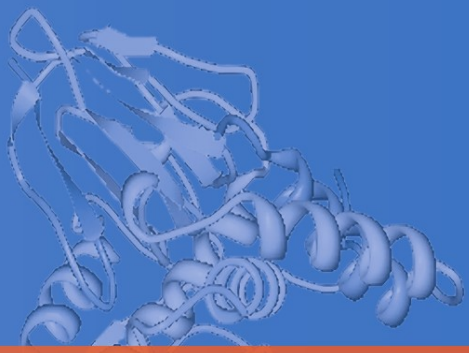
Subcellular Biochemistry 56



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Water Soluble Vitamins

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Preface

For many years, there were vague ideas that some diseases were due to dietary deficiencies. The discovery of vitamins in the early 1900s, their chemical characterization and the clarification of their metabolic functions are sequential aspects of a brilliant chapter in the history of modern nutritional sciences and medicine. The research in vitamins and deficiency-related pathologies has indeed seen a remarkable past, and the path to still better understanding is likely to continue far into the future.

Numerous observations led to the discovery of vitamins and their function, including some truly pioneering investigations that made history.

For example, perhaps one of the first randomized trials was done by James Lind (a British naval surgeon, 1716–1794) in 1747 on board the HMS Salisbury: he randomized 6 groups (2 sailors each) to either "a quart of cyder", or "two oranges and one lemon", or "half a pint sea water", or "twenty five gutts of elixir vitriol", or "two spoonfuls of vinegar", or "the bigness of a nutmeg of an electuray made of garlic, mustard seed, raddish root, Peruvian balsam and gum myrrh", -in addition to the regular diet. The idea was to find out which of the accepted antiscorbutic therapies really worked and the outcome was clear pointing towards the oranges and lemon-treatment (and cyder to some extent).

Another thrilling story refers to the clinical work of William Castle leading to the conclusion that there must be a reaction that takes place between an unknown intrinsic factor (IF) in the gastric juice and an unknown extrinsic factor in beef muscle. That extrinsic antianaemic factor turned out to be cobalamin (vitamin B12) and IF is needed for its absorption. Presumably, the gastric juice in pernicous anemia lacks IF.

Most of the mentioned work was done in the early 1900s. The period from 1912 to 1948 saw the isolation and identification of the variety of compounds and structures collectively termed vitamins. The name, derived from "vital-amines", indicates their elementary metabolic key functions in human metabolism. Indeed, severe deficiency is often associated with malfunction, symptoms and potentially fatal disease. The population-wide impact of overt deficiency-associated disease was so large that key discoveries in the field allowing prevention and leading to therapies were rewarded with several Nobel prizes.

The next logical step was synthetic mass production. Today, the general availability of vitamins to practically everyone and numerous national health programs have saved many lives and prevented complications. True (congenital) metabolic disorders and serious deficiency-associated diseases are rare and in general limited to particular geographic areas and high risk groups. Their identification is of utmost importance to make the best use of the appropriate vitamin's therapeutic potential. When used properly to treat or cure of diseases, vitamins are, indeed, "magical" substances. Due to their efficacy, they should therefore be regarded as drugs with effects and side effects to be weighted against each other. Sometimes a toxic dose is difficult to define and a longstanding misapprehension has led many people to believe that vitamins are harmless in any dose and, particularly in generous dose, can cure almost all of mankind's ills.

Today, it is not the previously fatal deficiency-associated diseases such as pernicious anemia or beri-beri that are in the focus of interest, but rather the relation of suboptimal vitamin bioavailability to chronic disease, which is much more difficult to observe and document. This is complicated by genetic susceptibility, lifestyle, and the presence or absence of health-compromising habits, such as smoking.

Vitamins are truly families of compounds, which include precursors and various bound forms, all with individual roles in metabolism and function. A more recent approach therefore searches for the components and optimal intake of a physiologically complete diet aiming at preventing disease and assuring optimal health. Any state of nutrition can be defined in theory, as deficient, marginal, satisfactory, excessive, or toxic. But in practice, this may prove to be extremely difficult requiring the development of appropriate tests and various status indicators.

In turn, the development and application of new and more sensitive and specific assays further enable us to look more closely into the many functions of vitamins.

At a national level, recommended daily allowances for vitamins become policy statements. Nutrition policy has far-reaching implications in the food industry, in agriculture, and in food provision programs. In fact, the broad availability of vitamins means that a great deal of work concentrates on aspects of overdosage and toxicology, interactions and also on regulations of direct and indirect intake. Water soluble vitamins are complex molecular structures and even today, many areas in vitamin biochemistry are not yet fully understood. Novel effects and functions of vitamins remain and continue to be discovered.

This book in your hands adds fantastic new insights into the biochemistry, metabolism, function, and therapeutic and diagnostic use of water soluble vitamins.

Serious clinical abnormalities including growth retardation, neurological disorders, and dermatological abnormalities occur in conditions of biotin (vitamin H) deficiency. Hamid M. Said (Irvine, California) presents the latest information on the biochemical, physiological, and clinical aspects of biotin nutrition.

Niacin is covered in two very interesting chapters. James B. Kirkland (Guelph, Ontario) explains the association between niacin status and genomic instability in bone marrow cells. The results are important for cancer patients, who tend to be niacin deficient, are exposed to large doses of genotoxic drugs, and suffer short-term bone marrow suppression and long-term development of secondary leukemias.

The recent identification of the nicotinic acid receptor has allowed distinction of the drug-like roles of nicotinic acid from its vitamin functions. The group of Elaine L. Jacobson (Tucson, Arizona) reviews niacin as an antidyslipidemic and cardioprotective drug.

The chapter on thiamin by Derrick Lonsdale (Westlake, Ohio) emphasizes beri-beri as the model for high calorie malnutrition and reviews the biochemistry of the three phosphorylated esters of thiamin and of their transporters. The pathophysiology of thiamin deficiency and the role of synthetic thiamin derivatives as therapeutic agents are discussed.

Riboflavin (vitamin B2) is essential for energy generation in the aerobic cell, through oxidative phosphorylation. Hilary J. Powers and co-workers (Sheffield, U.K.) provide insight into specific functions associated with cell fate determination, and review mechanisms and consequences of riboflavin depletion through effects on the expression of regulatory genes, exerted at both the transcriptional and proteomic level.

Sang Woon Choi (Boston, Mass.) and Simonetta Friso (Verona, Italy) and their co-workers review the evidence indicating a relationship between pyridoxin (vitamin B6) status and cancer as well as cardiovascular disease. Their discussion of the potential mechanisms of action is complemented by the chapter by Georg T. Wondrak (Tucson, Arizona), also from the Jacobson group, presenting insight to the structural basis of pyridoxin activity as a potent antioxidant, metal chelator, carbonyl scavenger and photosensitizer.

Novel aspects of the very complex biochemistry and metabolism of cobalamin (vitamin B12) are elaborated in three chapters. Bernhard Kräutler (Innsbruck, Austria) provides an in-depth survey of the physiological chemistry of cobalamin in the context of the metabolic transformation of cobalamin-derivatives and their use in cobalamin-dependent enzymes, whereas Sergey N. Fedosov (Aarhus, Denmark) reviews the molecular mechanisms of cobalamin transport with emphasis on interaction of corrinoids with the specific proteins and protein-receptor recognition. Practical novel aspects concerning early detection of cobalamin-related disorders are described including medical application of Cbl-conjugates, and purification of corrinoids from biological samples. To round out the subject, Wolfgang Herrmann (Homburg, Germany) suggests a revised definition of cobalamin deficiency.

Ascorbic acid (vitamin C) is covered in three further outstanding chapters. Mario C. De Tullio (Bari, Italy) focuses on largely unknown facets of the role of ascorbic acid in cell metabolism and its involvement in cell signalling and gene expression that are, at least in part, unrelated to antioxidant functions. John X. Wilson and F. Wu (Buffalo, New York) discuss in depth the role of ascorbic acid in correcting microvascular dysfunction and protecting capillary blood flow and arteriolar responsiveness in septic syndromes. Finally, neurons in the central nervous system (CNS) contain some of the highest ascorbic acid concentrations of mammalian tissues. James M. May (Nashville, Tenn.) reviews the role of the specific ascorbate transporter SVCT2 (Slc23a2) in regulating neuronal ascorbate homeostasis and the extent to which ascorbate affects brain function, including antioxidant protection,

peptide amidation, myelin formation, synaptic potentiation, and protection against glutamate toxicity in the CNS.

There is increasing research into the importance of folate-derived one-carbon units for DNA and histone methylation reactions, which exert crucial epigenetic control over cellular protein synthesis. Numerous polymorphisms have now been identified in folate related genes and it is thus becoming clear that genetic aspects of folate metabolism are wide-ranging and may touch on events as disparate as prenatal imprinting and cancer susceptibility. This topic is covered by Anne M. Molloy (Dublin, Ireland), who provides a detailed account of genetic aspects of folate metabolism. Willi Wonisch (Graz, Austria) and myself have elaborated on novel enzymatic and non-enzymatic antioxidative effects of folic acid and its reduced derivates that help explain the beneficial effects of folic acid supplementation in various clinical conditions. As an important clinical application J. D. Williams (Tucson, Arizona) reports on recent advances in research on folates in skin cancer and their potential in its prevention. And finally, J. Yang and Philipp Low (W. Lafayette, Indiana), expand upon themes introduced earlier and give a brilliant overview of the biology of the folate receptor (FR) and FR expression in immune cells with relevance for cancer and inflammatory and autoimmune disease. Their description of folate conjugates acting as drug carriers and diagnostic imaging tools holds strong promise for future successful efforts to cure certain types of cancer and chronic inflammatory disease.

This work in sum total so adds a new leaf to the exciting book on vitamin research that today's and tomorrows's scientists will continue to write.

I am deeply thankful to each of the contributing authors. All of them are highly esteemed and leading scientists very much dedicated to the demanding biochemistry of vitamins. They all have willingly agreed to share the latest research results from their respective working groups, laboratories and institutes. I truly thank them for all of their efforts and patience in the preparation of this book.

London, UK Olaf Stanger

Contents

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Chapter 1
Biotin: Biochemical, Physiological and Clinical Aspects

Hamid M. Said

Abstract Significant progress has been made in our understanding of the biochemical, physiological and nutritional aspects of the water-soluble vitamin biotin (vitamin H). It is well know now that biotin plays important roles in a variety of critical metabolic reactions in the cell, and thus, is essential for normal human health, growth and development. This is underscored by the serious clinical abnormalities that occur in conditions of biotin deficiency, which include, among other things, growth retardation, neurological disorders, and dermatological abnormalities (reviewed in 1). Studies in animals have also shown that biotin deficiency during pregnancy leads to embryonic growth retardation, congenital malformation and death (Watanabe 1983; Cooper and Brown 1958; Mock et al. 2003; Zempleni and Mock 2000). The aim of this chapter is to provide coverage of current knowledge of the biochemical, physiological, and clinical aspects of biotin nutrition. Many sections of this chapter have been the subject of excellent recent reviews by others (Wolf 2001; McMahon 2002; Mock 2004; Rodriguez-Melendez and Zempleni 2003; Said 2004; Said et al. 2000; Said and Seetheram 2006), and thus, for more information the reader is advised to consider these additional sources.

