to the interleukin-10 family. The antiviral effects of interferons are achieved by inhibition of viral replication within infected cells as well as by stimulation of cytotoxic lymphocytes and NK cells. All interferons can be induced by other cytokines such as IL-1, IL-2, TNF and COLONY-STIMULATING FACTORS. IFN-α and IFN-β are produced in many cell types—macrophages, fibroblasts, endothelial cells, osteoblasts, etc., being strongly induced by viruses and less strongly by other microorganisms and bacterial products. Interferons induce the expression of the major histocompatibility molecules (MHC I and II) that are involved in antigen presentation to T cells. IFNs also stimulate the expression of Fc receptors on GRANULOCYTES, promote the differentiation of myeloid cells and modulate the synthesis of cytokines. Interferon γ is primarily made by T lymphocytes (T helper type 1) which may suggest that it is more of an interleukin than an interferon. Indeed, it functions as an inhibitor of IL-4-dependent expression of low affinity IgE receptors, therefore inhibiting IgE synthesis.

Colony stimulating factors. These include *IL-3* and *GM-CSF* (granulocyte macrophage-colony stimulating factor) and several other cytokines. They regulate haematopoiesis, are chemotactic for neutrophils, as well as activating neutrophils and macrophages.

Anti-inflammatory cytokines. It is important to point out that apart from pro-inflammatory actions, some cytokines may inhibit inflammatory processes. These include *IL-1ra*, mentioned above, as well as *TGF-β* or the *IL-10 family* (including *IL10*, *IL19*, *IL20*, *IL22* and *IL24*).

9.5.2 Intracellular Signalling by Cytokine Receptors

Binding of cytokines to their receptors leads to the activation of cytoplasmic tyrosine kinases. Janus kinases (JAKs), a recently described family of four related cytoplasmic protein tyrosine kinases, further transfer cytokine signalling. There are four JAKs, JAK1, JAK2, JAK3 and TYK2, which transduct signals from cytokine receptors to effector mechanisms. On binding of the cytokine, JAKs bind to the receptor and mediate tyrosine KINASE activity and phosphorylation of the receptor and of receptor-associated JAKs (Fig. 9.4). The next step in signal transduction involves tyrosine phosphorylation of *signal transducers and activators of transcription* (STATs) in the cytoplasm. Upon activation, STATs become phosphorylated, form homodimers and migrate to the nucleus, where they bind to regulatory sequences in the promoters of cytokine-responsive genes, e.g. ICAM-1 or other cytokine genes. In summary, cytokine