Keywords Biotin · Vitamin H · Carboxylases · Gluconeogenesis · Fatty acids

1.1 Chemical Structure, Sources and Availability of Biotin

The chemical structure of the biotin molecule (see Fig. 1.1 for the structure of biotin and related/relevant compounds) is composed of two rings to which a valeric acid moiety is attached as a side chain. One of the rings contains a ureido group (N-CO-N) which is involved in the binding of the vitamin to avidin, a glycoprotein found in egg-white that has an extremely high binding affinity toward biotin (Green 1990). The other ring contains a tetrahydrothiophene group to which a valeric acid moiety is attached. While the biotin molecule can exist in eight stereoisomers, the

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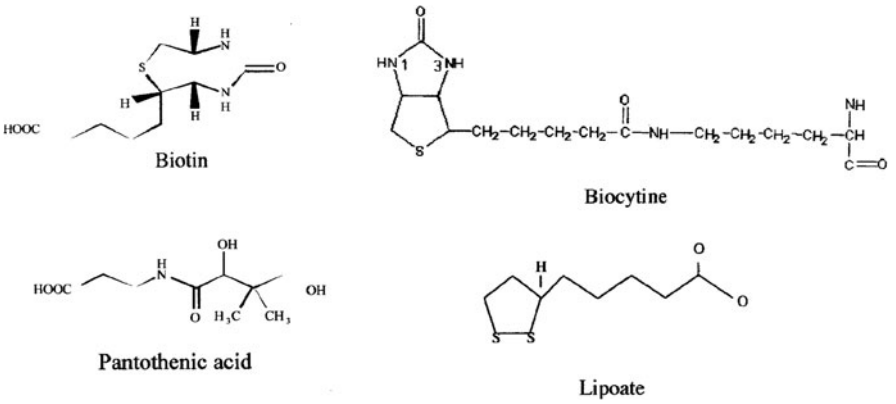

Fig. 1.1 Chemical structure of biotin and related compounds

D-biotin isomer is the only stereoisomer that is biologically active. Biotin has a pKa of 4.5, thus, it exists at physiological pHs mainly in the anionic de-protonated form.

Biotin is essential for all organisms. Animal cells cannot synthesize this micronutrient, but microorganisms (like certain bacteria and yeast) and plant cells can synthesize biotin endogenously (McMahon 2002). Biotin is widely distributed in foodstuff but at a level that is lower than that of other water-soluble vitamins (Combs 1992). Good sources for biotin include organ meat (like liver and kidney), egg yolk, some vegetables and cow's milk; poor sources include lean meat, cereal and fruits (Combs 1992; Harding and Crooks 1961). Bioavailability of dietary biotin varies from one food source to another; it is close to 100% in the case of corn, and as low as 5% in case of wheat (Combs 1992). In addition to the dietary source of biotin, humans have an additional source for this essential micronutrient, which is the bacterial source in the large intestine where the vitamin is produced and can be absorbed (see below). The relative contribution of this latter source of biotin toward overall body requirement of the vitamin is not well defined (Wolf 2001); however, it is becoming increasingly evident that this source of biotin does contribute to the host nutrition, and especially to the cellular nutrition of the localized colonocytes (see below).

1.2 Biotin Requirements and the Incidence of Biotin Deficiency

The recommended daily allowance of biotin has not been established due to the uncertainty about the contribution of the bacterially synthesized biotin in the human large intestine. However, a safe daily intake of the vitamin for both infants and adults has been estimated to be at 35 ug and 150–300 ug, respectively (National Research Council 1980). Biotin appears to be relatively nontoxic even at doses of greater than 60 mg/day for several months (Wolf 2001; Paul 1978; Watanabe 1996).

Biotin-deficiency and sub-optimal levels have been reported with increased frequency in recent years. Deficiency of biotin occurs in patients with inborn errors

of biotin metabolism (Wolf 2001; Sweetman and Nyhan 1986), in patients on long-term therapy with anticonvulsant agents (Krause et al. 1982a), and in patients on long-term parenteral nutrition (Krause et al. 1985; Forbes and Forbes 1997). Sub-optimal levels of biotin have been reported during pregnancy (Mock et al. 1997), in substantial numbers of alcoholics (Fennelly et al. 1969; Bonjour 1980), in patients with inflammatory bowel disease (Urabe et al. 1986; Banares et al. 1989), and in patients with seboric dermatitis and Leiner's disease (Nisenson 1957; Messaritakis et al. 1975).

1.3 Metabolic Role of Biotin

In mammals, biotin acts a cofactor for four carboxylases catalyzing the transfer of a carboxyl group to targeted substrates (reviewed in McMahon 2002; Mock 2004; Sweetman and Nyhan 1986; Dakshinamurti and Chauhan 1988). These carboxylases play a critical role in the intermediate metabolism of gluconeogenesis, fatty acid synthesis, and amino acid catabolism (McMahon 2002; Mock 2004; Sweetman and Nyhan 1986; Dakshinamurti and Chauhan 1988). The four biotin-dependent carboxylases are: pyruvate carboxylase (PC; EC 6.4.1.1), propionly-CoA carboxylase (PCC; EC 6.4.1.3), β-methylcrotonyl-CoA caboxylase (MCC; EC 6.4.1.4), and acetyl-CoA carboxylase (ACC; EC 6.4.1.2), with the latter enzyme existing in two genetically distinct forms one of which is in the cytosol (ACC1) and the other is in the mitochondria (ACC2). These carboxylases exist in the inactive apo-forms, which are converted to the active hollo-forms by the action of the enzyme holocarboxylase synthetase (HCLS; EC 6.3.4.10). The conversion occurs via a two-step, ATP-dependent reaction that involves the covalent attachment of a biotin molecule to a lysine residue in the carboxylases. This lysine moiety is located in a highly conserved domain common to all the biotin-dependent carboxylases. The HCLS exists in different cellular compartments including the nucleus, cytoplasm and mitochondria (Gravel and Narang 2005).

PC plays a role in the conversion of pyruvate to oxalate in the tricarboxylic acid cycle. Deficiency of this enzyme leads to lactic acidemia. PCC plays a role in the conversion of methylmalony-CoA to propionyl-CoA, which then becomes succinyl-CoA and enters into the tricarboxylic acid cycle. Deficiency of the latter enzyme leads to an increase in the excretion of the organic acids 3-hydroxypropionic acid and 2-methylcitric acid. MCC plays a role in the metabolism of leucine and deficiency of this enzyme leads to an increase in the urinary excretion of organic acids like 3-hydroxyisovaleric acid and 3-methylcrotonylglycine. ACC1 and ACC2 are involved in the generation of malonyl CoA.

In addition to the classical function of biotin described above, recent findings have indicated a role for the vitamin in histone modification (via biotinylation) (Gravel and Narang 2005; Ballard et al. 2002; Stanley et al. 2004). This function may be important for cell proliferation (Stanley et al. 2002) and DNA repair (Peteres et al. 2002) and may explain a number of findings on the effect of biotin on cell function. Both the HCLS and biotinidase (see latter) were suggested to play a role

in histones biotinylation (Gravel and Narang 2005; Ballard et al. 2002; Stanley et al. 2004). Patients with HCLS deficiency have very low histone biotinylation level in their lymphocytes (Narang et al. 2004).

Evidence for a role for biotin in the regulation of expression of a variety of genes has also been reported. An excellent review of this subject by Rodriguez-Melendez and Zempleni has recently been published (Rodriguez-Melendez and Zempleni 2003). Briefly, biotin appears to stimulate the level of expression of the insulin receptor (De La Vega and Stockert 2000), glucokinase (a key enzyme in glycolysis) (Chauhan and Dakshinamurti 1991, Dakshinamurti and Cheah-Tan 1968), and that of the human thiamin transporter-2 (Vlasova et al. 2005), while it suppresses the level of expression of the hepatic phosphoenolpyruvate carboxykinase (a key enzyme in gluconeogenesis; Dashinamurti and Li 1994). In addition, expression of the genes of the holocarboxylase synthetase and of the biotin-dependent carboxylases is affected by the prevailing level of biotin (Rodriguez-Melendez et al. 2001; Solorzano-Vargas et al. 2002; Wiedmann et al. 2003). Furthermore, the level of expression of the human sodium-multivitamin transporter (hSMVT, a major biotin transporter in different tissues, see latter) has also been shown to be modulated (in a tissue specific manner) by the prevailing level of biotin (Rodriguez-Melendez and Zempleni 2003; Crisp et al. 2004; Reidling and Said 2006; Balamurugan et al. 2005). Evidence has also been forthcoming demonstrating an effect for biotin on expression of oncogenes like N-myc, c-myb, N-ras and raf (Scheerger and Zempleni 2003). A post-transcriptional regulation of the asialoglycoprotein expression in hepatocytes by biotin has also been reported and is believed to be due to improper cell surface targeting of the glycoprotein (Collins et al. 1988). The mechanisms through which biotin exerts its above effects are not fully understood but could involve activation of soluble guanylate cyclase (by biotinyl-AMP), translocation of NF-kB to the nucleolus, and histone modifications via biotinylation (Rodriguez-Melendez and Zempleni 2003; Gravel and Narang 2005; Ballard et al. 2002; Stanley et al. 2004; Solorzano-Vargas et al. 2002).

Biotin also appears to play a role in normal immune functions including the production of antibodies, normal macrophage function, and the differentiation of T and B lymphocytes (Rabin 1983; Pruzansky and Axelrod 1955; Petrelli et al. 1981; Kung et al. 1979; Kumar and Axelrod 1978; Baéz-Saldaña et al. 1998). The vitamin may also be important for the normal function of natural killer cells as biotin supplementation alleviates the level of suppression in the activity of these cells in patients with Crohn's disease (Okabe et al. 1988). A role for biotin in cell proliferation has also been reported (Dakshinamurti et al. 1985; Mathey et al. 2002).

1.4 Biotin Catabolism

Biotin appears in the intact form in the urine and feces with the output being always higher than the daily intake of the vitamin. Some biotin, however, undergoes a limited-degree of catabolism to biotin sulfoxide (a process that takes place mainly in the liver; McCormick 1975) and to bisnorbiotin (a process that takes place

mainly in the mitochondria; McCormick and Wright 1971). These catabolic events appear to increase in pregnancy, with cigarette smoking, and following the use of anticonvulsant medications (Mock et al. 1997, 2002).

1.5 The Physiology of Biotin

The intestine and the kidney play important roles in regulating biotin body homeostasis via their involvement in the entry and the exit processes of the vitamin. Thus, significant attention has been paid toward the understanding of the mechanisms involved in biotin uptake by absorptive epithelial cells of these organs and how these processes are regulated at the cellular and molecular levels. In this section, we will describe in details the current knowledge of the mechanisms and regulation of the intestinal and renal transport process of biotin; more extensive reviews in the area also exist (Said 2004; Said et al. 2000; Said and Seetheram 2006). We will also provide a description of biotin transport into other tissues that are important in biotin metabolism/function.