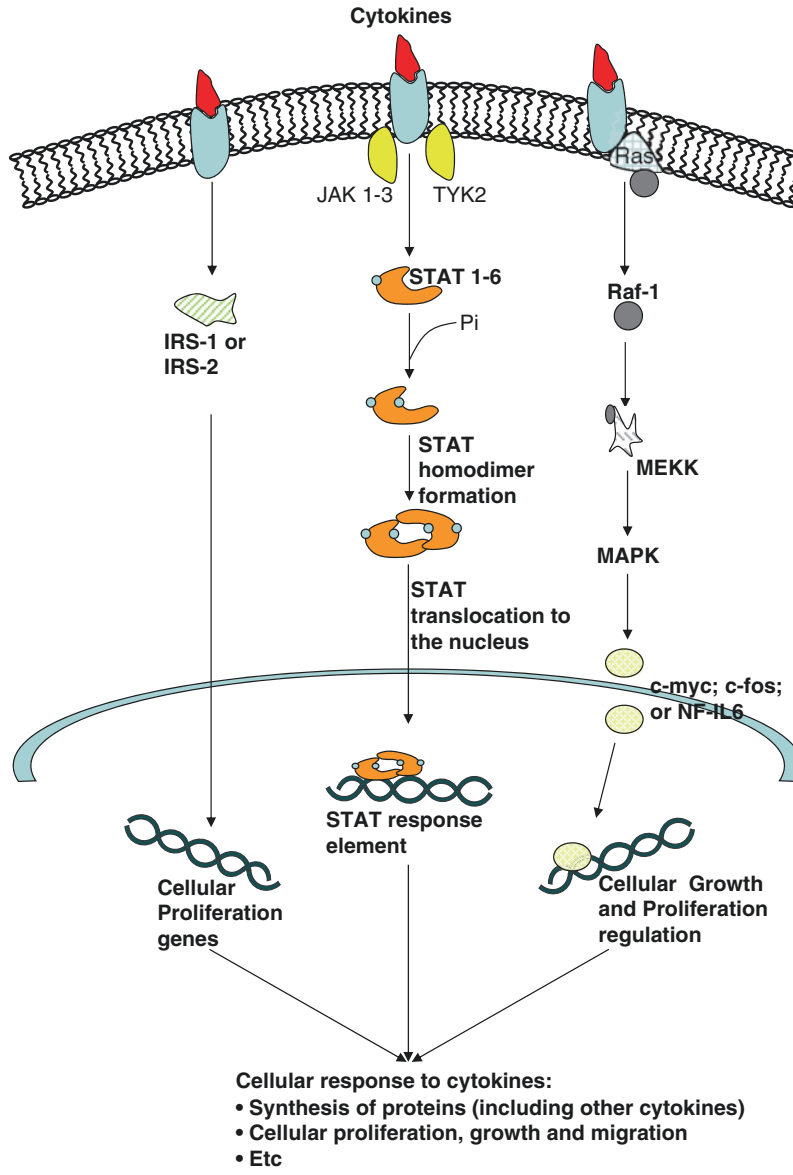

Fig. 9.4 Cytokine-induced intracellular signalling

signalling is based on a relatively small number of redundant tyrosine kinases. For instance, JAK-1 and JAK-3 transduct signals from cytokines such as IL-2 or IL-4, while JAK-2 is involved in IL-3, IL-6 and GM-CSF signalling. Similarly, the number of STATs is low when compared to the number of cytokines. Therefore, one can conclude that some additional mechanisms will guide different responses to various cytokines. An additional pathway used by many cytokine receptors includes *Ras*-dependent cascades. In this signal transduction cascade, Ras, Raf-1, Map/Erk kinase kinase (MEKK) and finally mitogen-activated protein kinases (MAPK) are sequentially activated and lead to regulation of cellular proliferation by growth factors and responses to IL-2 or IL-3. The activation of other signalling pathways, like insulin receptor substrates (IRS-1, IRS-2), can also mediate some other biological activities of cytokines, including proliferation and regulation of apoptosis. In conclusion, it becomes apparent

that different combinations of the signalling mechanisms described above will lead to many distinct responses to different cytokines.

9.6 Chemokines and Their Intracellular Signalling

CHEMOKINES are a family of 8–12 kDa molecules, which induce chemotaxis of monocytes, lymphocytes, neutrophils, other GRANULOCYTES as well as vascular smooth muscle cells and variety of other cells. There are 47 chemokines, sharing 30–60% homology. Chemokines are characterised by the presence of 3–4 conserved cysteine residues. The new classification of chemokines is based on the positioning of the N-terminal cysteine residues (Table 9.4). Chemokines are usually secreted proteins, except for fractalkine (CX3CL1) which is the only membrane-bound chemokine, acting as an adhesion molecule. Most chemokines play roles in recruiting and activating immune cells to the site of inflammation, while others are important in maintaining homeostasis within the immune system (housekeeping chemokines: CCL5, CCL17-19, 21, 22, 25, 27, 28, CXCL13, CXCL14). Homeostatic chemokines are expressed in an organ-specific manner, while inflammatory chemokines can be produced by multiple cell types.

Their activities are achieved through interaction with chemokine receptors. There are 18 chemokine receptors currently known; therefore, some receptors may bind several ligands, which lead to overlapping functions of known chemokines. Moreover, a single cell may express several chemokine receptors. One of the key features of chemokine receptors, owing to their heptahelical transmembrane structure, is their ability to signal through different intracellular signalling pathways. Binding of chemokine to the receptor leads to activation of Gα protein and binding of GTP. Gα subunit activates Src kinases and subsequently mitogen-activated protein kinases (MAPKs) and protein kinase B (PKB). During activation of Gα protein, a Gβγ complex is liberated and may independently lead to activation of PKB and MAPKs (via PI_3), PKC activation via phospholipase C (PLC) and finally through Pyk-2 [24]. These pathways lead to up-regulation of membrane INTEGRINS and initiate rolling and adhesion of cells as well as their conformational changes. Some of these intracellular pathways (in particular PLC activation) may then lead to an increase in intracellular calcium and its consequences, including DEGRANULATION, NOS activation, etc. within the target cells.

It is difficult to accurately describe the relative importance of individual chemokines. The largest number of studies was conducted on the actions of *IL-8* as the most important chemoattractant for polymorphonuclear leukocytes, although it appears late during the inflammatory response. Other well-investigated members of this family include CCL3 (*MIP-1α*) or *RANTES* (CCL5).

Apart from effects on chemotaxis, chemokines have direct and indirect effects on T-cell differentiation into T helper 1 or 2 subclasses, therefore regulating the nature of immune responses [23, 24].

Due to the critical role of chemokines in inflammation, interest has focused on potential therapeutic effects of inhibiting their activity. Both peptide antagonists and gene transfer approaches have been successfully used to inhibit inflammation in various animal models (e.g. allergic inflammation models or ApoE-knockout atherosclerosis prone mice).

Table 9.4 Classes of chemokines

Subfamily	Chemokines	Characteristics
C-X-C	CXCL 1-16, includes IL-8 (CXCL8)	First two cysteines separated by a variable amino acid
C-C	CCL1-28 (include MIP-1 MCP and RANTES)	First two cysteines are adjacent to each other
C	XCL 1 (lymphotactin) and XCL 2	Lacks first and third cysteine residue
CX3CL1	CX3CL1 (Fractalkine)	Two N-terminal cysteine residues separated by three variable amino acids

9.7 Neuropeptides

Neuropeptides are released from sensory neurons, and in some tissues, they contribute to inflammatory reactions. For example, substance P and other tachykinins produce smooth muscle contraction and mucus secretion, cause vasodilation and increase vascular permeability. "Calcitonin gene-related peptide" (CGRP) is a potent vasodilator, acting on CGRP receptors leading to activation of adenylate cyclase. The overall pattern of effects of tachykinins is similar, though not identical, to the pattern seen with kinins.

9.7.1 Tachykinins

The mammalian tachykinins comprise three related peptides: substance P (SP), neurokinin A (NKA) also called substance K and neurokinin B (NKB). They occur mainly in the nervous system, particularly in nociceptive sensory neurons and in enteric neurons. They are released as neurotransmitters, often in combination with other mediators. SP and NKA are encoded by the same gene and they have a similar distribution. Three distinct types of tachykinin receptor are known: NK_1, NK_2 and NK_3. They are selective for three endogenous tachykinins with the following affinity: SP>NKA>. NKB for NK_1, NKA>NKB>SP for NK_2 and NKB>NKA>SP for NK_3 receptor. Receptor cloning has shown that tachykinin receptors belong to a family of G-protein-coupled receptors. Several potent antagonists of NK_1 and NK_2 and NK_3 receptors have been discovered [25], and novel therapeutic agents for various disease states (e.g. pain, ASTHMA, arthritis, headache) may be developed.

CGRP differs from other tachykinins. It is coded for by the calcitonin gene which also codes for calcitonin itself. Differential splicing allows cells to produce either procalcitonin (expressed in thyroid cells) or pro-CGRP (expressed in neurons) from the same gene. CGRP is found in nonmyelinated sensory neurons, and it is a potent inducer of neurogenic inflammation.

9.8 Kinins

Kinins are polypeptides with vasodilator/hypotensive, thrombolytic, pro-inflammatory and algesic (painful) actions. The two best known kinins are bradykinin and kallidin, and they are referred to as plasma kinins. Since 1980, when Regoli and Barabe divided the kinin receptors into B_1 and B_2 classes, first- and second-generation kinin receptor antagonists have been developed, leading to a much better understanding of the actions of kinins.

Bradykinin is a nonapeptide; kallidin is a decapeptide and has an additional lysine residue at the amino-terminal position. These two peptides are formed from a class of α-2 globulins known as *kininogens* (Fig. 9.5). There are two *kininogens*: high molecular weight (*HMW*) and low molecular weight (*LMW*) kininogen which are products of a single gene that arises by alternative processing of mRNA. The highly specific proteases that release bradykinin and kallidin from the kininogens are termed *kallikreins*. Two distinct kallikreins, formed by different activation mechanisms from inactive *prekallikreins*, act on the kininogens. One of these is plasma kallikrein and the other is tissue kallikrein. LMW kininogen is a substrate only for the tissue kallikrein, and the product is kallidin, while HMW kininogen is cleaved by plasma and tissue kallikrein to yield bradykinin and kallidin, respectively.

Kallidin is similar in activity to bradykinin and need not be converted to the latter to exert its effects. However, some conversion of kallidin to bradykinin occurs in plasma due to the activity of plasma aminopeptidases.

The half-life of kinins in plasma is about 15 s, and concentrations of kinins found in the circulation are within the picomolar range. Bradykinin is inactivated by a group of enzymes known as *kininases*. The major catabolizing enzyme in the lung and in other vascular beds is *kininase II*, which is identical to peptidyl dipeptidase—known as angiotensin converting enzyme (ACE). *Kininase II* is inhibited by captopril, resulting in an increased concentration of circulating bradykinin, which contributes substantially to the

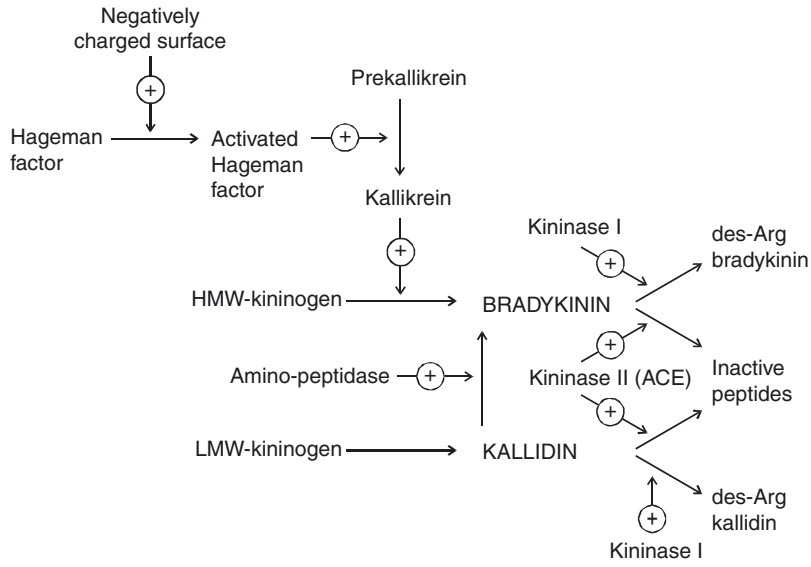

Fig. 9.5 The formation and metabolism of kinins

antihypertensive effect of captopril. On the other hand, *kininase I* is arginine carboxypeptidase, and it has a slower action than *kininase II*. It removes the carboxyl-terminal arginine residue producing des-Arg^{9}-bradykinin or des-Arg^{10}-kallidin, which are themselves potent B_1-kinin receptor agonists.

There are at least two distinct receptors for kinins, B_1 and B_2. The classical, constitutive bradykinin receptor, now designated the B_2 receptor, selectively binds bradykinin and kallidin and mediates a majority of the effects of bradykinin and kallidin in the absence of inflammation, such as the release of PGI_2 and NO from endothelial cells. On the other hand, inducible B_1 receptors are upregulated by inflammation. They bind des-Arg metabolites of bradykinin and kallidin. In contrast to B_1 receptors, the signalling mechanism of B_2 receptors has been well characterized. The B_2 receptor is coupled to G protein and activates both PLA_2 and PLC. While stimulation of the former liberates AA from phospholipids, with its subsequent oxidation to a variety of pro-inflammatory eicosanoids, the activation of PLC through IP_3 and DAG leads directly to pro-inflammatory effects.