1.5.1 Intestinal Absorption of Biotin

1.5.1.1 Digestion of Dietary Biotin

As mentioned earlier humans are exposed to two sources of biotin, the first being the dietary source, and the second being the bacterial source in the large intestine (Wrong et al. 1981). Dietary biotin exists in the free and protein-bound forms with the ratio of the two in a given dietary source being dependent on the type of that source (Lampen et al. 1942). Ingested protein-bound forms of biotin are first broken down by gastrointestinal proteases and peptidases to biocytin (biotinyl-L-lysine; Fig. 1.2) and biotin-oligopeptides (Wolf et al. 1984). These products are then further processed in the intestinal lumen (i.e., prior to absorption) to release the free biotin (Fig. 1.2). The latter process is performed enzymetically by the action of biotinidase (EC 3.5.1.12). The source of the involved intestinal biotinidase is believed to be the pancreas (Wolf et al. 1984). Clinical and experimental evidence have demonstrated the importance of the hydrolysis step of biocytin and biotin-oligopeptides to free biotin for the efficient absorption and optimal bioavailability of dietary biotin (Wolf et al. 1984; Said et al. 1993). The liberated biotin is then absorbed in the small intestine.

1.5.1.2 Mechanism of Intestinal Biotin Uptake

The mechanism of biotin uptake by the small intestine has been the subject of intense investigations and has been thoroughly reviewed in recent years (Said 2004; Said et al. 2000; Said and Seetheram 2006). Using a variety of intestinal in vitro and in vivo preparations, it has been well established that the intestinal biotin uptake

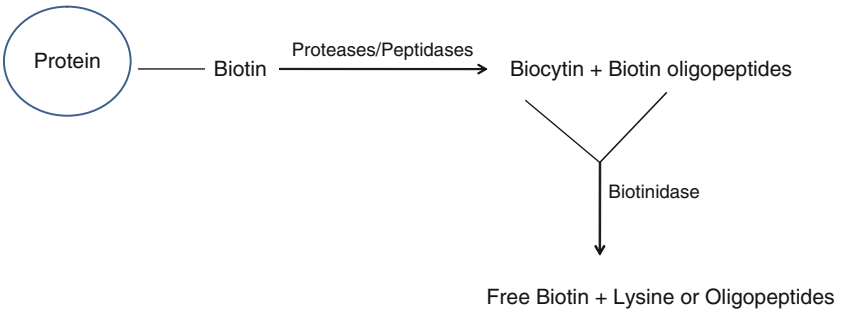

Fig. 1.2 Uptake of dietary biotin

process occurs via a Na+-dependent, carrier-mediated mechanism. This mechanism is inhibited by biotin structural analogues with a free carboxyl group at the valeric acid moiety of the biotin molecule (as in the case with desthiobiotin), but not by analogues with a blocked carboxyl group (as in the case of biocytin). Uptake of biotin in both human and animals is higher in the proximal compared to the distal part of the small intestine (Said et al. 1988; Said and Redha 1987). Functional and immunological studies have shown that the biotin Na+-dependent, carrier-mediated mechanism is expressed only at the apical membrane domain of the polarized enterocytes (Said et al. 1987, 1998; Said and Redha 1988b; Said and Derweesh 1991; Said 1991; Nabokina et al. 2003). This has been further confirmed by means of confocal imaging of living intestinal epithelial cells transfected with fluorescently tagged biotin transporter, SMVT (Fig. 1.4; Subramanian et al. 2006). The apical Na+-dependent biotin uptake system appears to be the rate-limiting step in the overall movement of biotin across the intestinal epithelial cells and is capable of transporting the substrate against a concentration gradient (Said et al. 1987; Said and Redha 1988a; Said and Derweesh 1991). The role of Na+ in biotin transport across the intestinal apical membrane domain is mediated via the inwardly directed Na+ gradient (which provides the needed energy for the transport of biotin against the concentration gradient) and not through the mere existence of Na+ in the incubation medium (Said et al. 1987) (Fig. 1.3). Internalized biotin then leaves the intestinal epithelial cells via the basolateral membrane by means of a Na+-independent, electrogenic, carrier-mediated mechanism (Said and Redha 1988c; Said 1991) (Fig. 1.3).

One of the interesting features of the Na+-dependent biotin uptake system is its ability to also transport two other functionally unrelated nutrients, namely pantothenic acid and lipoate (Fig. 1.1) (Said et al. 1998; Nabokina et al. 2003). Pantothenic acid is a member of the B family of water-soluble vitamins which is required for the biosynthesis of coenzyme A and acyl carrier proteins, and thus, it assumes important roles in carbohydrate, fat and protein metabolism. Lipoate is a potent intracellular and extracellular antioxidant in mammalian cells and is involved in the redox cycling of other antioxidants like vitamins C and E; it is also involved in regulating the intracellular level of glutathione. The ability of the intestinal biotin

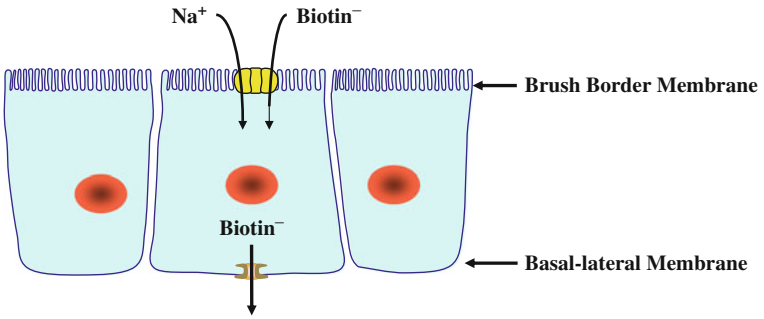

Fig. 1.3 Processing of dietary protein-bond biotin in the intestinal lumen

uptake system to also transport pantothenic acid and lipoate is not unique to the intestine but has also been observed in other cellular systems such as the brain, heart, placenta and kidney (Said 1991; Spector and Mock 1987; Beinlich et al. 1990; Grassl 1992). This ability increases the physiological and nutritional significance of the involved uptake system and was the basis for its naming as the sodium-dependent multivitamin transporter (SMVT).

With regards to the bacterially synthesized biotin, a substantial amount of this biotin exists in large intestinal lumen in the absorbable/unbound form (Wrong et al. 1981; Streit and Entcheva 2003). Also, in vivo studies in humans and animal models have shown that the large intestine is capable of absorbing luminal biotin (Barth et al. 1986; Brown and Rosenberg 1987; Sorrell et al. 1971). The mechanism involved in biotin uptake in the large intestine has also been the subject of investigation in recent years (Said et al. 1998). Using the human-derived colonic epithelial NCM460 cells as a model system for human colonocytes, studies have shown the existence of an efficient, Na+ -dependent, carrier-mediated mechanism for biotin uptake in these cells, which is again shared by pantothenic acid and lipoate (Said et al. 1998). These findings provided further evidence for the accessibility of the bacterially synthesized biotin into the human and its contribution toward the overall biotin pool, and especially that of the localized colonocytes.

1.5.1.3 Molecular Identity of the Intestinal Biotin Transport System

Cloning studies have determined the molecular identity of the intestinal SMVT of humans and of a number of animal models (Prasad et al. 1998; Wang et al. 1999; Chatterjee et al. 1999; and GenBank accession # AY572835). The human SMVT gene is located on chromosome 2p23 and consists of 17 exons (Wang et al. 1999). Significant sequence homology, at both the nucleotide and the amino acid levels, was found in the SMVT of the different mammalian species, and the polypeptide was predicted to have 12 trans-membrane domains with both of its ends (i.e., the N- and the C-terminal tails) oriented inwardly. The hSMVT polypeptide also appears to have a number of potential post-translational modification sites

including sites for phosphorylation by protein kinases and glycosylation sites. When expressed in heterologus systems, the cloned SMVT displayed Na+-dependency transport specificity for biotin, pantothenic acid and lipoate, and showed similar kinetic parameters to those observed for biotin uptake in the native intestine. The SMVT transcripts were shown to be expressed at a markedly higher level in the differentiated epithelial cells of the intestinal villi compared to those of the immature and undifferentiated cells of the crypt, a finding that corresponds with the higher level of biotin uptake in the former compared to the latter cells (Chatterjee et al. 1999). Distribution of the SMVT transcripts along the longitudinal axis of the intestine has also been delineated and shown to be similar in the different regions of the gut (Prasad et al. 1998; Wang et al. 1999; Chatterjee et al. 1999). This is in contrast to the observations of a higher biotin uptake in the proximal compared to the distal part of the small intestine, and the colon (Said et al. 1988; Said and Redha 1987). These findings suggest the possible involvement of specific post-translational modification(s) that may regulate biotin transport activity in the different regions of the intestinal tract. Cellular localization of the SMVT protein in human intestinal epithelial cells have also been examined using confocal imaging approach with results showing exclusive expression of the protein the apical membrane of polarized cells (Fig. 1.4; Unpublished observation from one laboratory). Other studies, have reported significant heterogeneity in the 5′ un-translated region of the rat SMVT, with four distinct variants (I, II, III, IV) being identified (Chatterjee et al.

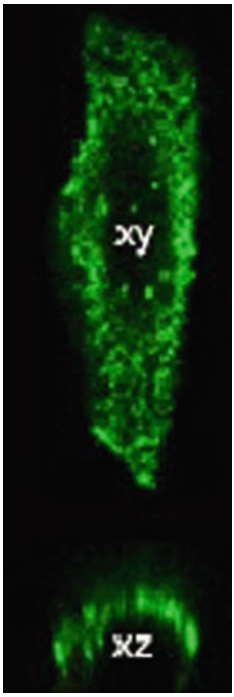

Fig. 1.4 Confocal imaging of live intestinal epithelial Caco-2 cells grown on filter showing exclusive expression of the human SMVT at the apical membrane domain. The XZ image shows exclusive expression of the human SMVT at the apical membrane domain of the polarized Caco-2 cells

1999); variant II was shown to be the predominant variant expressed in the intestinal tract (Chatterjee et al. 1999). A major role for the human SMVT system in intestinal carrier-mediated biotin uptake has been recently established with the use of a SMVT gene-specific siRNA approach (Balamurugan et al. 2003).

1.5.1.4 Regulation of the Intestinal Biotin Absorption Process

The intestinal biotin uptake process is regulated by a number of intracellular and extracellular factors. A role for intracellular protein kinase-C (PKC)- and Ca^{2+}/calmodulin-mediated pathways in the regulation of the intestinal biotin uptake process has been reported (Said 1999; Said et al. 1998). While each of these intracellular regulatory pathways was found to act via altering the activity (but not the affinity) of the SMVT system, they appeared to do so via different mechanisms (Said 1999; Said et al. 1998).

Extracellular biotin levels exert adaptive regulatory effects on the intestinal biotin uptake process in both humans and animal models (Reidling and Said 2006; Said et al. 1989a). Biotin deficiency leads to a specific and significant up-regulation in intestinal carrier-mediated biotin uptake, while biotin over-supplementation appears to have the opposite effect (ref). The up-regulation in intestinal biotin uptake observed in biotin deficiency occurs in association with a parallel increase in the level of SMVT protein and mRNA in intestinal epithelial cells with no changes in mRNA stability (Reidling and Said 2006). These findings suggest the involvement of transcriptional regulatory mechanism(s) in the observed adaptive regulatory effects in the intestinal biotin uptake process in biotin deficiency.

The intestinal biotin uptake process also undergoes developmental regulation via changes in the preferential site of biotin absorption and via changes in the kinetic parameters of the uptake process (Nabokina et al. 2003; Said and Redha 1988b). The latter changes involve the entry step of biotin across the apical membrane domain of the polarized intestinal epithelial cells and occurs in association with parallel changes in the level of SMVT protein and mRNA, as well as the transcription rate of the SMVT gene (Nabokina et al. 2003).

Recent studies using the human-derived intestinal epithelial Caco-2 cells as a model have also shown that the intestinal biotin uptake process is under differentiation-dependent regulation. Biotin uptake and the level of expression of hSMVT mRNA and protein as well as activity of the hSMVT promoter were found to be higher in post-confluent (differentiated) Caco-2 cells compared to pre-confluent (undifferentiated) cells (Reidling J and Said HM; unpublished observations). These findings clearly point to the possible involvement of transcriptional regulatory mechanism(s) in the differentiation-dependent regulation of the biotin uptake process.

Insight into the transcriptional regulation of the SMVT gene under basal and regulated conditions has also been forthcoming following the cloning and the characterization of the 5′-regulatory region of the human and rat SMVT genes (Chatterjee et al. 2001; Dey et al. 2002). In the case of the rat SMVT gene, three distinct promoters were identified (Chatterjee et al. 2001), while two promoters were

identified in the case of the human SMVT gene (Dey et al. 2002). In both cases activities of the cloned promoters were demonstrated by fusing the promoter fragments with the Firefly luciferase reporter gene followed by expression of the constructs in the appropriate cellular systems. Human promoter I was found to be more active than promoter II and required functional GKLF and AP-2 cis-regulatory elements for its activity in intestinal epithelial cells (Reidling and Said 2006). Activity of the cloned human SMVT promoter has also been confirmed in vivo in transgenic mice (Reidling and Said 2006).

1.5.1.5 Effect of Anti-Epileptic Drugs and Alcohol on Intestinal Biotin Uptake

As mentioned earlier, long-term use of anticonvulsant drugs leads to impairment in normal biotin status (Krause et al. 1982a, b). While the mechanism(s) involved in causing this abnormality is not fully clear, competitive inhibition of intestinal biotin uptake by these agents (Said et al. 1989b; Prasad and Ganapathy 2000) as well as accelerated biotin catabolism (Mock et al. 1997, 1998) and impairment renal reclamation of the vitamin (Chauhan and Dakshinamurti 1988) have all been reported. Similarly, the reduced blood biotin levels observed in alcoholics (Fennelly et al. 1969; Bonjour 1980) is believed to be, at least in part mediated via impairment in intestinal uptake of biotin (Said et al. 1990b).

1.5.2 Renal Uptake of Biotin

Circulating biotin undergoes filtration in the renal glomeruli. The vitamin is then salvaged via reabsorption by renal proximal tubular epithelial cells. Studies on the mechanism of renal biotin uptake have shown the involvement of a concentrative Na-dependent, carrier-mediated mechanism localized at the apical membrane domain of the polarized renal epithelial cells (Balamurugan et al. 2005; Baur and Baumgartner 1993; Baur et al. 1990; Podevin and Barbarat 1986). The system involved in renal biotin uptake is also shared by pantothenic acid and lipoate (Balamurugan et al. 2005) and is inhibited by biotin structural analogues with a free carboxyl group in the valeric acid moiety (like desthiobiotin), but not by analogues with a blocked carboxyl group (e.g., biocyin and biotin sulfoxide) (Balamurugan et al. 2005; Baur and Baumgartner 1993; Baur et al. 1990; Podevin and Barbarat 1986). Exit of biotin from the renal epithelial cells occurs via a carrier-mediated mechanism that is Na-independent and electrogenic in nature (Podevin and Barbarat 1986).

Regulation of the renal biotin uptake process has also been examined using the human-derived renal proximal epithelial HK-2 cells as in vitro model to human renal epithelial cells (Balamurugan et al. 2005). The results showed the process to be under the regulation of intracellular PKC- and Ca^{2+}/calmodulin (CaM)-mediated pathways. The renal biotin uptake process is also adaptively regulated by extracellular biotin levels via a mechanism that appears to be transcriptionally mediated (Balamurugan et al. 2005). Other studies have shown that native human renal

epithelial cells and the culture renal epithelial HK-2 cells to both express hSMVT at the protein and the mRNA levels (Balamurugan et al. 2005; Chatterjee et al. 1999). This hSMVT appears to be the main (if not the only) carrier-mediated mechanism for biotin uptake by renal epithelial cells as shown in studies utilizing gene silencing approach with gene-specific siRNA (Balamurugan et al. 2005).

An interesting finding with the renal biotin uptake process when compared to the intestinal uptake process is the finding that the former process does not appear to undergo developmental regulation (Nabokina et al. 2003). This conclusion is based on the observations that biotin uptake as well as the levels of the SMVT protein and mRNA is similar in suckling and adult rat kidney epithelial cells (Nabokina et al. 2003).

1.5.3 Biotin Uptake by the Liver

The liver plays an important role in normal biotin physiology and represents the major organ for biotin metabolism and utilization. While the liver contains the highest amount of biotin compared to other tissues, its capacity to store the vitamin is limited compared to other water-soluble vitamins like cobalamin, folate and riboflavin (Danford and Munro 1982). The liver extracts (from the portal circulation) a major portion of the newly absorbed biotin (Brown and Rosenberg 1987), and evidence exists that recycled biotin (i.e., the biotin that is generated as a result of degradation of holocarboxylase) is generated not in cells but in the extracellular compartment (Dakshinamurti and Chauhan 1988; Freytag and Utter 1983; Heard et al. 1985). Thus, the liver relies heavily on circulating and extracellular biotin for its needs, and on its transport across the hepatocyte basolateral membrane. Using isolated and cultured hepatocytes as well as purified liver basolateral membrane vesicle preparations of human and rat origin (Komro and McCormick 1985; Said et al. 1990a, 1992b, 1994), studies have shown the involvement of a concentrative, Na+-dependent carrier-mediated mechanism. This mechanism is sensitive to the effect of sulfhydryl group inhibitors and is inhibited by biotin structural analogues like desthiobiotin but not by biocytin. Human liver and the human-derived liver HepG2 cells both express the hSMVT at the protein and mRNA level (Balamurugan et al. 2003), and gene silencing studies with siRNA have shown that this system is the main biotin uptake system that operates in human hepatocytes (Balamurugan et al. 2003).

1.5.4 Biotin Transport Across the Blood Brain Barrier and the Placenta

Biotin transport across the blood brain barrier is carrier-mediated and is inhibited by probenecid and pantothenic acid but it is not affected by biocytin. This has been shown in vivo in the rat (Spector and Mock 1987) and in vitro using cultured

calf brain microvessel endothelial cells (Baur and Baumgartner 2000). The recent description of patients with a novel biotin-responsive basal ganglia disease (Ozand et al. 1998; Zeng et al. 2005), and a patient with sudden onset biotin-responsive coma (Mardach et al. 2002), underscore the importance of biotin for brain function.

The concentration of biotin in the plasma of human fetuses is many folds higher than that in the plasma of the mother, clearly suggesting the involvement of an efficient transport system that transports biotin in the direction of the fetus. The existence of such a system has indeed been demonstrated using different placental preparations (Karl and Fisher 1992; Schenker et al. 1993; Hu et al. 1994). This system transports biotin by a Na-dependent, carrier-mediated mechanism and the system is again shared with pantothenic acid and lipoate. These are characteristics of SMVT, which is expressed at a high level in the human placenta (Balamurugan et al. 2003).

1.5.5 Intracellular Transport of Biotin: Transport into the Mitochondria

Studies have shown that free biotin can be transported into the mitochondria for utilization in biotinylation reactions of apo carboxylases (Ahmad and Ahmad 1991). The mechanism involved in this transport has been studied using isolated liver mitochondria and shown to occur by an acid pH-dependent, non-mediated simple diffusion process (Said et al. 1992). It is believed that biotin enters the intramitochondrial space in the protonated (neutral) form, dissociates into its anionic form (pKa = 4.5) at the alkaline pH of the mitochondria, and becomes trapped within (Said et al. 1992).

1.6 Disorders of Biotin Metabolism and Physiology

As mentioned earlier, biotin in humans acts as a coenzyme to four carboxylases, and that these enzymes exist in the inactive apo forms which are converted to the holoactive forms by the action of HCLS by means of biotinylation (McMahon 2002; Mock 2004; Sweetman and Nyhan 1986; Dakshinamurti and Chauhan 1988). At the end of their functional life, the holocarboxylases undergo proteolytic degradation (in the lysosomes) that lead to the generation of biocytin and biotin-short peptides. Free biotin is then released from biocytin and biotin-oligopeptides via the action of biotinidase. The freed biotin is then reutilized by cells, i.e., recycled, in what is sometimes referred to as the "biotin cycle."

There are two major genetic disorders in biotin metabolism in humans, both of which lead to multiple carboxylase deficiency (reviewed in McMahon 2002; Mock 2004; Sweetman and Nyhan 1986). The first metabolic disorder leads to a defect in the process of biotinylation due to deficiency in HCLS. The second metabolic disorder leads to a defect in biotin recycling (release of free biotin from its

conjugated forms) due to deficiency in biotinidase. These two genetic disorders have also been classified according to the age at which their symptoms appear and are called early-onset (neonatal) multicarboxylase deficiency, and late-onset (juvenile) multiplecarboxylase deficiency syndromes, respectively.

HCLS deficiency is an autosomal recessive disorder. Patients with this disorder exhibit hypotonia, seizures, difficulties in breathing and in feeding, skin rash, and alopecia; in extreme conditions, patients may also exhibit developmental delay and coma. Metabolically, children affected by this disorder exhibit metabolic acidosis, organics aciduria and hyperammonemia. Multiple mutations in the HLCS gene have been identified and shown to lead to the generation of an enzyme with an impaired activity mainly due to decreased affinity. This condition can be diagnosed prenatally by determining the level of the relevant organic acids in the amniotic fluid, activity levels of the mitochondrial caboxylases in amniocytes, and by means of mutational analysis. This disorder can be treated with oral administration of pharmacological doses of biotin (10 mg/day), with most of the affected children demonstrating significant improvement.

Biotinidase deficiency is also an autosomal recessive disorder. The clinical symptoms associated with this disorder vary and include hypotonia, seizures, difficulty in breathing, ataxia, visual and hearing disturbances, skin rash, alopecia, immunological disturbances, and developmental delay. Metabolically, most of the affected children exhibit metabolic acidosis and organic aciduria. Multiple mutations in the biotinidase gene have been identified, which leads to the generation of an enzyme that is severely or partially incapable of recycling/re-utilization endogenous conjugated biotin; it is also incapable of releasing free biotin from its conjugated forms in the ingested food. Biotinidase deficiency can be diagnosed by measuring the biotinidase activity in the serum. Prenatally, the condition can also be diagnosed by means of mutational analysis. When diagnosed early all affected children can be successfully treated with oral administration of pharmacological doses of free biotin (5–20 mg).