During the last decade, the existence of other types of kinin receptors (B_3, B_4, B_5) has been suggested. However, recent studies indicate that some of them may actually represent functions of the B_2 receptor [15].

Kinins are among the most potent vasodilators known, acting on arteriolar beds of the heart, liver, skeletal muscle, kidney, intestines and ovaries. They are claimed to play a minor role in the regulation of blood pressure in health individuals, but they play a major vasodepressor regulatory role most likely mediated by arterial endothelium in hypertensive patients [26]. Indeed, kinins contract veins and non-vascular smooth muscle, such as gastrointestinal and bronchial muscle. Bradykinin and kallidin have similar contracting properties. At the level of the capillary circulation, kinins increase permeability and produce oedema. Stimulation of B_1 receptors on inflammatory cells such as macrophages can elicit the production of the inflammatory mediators such as IL-1 and TNF-α [27]. Kinins are also potent pain-inducing agents in both the viscera and skin. In acute pain, B_2 receptors mediate bradykinin-induced algesia. The pain of chronic inflammation appears to involve an increased expression of B_1 receptors.

As in the case of other autacoids, the therapeutic interest in kinins has focused particularly on attempts to modulate their formation or metabolism in vivo [28]. Blockade of kinin formation with a kallikrein inhibitor, aprotynin (Trasylol),

has been used with some success to treat acute pancreatitis, carcinoid syndrome or Crohn disease. Experimentally, progress has been made in the development of selective antagonists of kinins. Currently, they are not available for clinical use. However, recent studies indicate that kinin receptor antagonists might be useful for the treatment of patients with septic shock, pancreatitis-induced hypotension bronchial ASTHMA and rhinovirus-induced symptoms and in fighting pain.

9.9 Nitric Oxide

In animal tissues, nitric oxide (NO) is generated enzymatically by NO synthases (NOS). The three NOS isoenzymes (neuronal, endothelial and inducible) are flavoproteins which contain tetrahydrobiopterin and haem, and they are homologous with cytochrome p 450 reductase [29]. Isoenzymes of NOS act as dioxygenases using molecular oxygen and NADPH to transform L-arginine to L-citrulline and NO (Fig. 9.6). NO formed by endothelial constitutive NOS (eNOS) is responsible for maintaining low vascular tone and preventing leukocytes and platelets from adhering to the vascular wall. eNOS is also found in renal mesangial cells. NO formed by neuronal constitutive NOS (nNOS) acts as a neuromodulator or neuromediator in some central neurons and in peripheral "non-adrenergic non-cholinergic" (NANC) nerve endings. NO formed by inducible NOS (iNOS) in macrophages and other cells plays a role in the inflammatory response.

NO was discovered by Furchgott and Zawadzki as "endothelium-derived relaxing factor" (EDRF) [30]. It soon became obvious that EDRF, like nitroglycerine, activates soluble guanylate cyclase in vascular smooth muscle by binding to its active haem centre. The rise in cyclic GMP achieved is responsible for vasodilatation and for other physiological regulatory functions of NO.

The activities of constitutive nNOS and eNOS are controlled by intracellular calcium/calmodulin levels. For instance, nNOS in central neurons is activated by glutamate binding to NMDA

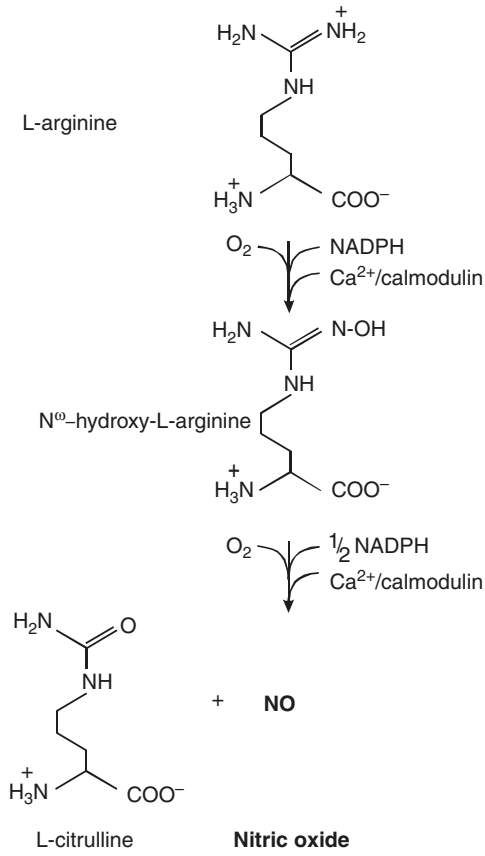

Fig. 9.6 The synthesis and metabolism of nitric oxide (NO)

receptors with a subsequent rise in $[Ca^{2+}]i$ due to opening of voltage calcium channels, whereas eNOS is activated by blood shear stress or stimulation of endothelial muscarinic, purinergic, kinin, substance P or thrombin receptors. This triggers an increase in $[Ca^{2+}]i$ at the expense of the release of Ca^{2+} from endoplasmic reticulum.

Calcium ionophores (e.g. A23187) and polycations (e.g. poly-L-lysine) cause a rise in $[Ca^{2+}]i$ and activate eNOS, thereby bypassing the receptor mechanisms.