Another inherited disorder related to biotin is the recently described biotin-responsive basal ganglia disease (BRGD). BBGD is a recessive disorder with childhood onset that exhibit subacute encephalopathy, confusion, dysarthria, disphagia with occasional external opthalmopelgia or supranuclear facial nerve palsy (Ozand et al. 1998; Zeng et al. 2005). The condition could progress to severe cogwheel rigidity, dystonia and quadriparesis, and even death if untreated. Patients with this disorder respond well to pharmacological doses of biotin (5–10 mg/kg/day), but the symptoms re-appear within a month if biotin supplementation is discontinued (Ozand et al. 1998; Zeng et al. 2005). Recent studies have shown that the cause of BRDG is mutations in the SLC19A3 gene, a member of the solute transporter family of genes (Zeng et al. 2005). More recent studies, however, have shown that the product of the SLC19A3 is not a biotin transporter but rather a specific membrane transporter for the water-soluble vitamin thiamin (Subramanian et al. 2006). Thus, it is still unclear at this stage how a disease like BRGD with mutations in a thiamin transporter responds to biotin over-supplementation, and further studies are needed to understand the connection(s).

A biotin-dependent sudden onset coma in an 18 months old child has also been recently described and is believed to be due to a defect in a biotin transport other than SMVT (Mardach et al. 2002). The patient in this case did not have holocarboxylase synthetase or biotinidase deficiencies, nor did the child appear to have nutritional biotin deficiency or accelerated biotin catabolism. The actual cause behind this case is under further investigation.

1.7 Concluding Remarks

Significant progress has occurred in recent years in our understanding of the physiology, biochemistry and nutritional roles of biotin. Despite that, there is a clear need for better understanding of the effects of suboptimal (marginal) biotin status on cellular metabolism and on human health and well-being. Additional effort is also needed for better appreciation of the degree of occurrence of suboptimal biotin status in the general population. There is also a need for better understanding of the nature of the molecular and biochemical defects in children who respond to biotin over-supplementation yet they are neither biotinidase nor holocarboxylase deficient. Finally a better understanding of the biotin physiology at the integrated whole animal level in vivo is required.

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Chapter 2
Niacin Status and Genomic Instability in Bone Marrow Cells; Mechanisms Favoring the Progression of Leukemogenesis

James B. Kirkland

Abstract Niacin deficiency causes dramatic genomic instability in bone marrow cells in an in vivo rat model. The end result is seen in the increased incidence of sister chromatid exchanges, micronuclei, chromosomal aberrations and the eventual development of nitrosourea-induced leukemias. From a mechanistic perspective, niacin deficiency delays excision repair and causes double strand break accumulation, which in turn favor chromosome breaks and translocations. Niacin deficiency also impairs cell cycle arrest and apoptosis in response to DNA damage, which combine to encourage the survival of cells with leukemogenic potential. Niacin deficiency also enhances the level of oxidant damage found in cellular proteins and DNA, but not through depression of GSH levels. Pharmacological supplementation of niacin decreases the development of nitrosourea-induced leukemias, while short term effects of high niacin intake include a large increase in cellular NAD+ and poly(ADP-ribose) content and enhanced apoptosis. These results are important to cancer patients, which tend to be niacin deficient, are exposed to large doses of genotoxic drugs, and suffer short-term bone marrow suppression and long-term development of secondary leukemias. The data from our rat model suggest that niacin supplementation of cancer patients may decrease the severity of short and long-term side effects, and may also improve tumor cell killing through activation of poly(ADP-ribose)-dependent apoptosis pathways.

Keywords Niacin · NAD+ · Poly(ADP-ribose) · Genomic instability · Leukemogenesis

2.1 Niacin and Genomic Stability, Underlying Mechanisms

The human disease of niacin deficiency, pellagra, is characterized by diarrhea, dementia and sun-sensitive dermatitis, the last of which is suggestive of problems with DNA repair (Kirkland 2007). In the last few years there has been an explosion

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of knowledge in the area of niacin and its role in DNA function (Kirkland 2003, 2007; Kirkland et al. 2007). Niacin has been viewed traditionally as a central player in catabolic and synthetic pathways through a multitude of redox reactions based on NAD and NADP cofactors. A new appreciation of niacin function in DNA stability has come with the knowledge that NAD+ participates in a wide variety of ADP-ribosylation reactions. While redox reactions cycle pyridine nucleotides between oxidized and reduced forms, ADP-ribosylation reactions cleave NAD+, releasing nicotinamide and using the ADP-ribose moiety in a variety of interesting reactions. These reactions can be roughly divided into four categories, including; poly (ADPribose) synthesis, mono ADP-ribosylation, cyclic ADP-ribose formation and NAD-dependent deacetylation reactions (sirtuins).

2.1.1 Poly(ADP-Ribose) Polymerases

In the last few years rapid progress has been made in the study of poly(ADP-ribose) metabolism (Burkle 2006; Gagne et al. 2006; Schreiber et al. 2006). It is now recognized that 17 genes in humans have the potential to produce enzymes that synthesize poly(ADP-ribose), and catalytic activity has been proven in eight of these (Schreiber et al. 2006). PARP-1 has been recognized for several decades, and makes the majority of cellular poly(ADP-ribose). It is a nuclear protein that binds to and is catalytically activated by DNA strand breaks, creating one of the earliest metabolic signals at a site of DNA damage (Huber et al. 2004). PARP-2 has functional similarities to PARP-1; animals with either gene deleted experience genomic instability while deletion of both genes is embryonic lethal (Menissier et al. 2003). PARP-3 associates with the daughter centriole during cell division (Schreiber et al. 2006). Tankyrases one and two synthesize poly(ADP-ribose) at the telomere, opening the structure and allowing telomerase to maintain the length and stability of the chromosome terminus (Sbodio et al. 2002). vPARP is associated with vault particles and may play a role in multidrug resistance (Kickhoefer et al. 1999). PARP-10 interacts with c-Myc and inhibits cell transformation (Yu et al. 2005). tiPARP is induced by dioxin, with uncertain effects on metabolism (Ma et al. 2001).

While various individual functions have been described, it is also clear that PARP genes function together in the maintenance of genomic stability. PARP-1 and 2 have clear interactive roles in base excision repair of DNA, and likely function in other forms of DNA repair (Huber et al. 2004). PARP-1 and 3, tankyrase-1 and vPARP localize to centrosomes and mitotic spindle apparatus, while PARP-1 and 2 localize to centromeres, demonstrating an integrated role of poly(ADPribose) metabolism in chromosome segregation (Schreiber et al. 2006).

There is an extensive literature describing the complex mechanisms by which PARP enzymes accomplish these varied tasks (Burkle 2006; Gagne et al. 2006; Schreiber et al. 2006), and these will be described only briefly below:

(i) Poly(ADP-ribose) is negatively charged, as is DNA, and these two molecules repel one another. When histones are poly(ADP-ribosyl)ated they lose their

affinity for DNA and this leads to a local chromatin relaxation. PARP-1 and 2 become automodified, and this eventually pushes them away from the site of damage, allowing repair to finish. Also, clouds of poly(ADPribose) around sites of DNA damage likely repel other free ends of DNA and prevent nonhomologous translocation events.

(ii) Proteins have specific high-affinity binding sites for poly(ADP-ribose), which draws them to sites of DNA damage, where they may participate in repair reactions or initiate signaling pathways for inflammatory or apoptotic responses (Malanga and Althaus 2005). This also contributes to local chromosome relaxation, as histones have high affinity sites, and are pulled out of chromatin onto the polymer bound to automodified PARP-1/2. Other proteins involved in DNA repair, cell cycle arrest, apoptosis and inflammation (topoisomerases, DNAPK, NF-kB, XRCC1, p53) are drawn to the site of injury through high affinity poly(ADP-ribose) binding (Malanga and Althaus 2005).

(iii) Covalent poly(ADP-ribosyl)ation of enzymes, transcription factors and signaling molecules can increase or decrease their activities, controlling the complex stress response of the cell, directing repair activities and survival decisions. Acceptor proteins for poly(ADP-ribose) addition include DNA topoisomerase, polymerase and ligase enzymes (D'Amours et al. 1999).

2.1.2 Sirtuins

There has been great interest in the sirtuin gene family since the finding that Sir2 (SirT in humans) is central to the extension of lifespan in response to caloric restriction, and that lifespan extension can be mimicked by the sirtuin activator resveratrol (Kruszewski and Szumiel 2005). Of interest, Sir2 is an NAD-dependent deacetylase, which removes acetyl groups from key proteins like histones and p53, leading to changes in chromatin structure and the cellular stress response. The deacetylation is an ADP-ribosylation reaction, producing acetyl-ADP-ribose as an end product. The sirtuins provide a mechanistic connection between cellular energy status, regulation of DNA structure and metabolism, and genomic stability and lifespan. Sir2 and PARP-1/2 may have close interactions, based on competition for NAD+ and inhibition of Sir2 by nicotinamide. It has been suggested that cell survival is dependent on the balance of PARP versus sirtuin activity, with PARP directing apoptotic signals and sirtuins promoting survival (Kruszewski and Szumiel 2005). It is clear that PARP and sirtuin enzymes play strong integrated roles in the maintenance of genomic stability in eukaryotes.

2.1.3 Mono ADP-Ribosyltransferases and ADP-Ribosyl cyclases

While PARP enzymes and sirtuins provide the most plausible links between niacin status and genomic stability, there are also potential effects of mono ADP-ribosylation reactions and cyclic ADP-ribose metabolism. Mono ADP-ribosylation

is represents the post-translational modification of cellular proteins with a single ADP-ribose unit derived from NAD+. There are many different transferases and protein substrates involved, and diverse processes are controlled, from immune response, to cell adhesion and G-protein signaling (Di Girolamo et al. 2005). Cyclic ADP-ribose causes release of intracellular calcium stores, controlling functions in all cell types, from nerve conductance, to muscle contraction to insulin secretion (Guse 2005). There is potential for alterations in mono ADP-ribosylation and cyclic ADP-ribose metabolism to impact on genomic stability, though control of cell cycle and other signaling pathways. For example, the nuclear envelope contains calcium stores that are released by cyclic ADP-ribose, and another niacin based molecule, nicotinic acid adenine dinucleotide phosphate, exerting control over nuclear processes such as DNA replication and cell cycle checkpoints (Gerasimenko and Gerasimenko 2004). Basic redox metabolism could also be altered by niacin deficiency leading to genomic instability. One example of this would be the maintenance of reduced glutathione (GSH) levels using NADPH as a substrate.

2.2 Niacin Deficiency in a Whole Animal Model

Early experiments on NAD and genotoxic stress made use of cell culture models, which allowed for decreases in cellular NAD+ in excess of 90% (Durkacz et al. 1980; Jacobson et al. 1992). Of interest, normal cell growth and division were maintained in these models until DNA-damaging agents were added, suggesting that essential redox functions are relatively protected during niacin depletion. Competition for limiting NAD+ pools will occur at the subcellular level, but in whole animal models there will also be competition between tissues. The specific reactions that lose this tissue and subcellular competition for NAD+ will determine the pathologies that arise as the deficiency progresses. The end result may differ depending on the age, sex and species of animal model, and depending on the external stresses that are applied during deficiency. This was observed historically in human populations, where working class pellagrins would tend to present with sun-sensitive dermatitis, while wealthier, less sun-exposed patients often presented with dementia, and the full triad of dermatitis, dementia and diarrhea was observed less frequently (Kirkland 2007).

In our early work on niacin deficiency in rats, we found that liver and lung tissues have very high basal levels of NAD+, are relatively resistant to niacin depletion, and are not sensitized to genotoxic damage during niacin deficiency (Rawling et al. 1994, 1995, 1996). Noting that the symptoms of pellagra tend to appear in highly proliferative tissues like the skin and intestinal tract, we began to study bone marrow tissue. We have found that bone marrow is the most sensitive tissue that we have characterized in our model, with decreases in NAD+ of 80%, an almost complete loss of basal poly(ADP-ribose) and a large inhibition of nitrosourea-induced poly(ADP-ribose) formation (Fig. 2.1) (Boyonoski et al. 2002a). Bone marrow poly(ADP-ribose) is also very sensitive to high niacin intake,

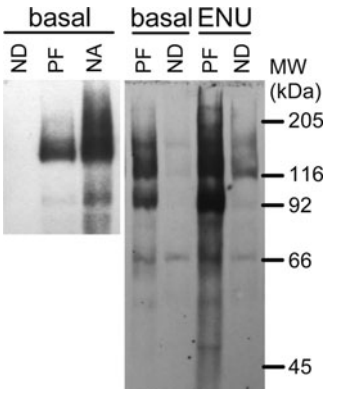

Fig. 2.1 The effect of niacin status on bone marrow poly(ADP-ribose) levels. ND, niacin deficient, 0 mg added dietary niacin; PF, pair-fed control, control diet with 30 mg nicotinic acid/kg diet, fed in identical quantities to ND; NA, high niacin diet, 4,000 mg nicotinic acid/kg diet, also pair-fed to ND. Ethylnitrosourea (ENU) is used to induce DNA damage. Cellular proteins are run using SDS PAGE, transferred and blotted with anti-poly(ADP-ribose) polyclonal Ab 96–10 (Dr. Guy Poirier)

as basal and damage induced poly(ADP-ribose) levels are much higher when rats are fed 4,000 mg nicotinic acid/kg diet compared to the 30 mg/kg diet found in the control diet (Fig. 2.1) (Boyonoski et al. 2002a). In total, bone marrow NAD+ and poly(ADP-ribose) concentrations vary 30- and 65-fold, respectively, going from niacin deficient to pharmacologically supplemented status (Boyonoski et al. 2002a). The majority of polymer appears to be bound to the 116 kDa PARP-1 and its 97 kDa apoptotic fragment (Boyonoski et al. 2002a).

The next question to be addressed was whether the diet-induced changes in NAD+ and poly(ADP-ribose) where associated with pathological endpoints associated with genomic instability. From a human clinical perspective, bone marrow progenitors experience the critical side effects of most types of cancer chemotherapy, leading to dose-limiting bone marrow suppression in the short term, and the development of secondary leukemias over time (Terada 2006). We set out to model the stress of chemotherapy on the bone marrow by treating Long-Evans rats with 12 doses of ethylnitrosourea (ENU), spread over a Langley et al. (2002) day period. Long-Evans rats tend to develop non-lymphocytic leukemias in response to alkylating agents (Shisa and Hiai 1985), similar to human cancer patients (Tucker 1993). Rats that were niacin deficient during ENU treatment displayed more severe short term bone marrow suppression (Boyonoski et al. 2000). We also collected long-term cancer data from these cohorts (Boyonoski et al. 2002a, b), but the results were compromised by excessively rapid appearance of cancers and differences in the basal diets between deficient and pharmacological models. We have recently repeated this experiment using 6 doses of ENU and have found that niacin status has a significant impact on nitrosourea-induced leukemogenesis (Fig. 2.2) (Bartleman et al. 2008). While non-leukemia cancers were not affected by diet, the incidence

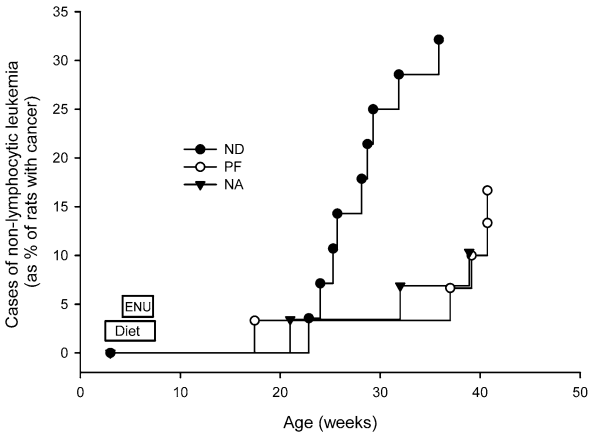

Fig. 2.2 The effect of niacin status during exposure to ENU on the long term development of non-lymphocytic leukemias in Long-Evans rats. ND, niacin deficient, 0 mg added dietary niacin; PF, pair-fed control, control diet with 30 mg nicotinic acid/kg diet, fed in identical quantities to ND; NA, high niacin diet, 4,000 mg nicotinic acid/kg diet, also pair-fed to ND. Ethylnitrosourea (ENU) is used to induce DNA damage. Rats consumed experimental diets and were exposed to ENU between 3 and 7 weeks of age, after which all three groups consumed control diet (AIN93M) and were observed for development of cancer

of non-lymphocytic leukemias was very sensitive to niacin status. While there were not any significant survival differences between the adequate and pharmacological niacin groups, the pharmacological niacin intake group did provide the greatest degree of protection, relative to niacin deficient rats, in the incidence of total cancers, total leukemias and non-lymphocytic leukemias (Bartleman et al. 2008).

Given the close connection between PARP-1/2 function and base excision repair (BER) (Malanga and Althaus 2005), we sought to characterize the impact of niacin deficiency on the repair of alkylation injury in our model of niacin and leukemogenesis. ENU was an ideal compound to initiate the damage response since it decomposes rapidly and spontaneously to produce reactive ethyl ions that damage DNA in an even pattern across tissues, independently of phase I or II xenobiotic metabolism. These small ethyl adducts are then mainly resolved by BER. DNA excision repair pathways involve incision, excision, repair synthesis and ligation (Seeberg et al. 1995). During base excision repair (BER), the removal of altered bases is initiated by DNA glycosylases, creating an abasic (AB) site, but not a strand break. The phosphate group 5′ to the AB site is incised by endonuclease, or the 3′ site is cleaved by lyase. This creates the strand break that is recognized by PARP-1 and 2, initiating the synthesis of poly(ADP-ribose), leading to changes in local chromatin structure, and the protein:protein and protein:poly(ADP-ribose) interactions that help to organize the repair process. The deoxyribose phosphate is excised by phophodiesterase, and DNA polymerases fill the resulting gap. The final nick is sealed by DNA ligase. At some point, the synthesis of poly(ADP-ribose) on PARP enzymes becomes extensive enough that anionic repulsion allows it to

leave the strand break to allow completion of repair. The ability of catalytically inactive PARP-1 to block excision repair has been demonstrated through NAD+ removal (Durkacz et al. 1980; Satoh et al. 1993), competitive inhibitor treatment and mutagenesis of the catalytic domain (D'Amours et al. 1999). We examined repair kinetics using the alkaline comet assay during the 36 h following a single dose of ethylnitrosourea (ENU) (30 mg/kg bw). The comet assay uses carefully isolated bone marrow cells, which are suspended in soft agar and incubated at alkaline pH. The slides are then electrophoresed, causing loose strands of DNA to migrate out of the nucleus, forming a comet-like image. The current thinking is that single strand breaks cause a loss of supercoiling between sites of attachment to the nuclear matrix, allowing loops of DNA to migrate with the electrical current. The alkaline assay is fully sensitive to existing single strand breaks, but is also partially sensitive to AB sites and some other DNA lesions. The comet images are analyzed by computer, generating a proportionate index of tail versus nuclear DNA, referred to as mean tail moment (MTM). In our time course, there was no effect of ND on MTM before ENU treatment, or on the development of strand breaks between 0 and 8 h after ENU (Fig. 2.3) (Kostecki et al. 2007). This indicates that the ENU is causing similar levels of DNA alkylation, and that subsequent differences are due to changes in DNA repair processes. The peaks at 4 and 9 h likely represent formation of AB sites and strand breaks, respectively, through the action of glycosylases

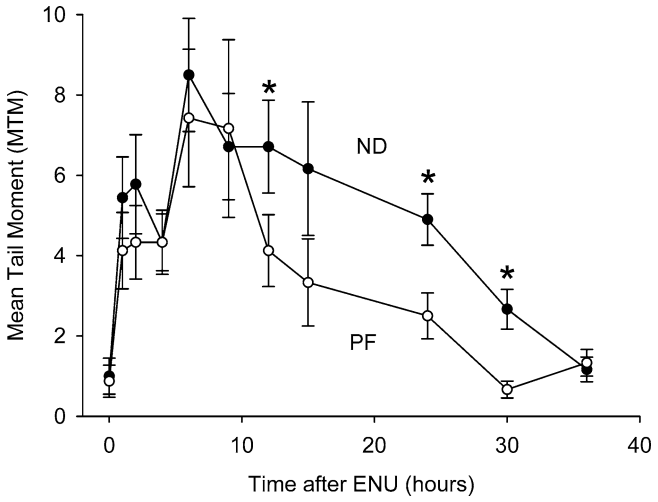

Fig. 2.3 The effect of niacin deficiency and ethylnitrosourea treatment on DNA strand breaks in bone marrow cells. Weanling Long-Evans rats were fed niacin deficient (ND) or pair fed niacin replete control diet (PF) for 3 weeks. Rats were gavaged with ethylnitrosourea (ENU, 30 mg/kg bw in pH 4 water) at 10:00 am. Time 0 rats were gavaged with pH 4 water and killed at the 3 h time point, to assess the influence of vehicle and procedures. ENU treated ND and PF rats were killed at the times indicated, and the bone marrow cells from the femurs were blindly analyzed by the alkaline comet assay. $n = 6$–14. *ND significantly greater than PF ($p < 0.05$, t-test)

and endonucleases. These processes are not affected by niacin deficiency. In contrast, repair kinetics between 12 and 30 h were significantly delayed by ND, with a doubling of area under the MTM curve during this period (Fig. 2.3).

It is logical to look at the lack of basal and damage-induced poly(ADP-ribose) synthesis in Fig. 2.1 and postulate that the observed delay in repair kinetics represents the effect of PARP-1 and/or 2 binding to strand breaks and blocking the completion of the repair process due to the lack of automodification. This mechanism has been demonstrated in many in vitro systems using NAD deprivation, competitive inhibitors or catalytically inactive mutants to produce dominant-negative forms of PARP-1 that block the later stages of BER (D'Amours et al. 1999). This may well be occurring in our in vivo model, but PARP enzymes play other roles in the BER process that should be considered. The local chromatin relaxation induced by poly(ADP-ribose) formation around strand breaks is likely decreased, and this may delay the completion of repair. PARP-1/2 activity draws XRCC1 to DNA strand breaks, where it acts as a scaffold for the repair machinery (Malanga and Althaus 2005). PARP-1 also attaches poly(ADP-ribose) covalently to DNA protein kinase, topoisomerases, polymerases and ligase (D'Amours et al. 1999), and these may all suffer functionally during niacin deficiency, leading to delayed excision repair.