In contrast to the constitutive isoforms of NOS, iNOS does not require a rise in $[Ca^{2+}]i$ to initiate its activity. In macrophages, monocytes and other cells, the induction of iNOS and the presence of L-arginine are sufficient to initiate the generation of NO. Induction of iNOS can be initiated by IFN-γ, TNF-α or IL-1. However, the

best recognized inducer is lipopolysaccharide (LPS) or endotoxin from *Escherichia coli* which is known to be responsible for the development of systemic inflammatory response syndrome (SIRS) in the course of sepsis due to gram-negative bacteria. Myeloid cells express a receptor for LPS on their cell membrane, m-CD14 protein. LPS, using an "LPS binding protein" (LBP), is anchored to m-CD14 and then triggers a chain of protein phosphorylation which eventually leads to the activation of the major transcription protein NF-κ-B. This is responsible for transcription of the message to make iNOS. In cells which lack m-CD14, the induction of iNOS is achieved by a complex of soluble s-CD14 with LBP and LPS itself. In a similar manner, LPS can also induce COX-2. Although NO fulfils more paracrine than autoendocrine functions, in the case of iNOS, large amounts of locally formed NO may inhibit iNOS itself as well as COX-2, in a negative feedback reaction. Glucocorticosteroids and some cytokines, such as TGF-β, IL-4 or IL-10, inhibit the induction of iNOS.

9.9.1 Nitric Oxide as an Effector of Inflammation

Kinetics of nitric oxide production by iNOS differ greatly from production by eNOS or nNOS (Fig. 9.7) [31]. Inducible NOS produces very large, toxic amounts of NO in a sustained manner, whereas constitutive NOS isoforms produce NO within seconds, and its activities are direct and short acting. There are multiple intracellular mechanisms through which nitric oxide may act as an inflammatory mediator [32]. Low levels of NO produced by constitutive synthases primarily interact directly with positively charged metal ions of guanylate cyclase, cytochrome p450 and NOS itself. Activation of guanylate cyclase leads to an increase in intracellular cyclic guanosine monophosphate (cGMP), which in turn activates cGMP-dependent protein kinases which mediate NO actions including vasorelaxation, increase of vascular permeability, as well as anti-proliferative, anti-platelet and antioxidant effects of nitric oxide. Recent data have also indicated that NO

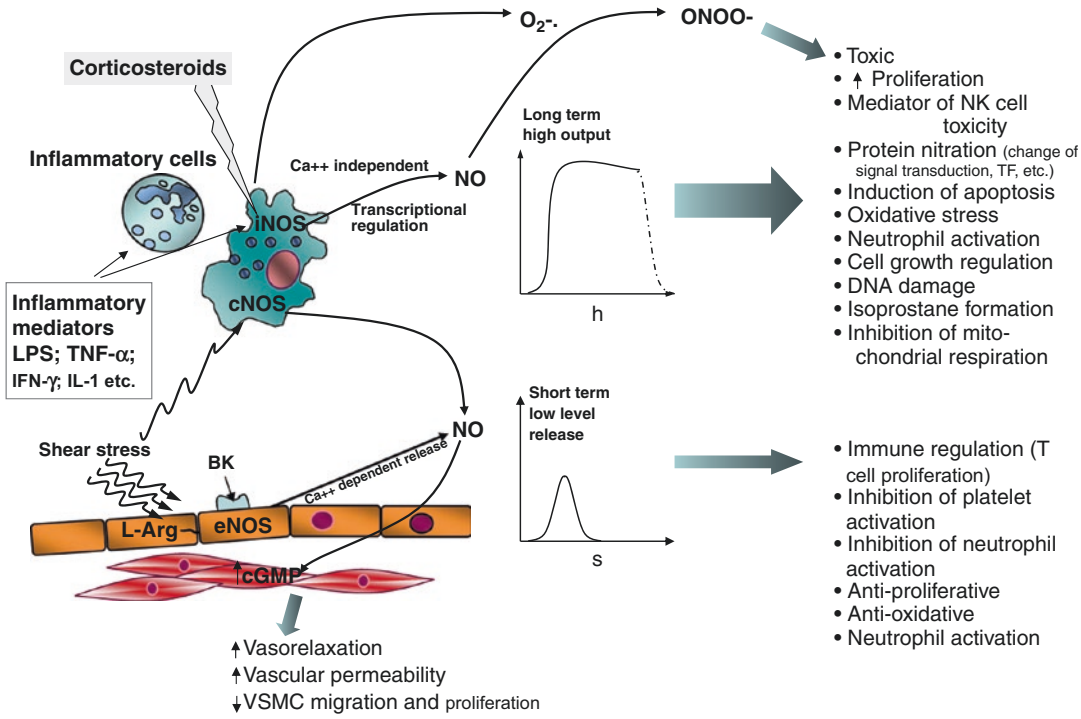

Fig. 9.7 Differences between kinetics of nitric oxide generation by eNOS and iNOS

produced by constitutive NOS enzymes may be involved in immune regulation of T helper cell proliferation and cytokine production.