O6-alkylguanine is a lesion that is not recognized by the glycosylases of BER. In recognition of its significant mutagenicity, there is a specific protein, O6-alkylguanine-DNAalkyltransferase (AGT), which is devoted to detecting and removing these lesions. AGT transfers the alkyl group to itself and is then degraded, at a metabolic cost of about 850 ATP for each lesion that is repaired. During niacin deficiency the removal of O6-ethylguanine is delayed, presumably by a lower availability of AGT, which may be due to a small drop in mRNA levels (Kostecki et al. 2007). There is no obvious reason for a drop in expression, although decreased ADPribosylation activity may be impairing the damage-induced signaling pathways that control AGT expression. Of interest, resveratrol has been shown to induce MGMT activity (Niture et al. 2007), and it is also known as an activator Sir2 catalytic activity (Wood et al. 2004), demonstrating one possible link between NAD+ pools and AGT expression. If O6-ethlyguanine persists through DNA replication, it may cause point mutations or it may be recognized by the mismatch repair system (MMS), which removes a long patch of the newly synthesized strand, and replaces it, without resolving the lesion. This continuing futile cycle of repair leads to double strand breaks which lead to chromosome breaks, translocations and other aberrations (Margison et al. 2002).

Chromosome breaks and translocations are key players in carcinogenesis, and they are dependent on the formation of double strand breaks in DNA. However, these lesions are increased by many forms of DNA damage which are known to cause single strand breaks, either directly (some forms of radiation) or indirectly through pathways like BER. In this model of simple alkylation damage, double strand breaks could form by several mechanisms, including multiple nearby single strand breaks, stalled replication forks (Cox et al. 2000), futile cycles of mismatch repair (as described above) and the blockage of strand break repair by catalytically-inactive PARP-1 or 2. Double strand breaks can be measured using the neutral comet

assay. Neutral MTM was nearly doubled in ND bone marrow cells Langley et al. (2002) hours following ENU treatment, indicating an accumulation of double strand breaks (Kostecki et al. 2007). PARP-1 has been shown to be required for efficient resolution of stalled replication forks (Yang et al. 2004). We have also shown that niacin deficiency impairs damage-induced cell cycle arrest in our model (Spronck et al. 2007). Poorly resolved replication forks proceeding into DNA replication are likely to produce double strand breaks (Cox et al. 2000). Double strand breaks may also accumulate due to a lack of repair, and PARP enzymes have been sown to participate in various forms of double strand break repair (Audebert et al. 2004; Frosina et al. 1996).

The traditional analysis of metaphase spreads for chromosome aberrations should reveal differences if there are more double strand breaks in niacin deficient bone marrow cells, and this is what we have observed (Kostecki et al. 2007). Figure 2.4a shows examples of metaphase spreads from normal and niacin deficient bone marrow cells. Figure 2.4b shows the results of blinded analysis of metaphase arrests for chromosome breaks, which are breaks that appear in both sister chromatids. There was a dramatic (4-fold) increase in chromosome breaks caused by niacin deficiency alone. Niacin deficiency enhanced the actual number of additional breaks caused by ENU, although not the proportion.

Other short-term biomarkers of genomic instability are also increased in this model. We found that sister chromatid exchanges and micronuclei were increased by 3- and 6-fold, respectively, by niacin deficiency alone (Spronck and Kirkland

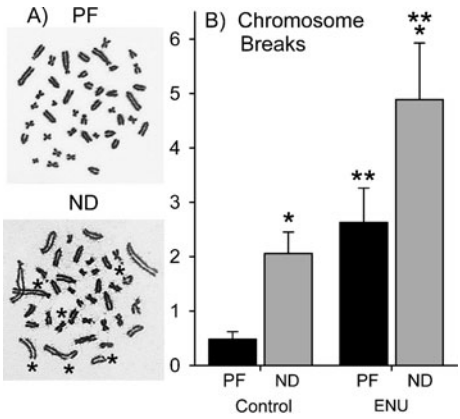

Fig. 2.4 The effect of niacin deficiency and ENU treatment on Chromosome Breaks. Weanling Long-Evans rats were fed niacin deficient (ND) or pair fed niacin replete control diet (PF) for 3 weeks. Rats were gavaged with ethylnitrosourea (ENU, 30 mg/kg bw in pH 4 water) at 10:00 am. Control rats were gavaged with pH 4 water to assess the influence of vehicle and procedures. Four hours following the ENU treatment, rats were injected with colcemid (0.6 mg/kg bw, i.p.) and killed 2 h later. (**a**) Metaphase arrests from PF and ND bone marrow cells (*site of chromatid or chromosome break), and (**b**) Blinded analysis of chromosome breaks. $n = 9–11$, 50 spreads per animal. *significant effect of diet, **significant effect of ENU ($p < 0.05$, t-test)

2002). Pharmacological niacin intake offered no additional benefit using these endpoints (Spronck and Kirkland 2002). Sister chromatid exchanges represent homologous recombination events that do not cause damage per se, but they generally correlate with increased non-homologous recombination events, which underlie chromosomal translocations. Essentially all models of PARP disruption have caused increased sister chromatid exchanges. The bone marrow micronucleus assay is a simple measure of genomic instability, and one that is very sensitive to niacin deficiency. The micronucleus is easily observed in polychromatic erythrocytes, which have recently extruded their nucleus. Micronuclei may arise from clastogenic events, which cause a chromosome fragment lacking a centromere. Conversely, an aneugenic micronucleus is complete and has been left behind due to some failure of the sorting mechanisms, either the physical microtubular structures, or the checkpoint signals that ensure all chromosomes are attached. Thus the micronucleus assay is sensitive to many defects in DNA repair, checkpoint control or chromosome sorting. In looking at total numbers of micronuclei, niacin deficiency alone caused a large increase in micronuclei, and further increased the response to ENU treatment (Fig. 2.5) (Kostecki et al. 2007). It would be quite interesting, from a mechanistic perspective, to know which type of micronuclei are being produced. I would speculate that the numerous micronuclei caused by niacin deficiency alone are predominantly aneugenic. This is based on the fact that we do not see evidence that

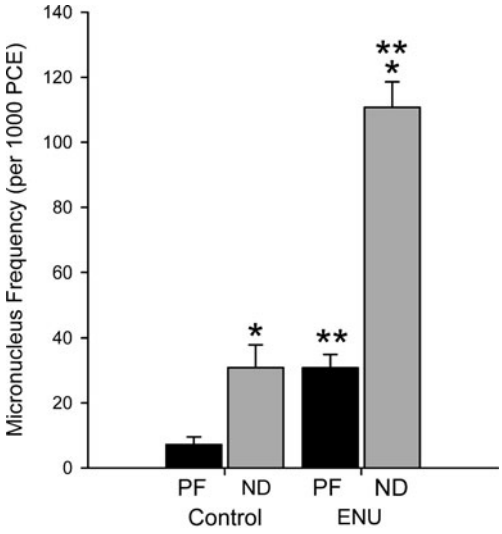

Fig. 2.5 The effect of niacin deficiency and ENU treatment on the frequency of micronuclei (MN) in bone marrow polychromatic erythrocytes (PCE). Weanling Long-Evans rats were fed niacin deficient (ND) or pair fed niacin replete control diet (PF) for 3 weeks. Rats were gavaged with ethylnitrosourea (ENU, 30 mg/kg bw in pH 4 water) at 10:00 am. Control rats were gavaged with pH 4 water to assess the influence of vehicle and procedures. Twenty four hours after ENU treatment bone marrows cells were processed to view MN in PCE. 2000 PCE were evaluated for each sample ($n = 8$–13). *significant effect of diet, **significant effect of ENU ($p < 0.05$, t-test)

niacin deficiency alone causes a significant excess of strand breaks using the comet assay (Fig. 2.3, time 0) or chromatid gaps as end points (Kostecki et al. 2007). Conversely, ENU-induced micronuclei are likely to be clastogenic in nature since they are being induced by a DNA-breaking compound. It seems probable that niacin deficiency is favoring the formation of micronuclei through changes in both DNA repair and chromosome sorting.

Tankyrases I and II are PARP enzymes that associate with telomeres, which are repetitive sequences at the ends of chromosomes. Normal DNA replication causes a small loss of telomeric DNA with each cell division, and the erosion of these areas plays an important role in cell senescence. Telomerase is an enzyme that extends the telomere to allow continued cell division. Tankyrase enzymes use NAD+ to add poly(ADP-ribose) to certain telomere binding proteins, causing them to dissociate and enable telomerase activity. This represents another connection between cellular NAD+ status and chromosome function and stability. When the telomeres erode, they tend to become instable and participate in translocation events. During niacin deficiency one would predict that telomere erosion would occur, and we have preliminary results that suggest this is true (unpublished data).

DNA damage and genomic instability can also be induced through oxidant stress. Oxidant stress results from an imbalance between the formation and protective metabolism of reactive oxygen species. Reactive oxygen is an unavoidable aspect of aerobic metabolism, and also plays specialized roles in metabolism and cell signaling. An excess of reactive oxygen species leads to DNA damage through the formation of adducts such as 8-oxo-deoxyguanosine (8-oxodG). Niacin deficiency could lead to oxidant stress through decreased levels of NADPH, limiting the regeneration of GSH, and thus an inhibition of glutathione peroxidase activity. We have found that niacin deficient bone marrow cells display increased levels of oxidant damage to both cellular proteins and DNA, as reflected by 8-oxodG and protein carbonyl formation (Tang et al. 2008). However, there was no effect of niacin status on NADPH or GSH levels, indicating that the activity of glutathione peroxidase was not being limited directly by niacin deficiency. Inflammation can also cause oxidant stress, but cDNA microarray analysis did not reveal any changes in gene expression that were suggestive of enhanced inflammatory signaling (Tang et al. 2008). Not all increases in reactive oxygen represent cellular stress or injury. NAD(P)H, or mitogenic, oxidases produce reactive oxygen species that act as signals for cell division, adhesion and survival (through inhibition of apoptosis) (Terada 2006). This is similar to what we observe in niacin deficient bone marrow cells. Mitochondria use reactive oxidants as signals to control cellular energetics (Terada 2006), and this process could be disrupted directly or indirectly by changes in niacin status. The causes and impact of oxidant damage during niacin deficiency are uncertain and require further research.