During the course of an inflammatory response, the large amounts of NO formed by iNOS surpass the physiological amounts of NO which are usually made by nNOS or eNOS. The functions of iNOS-derived NO are also different. In immunologically or chemically activated macrophages, NO kills microorganisms and destroy macromolecules. NO, formed by constitutive isoforms of NOS, is stored as a nitrosothiol in albumin and acts physiologically as *N*-nitrosoglutathione and *N*-nitrosocysteine. Eventually, within a few seconds, NO is oxidized to nitrites or nitrates. Large amounts of "inflammatory NO" from myeloid cells are usually generated side by side with large amounts of superoxide anion (O_2^-). These two can form peroxynitrite (ONOO-) which mediates the cytotoxic effects of NO, such as DNA damage, LDL oxidation, isoprostane formation, tyrosine nitration, inhibition of aconitase and mitochondrial respiration. The discovery of this reaction opens new possibilities for the therapeutic use of superoxide dismutase (SOD). Indeed superoxide dismutase mimetics have been successfully used to limit the extent of inflammation. Interestingly, overstimulation of NMDA receptors by glutamate may activate nNOS to such an extent that NO itself exerts neurotoxic properties. NO formed by eNOS seems to be mostly cytoprotective, possibly due to its unusual redox properties.

Large amounts of NO and ONOO- may target numerous proteins and enzymes critical for cell survival and signalling. These include signalling molecules involved in cytokine signalling like JAK or STAT proteins, NKκB/IκB pathway as well as MAPK, some G proteins and transcription factors. Nitration of cysteines in these proteins may lead to their activation or inactivation.

NO is scavenged by haemoglobin, methylene blue and pyocyanin from *Pseudomonas coereleus*. These last two are also claimed to be inhibitors of guanylate cyclase. Glucocorticoids selectively inhibit the expression of iNOS. Arginine analogues, such as L-N^G-monomethyl arginine (L-NMMA) and L-N^G-nitroarginine methyl ester (L-NAME), inhibit inducible and constitutive NOS isoforms nonselectively. Selective iNOS inhibitors (e.g. alkylisothioureas or aminoguanidines) are being intensively investigated in the hope that selective inhibition of iNOS may prevent development of SIRS (systemic inflammatory response syndrome) or MODS (multiple organ dysfunction syndrome). Indeed, overproduction of NO by iNOS during septicaemia is claimed to be responsible for irreversible arterial hypotension, vasoplegia (loss of responses to noradrenaline), lactic acidosis, suffocation of tissues, their necrosis and apoptosis. However, it is important to remember that NO made by iNOS is of benefit to the host defence reaction by contributing to microbial killing.

Moreover, NO generated by eNOS is essential to maintain tissue perfusion with blood, to offer cytoprotection in the pulmonary and coronary circulation against toxic lipids which are released by LPS and to preserve red cell deformability which becomes reduced in septicaemia [33]. Preliminary clinical experience with L-NMMA has been reasonably encouraging, as long as a low dose of the NOS inhibitor is used. In animal models of endotoxic shock, nonselective NOS inhibitors were reported to decrease cardiac output, to increase pulmonary pressure, to decrease nutritional flow to organs, to damage gastric mucosa and to increase mortality rate. On the other hand, inhalation of NO gas (10 ppm) in septic patients has been found to prevent the mismatch of the ventilation/perfusion ratio in their lung. The exact role of NO in various stages of sepsis, SIRS and MODS still awaits further elucidation and evaluation.

9.9.2 Nitric Oxide in Immune Regulation

The exact role of NO in immune regulation is also unclear. Initial mouse studies suggested that ANTIGEN PRESENTING CELL-DERIVED NO may inhibit T-cell proliferation, particularly of the Th1 subset of T helper cells. Mouse Th1

cells were also shown to produce NO, suggesting that the above mechanism is a part of a negative feedback process. In this way, NO would inhibit Th1 and therefore promote Th2 type cytokine responses leading to humoral and allergic responses. Subsequent studies, however, indicate that both Th1 and Th2 produce similar amounts of NO, and both subsets respond similarly to nitric oxide. NO-induced changes in lymphocyte proliferation seem to be dependent more on the effects on the cell cycle proteins than due to changes in cytokine profile [31].

It is also important to recognize that cells which produce NO protect themselves against its toxic actions [34]. Recent studies show that GSH-GSSG anti-oxidative systems protect macrophages against large amounts of NO generated by iNOS. In addition, endothelial cells appear not to be primary responders to NO produced by eNOS because increases in intracellular calcium which mediate eNOS activation are also able to inhibit guanylate cyclase activity.

9.10 Reactive Oxygen Species

Reactive oxygen species (ROS) production plays an important role in modulation of inflammatory reactions. Major ROS produced within the cell are superoxide anion, hydrogen peroxide and hydroxyl radical [32]. Extracellular release of large amounts of superoxide anion produced by the respiratory burst in leukocytes is an important mechanism of pathogen killing and also leads to endothelial damage resulting in an increased vascular permeability as well as cellular death. However vast evidence has implicated intracellular ROS production as a key player in modulation of the release of other mediators of inflammation. This is related mainly to the constitutive expression of NAD(P)H oxidases (termed NOXs—non-phagocytic oxidases) in various tissues [31]. ROS produced by this family of enzymes can regulate adhesion molecule expression on endothelium and inflammatory cells, thus regulating cellular recruitment to the sites of inflammation. They also increase chemokine and cytokine expression. At least some of these effects result from the ability of ROS (in particular H_2O_2) to stimulate MAP kinase activities which lead to activation of several transcription factors. It is possible that intracellular ROS may act as second messengers in inflammatory signal transduction [31].

Inflammatory cytokines (like TNF-α) may in turn increase NAD(P)H oxidase activity and expression which closes the vicious circle of inflammation. While loss of NAD(P)H oxidase activity in cells leads to diminished inflammation in the vascular wall, several humoral factors may affect constitutive NAD(P)H oxidase expression in the vascular wall and therefore intracellular ROS production. These include angiotensin II, endothelins, high glucose or high cholesterol levels. Their effects on baseline ROS production may therefore mediate modulatory effects of these factors on inflammation which traditionally were not associated with inflammation. Interestingly, T and B LYMPHOCYTES at various stages of their development and activation express NADPH oxidases, mainly classical gp91phox containing NADPH oxidase, Nox2 (mature T cells), and a calcium-dependent Nox5 (during development).

Accordingly, attempts have been undertaken to inhibit intracellular ROS production in order to limit inflammatory responses. Apocynin, an NAD(P)H oxidase activation inhibitor, has been successfully used in limiting inflammation in animal models of rheumatoid arthritis, while decoy peptides preventing association of NAD(P)H oxidase subunits were shown to be effective in inflammation related to atherosclerosis.

9.11 Amines

Histamine, 2-(4-imidazolyl)-ethyl-amine, is an essential biological amine in inflammation and allergy. It is found mostly in the lung, skin and in the gastrointestinal tract. It is stored together with macroheparin in granules of mastocytes or basophils (0.01–0.2 mol per cell), from which it is released when COMPLEMENT components C3a and C5a interact with specific receptors or when antigen interacts with cell-fixed IgE.

These trigger a secretory process that is initiated by a rise in cytoplasmic Ca^{2+} from intracellular stores. Morphine and tubocurarine release histamine by a non-receptor action. Agents which increase cAMP formation inhibit histamine secretion, so it is postulated that, in these cells, c-AMP-dependent protein kinase is an intracellular restraining mechanism. Replenishment of the histamine content of MAST CELLS or basophils after secretion is a slow process, whereas turnover of histamine in the gastric histaminocyte is very rapid.

Histamine is synthesized from histidine by a specific decarboxylase and metabolized by histaminases and/or by imidazole N-methyltransferase. Histamine exerts its effects by acting on H_1-, H_2- or H_3 receptors on target cells [35]. It stimulates gastric secretion (H_2), contracts most of the smooth muscle other than that of blood vessels (H_1), causes vasodilatation (H_1) and increases vascular permeability by acting on the postcapillary venules [36]. Injected intradermally, histamine causes the triple response: local vasodilatation and wheal by a direct action on blood vessels and the surrounding flare which is due to vasodilatation resulting from an axon reflex in sensory nerves, thereby releasing a peptide mediator [36]. Of many functions of histamine, the stimulation of gastric acid secretion and mediation of type 1 hypersensitivity, such as urinary and hay fever, are among the most important. The full physiological significance of the H3 receptor has yet to be established [37]. Histamine may also be involved in T helper cell immune regulation (extensively reviewed in [38]).

5-Hydroxytryptamine (*5-HT*, *serotonin*) was originally isolated and characterized as a vasoconstrictor released from platelets in clotting blood. 5-HT occurs in chromaffin cells and enteric neurons of the gastrointestinal tract, in platelets and in the central nervous system. It is often stored together with various peptide hormones, such as somatostatin, substance P or "vasoactive intestinal polypeptide" (VIP). The biosynthesis and metabolism of 5-HT closely parallel that of catecholamines, except the precursor for decarboxylase of aromatic amino acids is 5-hydroxytryptophan instead of tyrosine (Fig. 9.8). 5-HT is inactivated mainly by the monoamine oxidases A or B (MAO A or B) to 5-hydroxyindoleacetic acid (5-HIAA) which is excreted in the urine. Some 5-HT is methylated to 5-methoxytryptamine, which is claimed to be involved in the pathogenesis of affective disorders.

The actions of 5-HT are numerous and complex, showing considerable variation between species [39]. For instance, in the inflammatory response, 5-HT seems to be more important in rats than in humans. 5-HT is known to increase gastrointestinal motility and to contract bronchi, uterus and arteries, although 5-HT may also act as a vasodilator through endothelial release of NO. In some species, 5-HT stimulates platelet aggregation, increases microvascular permeability and stimulates peripheral nociceptive nerve endings. A plethora of pathophysiological

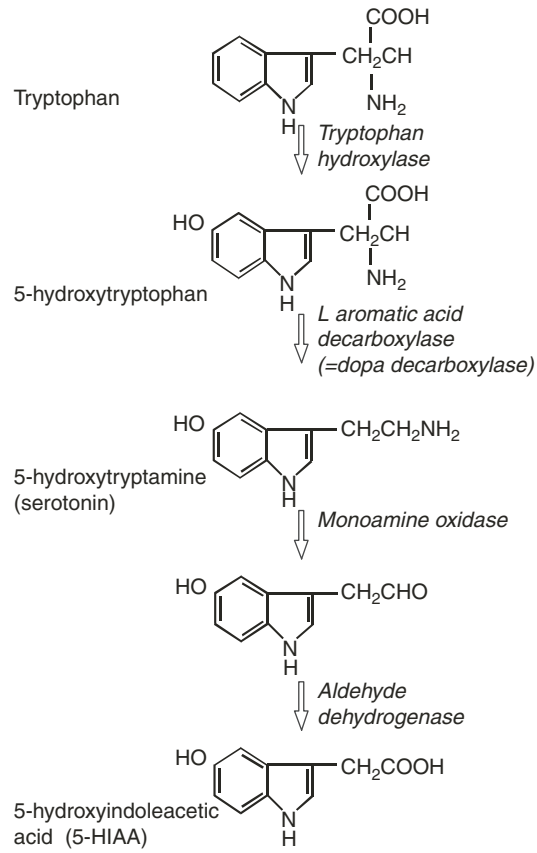

Fig. 9.8 The synthesis and breakdown of 5-HT

functions proposed for 5-HT includes control of peristalsis, vomiting, haemostasis, inflammation and sensitization of nociceptors by peripheral mechanisms or control of appetite, sleep, mood, stereotyped behaviour and pain perception by central mechanisms. Clinically, disturbances in the 5-HT regulation system have been proposed in migraine, carcinoid syndrome, mood disorders and anxiety [39].

These diverse actions of 5-HT are not mediated through one type of receptor. The amino acid sequence for many 5-HT receptor subtypes has been determined by cloning, and the transduction mechanisms to which these receptors are coupled have been explained. The four basic types of receptors are $5\text{-HT}_{1\text{-}4}$, 5-HT_1 and 5-HT_2 receptors which are further subdivided into A, B and C subtypes [40]. Types 1, 2 and 4 are G-protein-coupled receptors, while type 3 is a ligand-gated cation channel. 5-HT_1 receptors occur mainly in the CNS (all subtypes) and in blood vessels (5-HT_{1D} subtype). 5-HT_{1B} and 5-HT_{1D} receptors appear to be involved, at least in part, in the modulation of neurogenically induced (following electrical, chemical or mechanical depolarization of sensory nerves) vascular inflammation. 5-HT_2 receptors (5-HT_{2A} subtype being functionally the most important) are distributed more in the periphery than in the CNS, and they are linked to phospholipase C which catalyses phosphatidylinositol hydrolysis. The role of 5-HT_2 receptors in normal physiological processes is probably a minor one, but it becomes more prominent in pathological conditions, such as ASTHMA, inflammation or vascular thrombosis. 5-HT_3 receptors occur particularly on nociceptive sensory neurons and on autonomic and enteric neurons, on which 5-HT exerts an excitatory effect and evokes pain when injected locally.

Catecholamines. It has become increasingly recognized that the release of catecholamines at autonomic nerve endings and from the adrenal medulla may modulate the function of immunocompetent cells. The major lymphoid organs (spleen, lymph nodes, thymus and intestinal Peyer's patches) are extensively supplied by noradrenergic sympathetic nerve fibres. Sympathetic nervous system innervation of these lymphoid organs as well as the presence of adrenergic and dopamine receptors on immune cells provide the channels for noradrenergic signalling to lymphocytes and macrophages by sympathetic nerves [41]. Catecholamines have a wide range of direct effects on immune cells, particularly on macrophages and lymphocytes. Stimulation of β-adrenergic receptors on LPS-pretreated macrophages prevents the expression and release of pro-inflammatory TNF-α and IL-1, while the release of anti-inflammatory IL-10 is augmented. On the other hand, α-adrenergic stimulation augments phagocytic and tumouricidal activity of macrophages. Catecholamines acting through β-adrenergic and dopaminergic receptors, which are linked to adenylate cyclase through cyclic-AMP, modulate the function of immune cells. An increase in intracellular cyclic-AMP inhibits lymphocyte proliferation and production of pro-inflammatory cytokines. The demonstration of the presence of α_2-, β-adrenergic, D1 and D2 receptors on various immune cells has recently provided the basis for regulation of cytokine production, specifically interleukins and TNF, by these receptors in response to LPS [41]. Vasopressor and inotropic catecholamines seem to have potent immunomodulating properties which, as yet, have not been adequately explored and may contribute to the therapeutic effects of dobutamine or dopexamine in the treatment of septic shock and SIRS.

9.12 Summary

Inflammation is a protective response of the macroorganism to injury caused by trauma, noxious chemicals or microbiological toxins. This response is intended to inactivate or destroy invading organisms, remove irritants and set the stage for tissue repair. The inflammatory response consists of immunological and non-immunological reactions. The latter are triggered by the release from injured tissues and migrating cells of lipid-derived autacoids, such as eicosanoids or "platelet-activating factor" (PAF); large peptides, such as interleukin-1 and cytokines; small peptides, such as bradykinin; and amines, such as histamine 5-hydroxytryptamine. These constitute

the chemical network of the inflammatory response and result in clinical and pathological manifestations of inflammation.

Prostanoids, as autacoids, are involved in virtually every stage of inflammation. They regulate vascular tone and permeability (PGs), induce platelet aggregation and thrombus formation (TX) and are involved in the pathogenesis of pain and fever (PGs) accompanying inflammation. The recently discovered lipoxins are important regulators of inflammatory reactions. PAF, cytokine and chemokine groups as well as kinins also play crucial pro-inflammatory roles. Recent studies have shed more light on our understanding of intracellular signalling mechanisms involved in the responses to pro-inflammatory cytokines such as IL-1, TNF, TGF and interferons. Toll-like receptors contribute to the mediation of effects of components of microorganisms on innate and adaptive immunity.

Nitric oxide and reactive oxygen species not only act as important effectors, causing damage to invading microorganisms (NO from iNOS or superoxide anion), but may also be very important in immunoregulation, in part by regulating redox-sensitive genes. Co-ordinated pharmacological interventions, which would modify different parallel pathways in the inflammatory cascade, are needed to treat inflammatory diseases.

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