There are many aspects to genomic stability beyond the correct replication and repair of DNA and the sorting of chromosomes. These processes can be disrupted indirectly by improper control of the cell cycle. A failure to maintain genomic stability is also mitigated by the process of apoptosis, and defects in these pathways allow damaged cells to survive and progress to leukemias. One protein that plays

a key role in coordinating DNA repair, cell cycle arrest and apoptosis is p53. This protein plays a strong role in tumor suppression and is mutated or downregulated in most human cancers. p53 is regulated by several NAD-dependent processes. It has been reported as a covalent acceptor of poly(ADP-ribose), via PARP-1 activation (Won et al. 2006). It also has a high affinity binding site for non-covalent interactions with poly(ADP-ribose) (Malanga and Althaus 2005) and it is deacetylated by Sir2 (Langley et al. 2002). The end result of these interactions is uncertain and there is a lack of consensus on the role of ADP-ribosylation in the regulation of p53 function. We have examined p53 expression in bone marrow cells in our model, and have found that niacin deficiency causes accumulation of two slow mobility forms of p53 (Spronck et al. 2007). We have determined that they are not due to alternate splicing of the mRNA (Spronck et al. 2007). The change in mobility is suggestive of one or two ubiquitination sites, and may indicate a defect in proteasome function (Won et al. 2006), but further work is needed to characterize these changes, and determine if they occur in other species.

If p53 function is disrupted there can be impairment of cell cycle arrest and apoptotic signaling. We found that niacin deficient bone marrow cells displayed an altered response to etoposide-induced DNA damage, with a blunting of the G1 arrest, and a greater portion of cells staying in S-phase (active DNA synthesis). In addition, there was an impaired apoptotic response in niacin deficient cells. Of interest, this is one of the few biomarkers that we have found to be altered by pharmacological supplementation of niacin relative to adequate niacin intake. The progressive increase in apoptotic frequency from niacin deficient through adequate to pharmacological intake correlates with cellular NAD+ and poly(ADP-ribose) content in these groups (Spronck et al. 2007). This relationship is also reflected by the eventual development of ENU-induced leukemias (Bartleman et al. 2008). Essentially all of the other intermediate endpoints that we have examined have not shown additional benefit of pharmacological supplementation, suggesting that regulation of apoptosis may be a key aspect of the impact of dietary niacin on leukemogenesis. There are a number of possible connections between niacin status and apoptosis, but the most compelling of these involves the activation of apoptosis-inducing factor (AIF) (Porter and Urbano 2006). AIF is a 67 kDa flavoprotein that appears to play important roles in mitochondrial metabolism of normal cells and in apoptosis of injured cells (similar to cytochrome c). Decreased AIF levels are associated with impaired mitochondrial oxidative phosphorylation and increased neuronal cell death in Harlequin mice (Porter and Urbano 2006). Conversely, following cell injury, AIF may be released from the mitochondria after PARP-1 is activated in the nucleus (Yu et al. 2006). It can then translocate to the nucleus and initiate or contribute to apoptotic cell death.

Most of this is not well understood, especially the mechanisms by which nuclear formation of poly(ADP-ribose) leads to AIF release from mitochondria. It does provide an interesting explanation for the impact of niacin status on apoptosis in bone marrow cells. The fact that cell cycle arrest and p53 expression patterns are not changed between control and pharmacological niacin intake, while

poly(ADP-ribose) and apoptosis are changed, suggests that high polymer levels during niacin megadosing help to favor AIF-induced apoptosis.

For the cancer patient, the critical relationship is between toxicity and efficacy of the chemotherapy treatment. Many side effects, especially those that may happen in the long term, are tolerated to achieve treatment efficacy. Correcting niacin status may not be clinically beneficial if tumor cells are also protected from the toxicity of the chemotherapy drugs. Optimally, niacin supplementation could protect the bone marrow and increase tumor cell killing, and there are several indications that this could be true. Tumors evolve protective mechanisms against endogenous controls over cell division by downregulation of apoptosis pathways, and these changes also protect them from chemotherapy drugs. When a cancer patient is niacin deficient, it is likely that the tumor cells will have decreased NAD levels and experience some of the physiological changes that we observe in the bone marrow, including depression of apoptosis, decreased control over the cell cycle and increased genomic instability. These are all effects that increase the malignant potential of a tumor, and/or make it more resistant to chemotherapy. One intriguing possibility is that pharmacological niacin intake could increase poly(ADP-ribose) synthesis in tumor cells, activating the AIF-dependent apoptotic pathway and increasing treatment efficacy. Tumors tend to display decreased mitochondrial metabolism and become more dependent on anaerobic glycolysis (Bonnet et al. 2007). This has been shown to inhibit apoptosis and contribute to chemoresistance. Dichloroacetate (DCA) activates pyruvate dehydrogenase, increasing mitochondrial metabolism and sensitizing cancer cells and tumors in vivo to chemotherapy (Bonnet et al. 2007). We have recently measured mitochondrial NAD+ in bone marrow cells, and found that they are not protected during niacin deficiency, depleting to the same extent as whole cell pools (unpublished data). Niacin supplementation may work in a similar fashion to DCA treatment, and may provide additive or synergistic increases in tumor cell killing, by supporting the increase in mitochondrial metabolism and driving up the poly(ADP-ribose) dependent activation of AIF.

2.3 Summary

Niacin deficiency decreases bone marrow NAD+ and poly(ADP-ribose) levels, disrupts cell cycle regulation, depresses apoptosis and enhances multiple forms of genomic instability, leading to bone marrow suppression and leukemogenesis in response to alkylating agents. Pharmacological supplementation with niacin increases bone marrow NAD+ and poly(ADPribose) levels, enhances apoptosis and decreases leukemogenesis. The next stage of research in this area should be to characterize the effect of dietary niacin status on the response of implanted tumors to chemotherapy in various animal models. This will allow a decision to be made on whether aggressive supplements of niacin during therapy are likely to benefit the balance between efficacy and toxicity in human cancer patients.

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Chapter 3
Niacin: Vitamin and Antidyslipidemic Drug

Elaine L. Jacobson, H. Kim, M. Kim, and M.K. Jacobson

Abstract Niacin is defined collectively as nicotinamide and nicotinic acid, both of which fulfill the vitamin functions of niacin carried out by the bioactive forms NAD(P). In the last few decades numerous new enzymes that consume NAD(P) as substrates have been identified. The functions of these enzymes are emerging as exciting paradigm shifts, even though they are in early stages of discovery. The recent identification of the nicotinic acid receptor has allowed distinction of the drug-like roles of nicotinic acid from its vitamin functions, specifically in modulating blood lipid levels and undesirable side effects such as skin vasodilation and the more rare hepatic toxicities. This information has led to a new strategy for drug delivery for niacin, which, if successful, could have a major impact on human health through decreasing risk for cardiovascular disease. Understanding the many other effects of niacin has much broader potential for disease intervention and treatment in numerous diseases including cancer.

Keywords Cardiovascular diseases · Clinical trial · HDL · LDL · Modulating blood lipids · Niacin · Nicotinamide · Nicotinic acid · Risk for triglycerides · Vasodilation · Topical delivery strategy

3.1 Niacin's Multifaceted Metabolism

Nicotinic acid and nicotinamide, collectively referred to as niacin, were identified as compounds that prevent and cure the dietary deficiency disease, pellagra (Elvehjem et al. 1938). Since then the identification of functions of niacin and it's active metabolites, NAD(H) and NADP(H), in cell metabolism constitute a large metabolome in and of itself [Fig. 3.1, reviewed in (Hassa et al. 2006; Ziegler 2000)].

Both nicotinic acid and nicotinamide function as vitamins, supporting the biosynthesis of NAD(P)(H) (Fig. 3.1). The de novo pathway from tryptophan to NAD is not functional in most human tissues and may not be significant in

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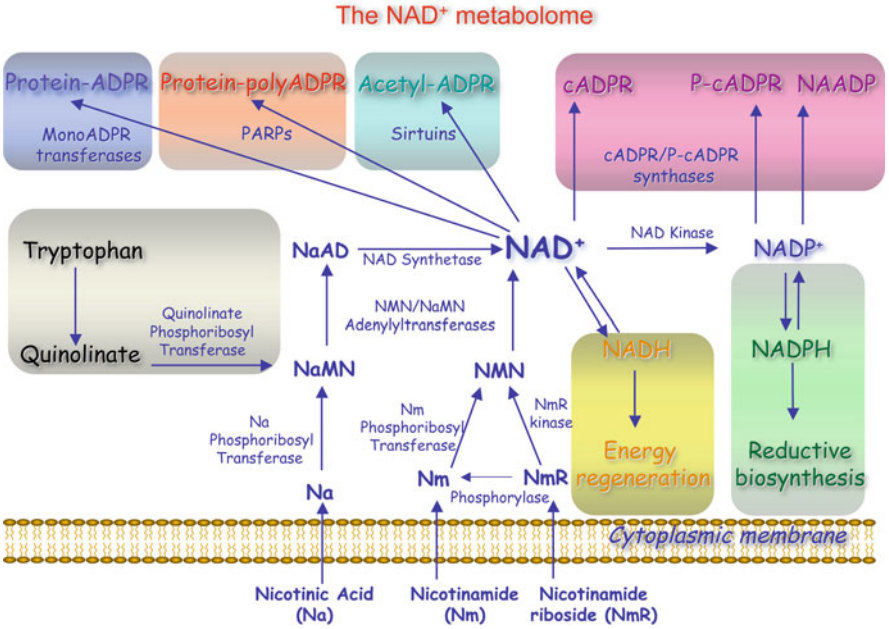

Fig. 3.1 The vitamin functions of niacin

humans, at least under partial dietary restriction (Fu et al. 1989). NAD+/NADH play crucial roles in energy generation and in regulating numerous enzymes of energy metabolism, while NADPH is essential for reductive biosynthesis and maintenance of the antioxidant status of the cell. In more recent decades, numerous enzyme activities have been identified that consume NAD+ or NADP+ as a substrate for post-translational modification of proteins and to synthesize small signaling molecules. Multiple classes of mono-ADP-ribosyl transferases have been identified that have specificity for unique proteins at a specific amino acid side chain, e.g., arginine, cysteine, histidine, etc. While the chemistry and biochemistry of these modifications have evolved, understanding the biological impact of these modifications is an early science. Multiple poly (ADP-ribosyl) polymerases (PARPs) have been discovered and shown to consume NAD+ to regulate many cellular functions that involve assembly and disassembly of protein-nucleic acid complexes, including DNA repair complexes, mitotic spindles, telomeres, etc. NAD+ dependent protein deacetylases (SIRTs) have emerged more recently with seven putative gene products identified to date in human tissues. These enzymes have been implicated in many cellular regulatory events from effecting longevity to regulating specific enzymes of energy metabolism such as glutamate dehydrogenase (Haigis et al. 2006). Finally, ADP-ribosyl transferases that catalyze an internal transfer within the ADP-ribose moiety to form the small signaling molecules, cyclic ADP-ribose (cADPR) and phospho-cyclic-ADP-ribose (Pc-ADPR) appear to effect numerous regulatory roles, many of them involving calcium signaling, however the functions of these enzymes also are poorly understood. The enzymes/pathways mentioned here and noted in

Fig. 3.1 have been briefly mentioned only to illustrate the large number of functions niacin effects as a vitamin. It is important to note that the recent discovery of the nicotinic acid receptor separates the vitamin functions of these two forms of niacin from drug-like functions, since only nicotinic acid can bind the receptor [reviewed in (Offermanns 2006)].

3.2 Niacin's Potential and Pitfalls as an Antidyslipidemic Drug

Blood levels of different lipoprotein classes are strongly associated with the risk of cardiovascular diseases leading to death by heart attack and stroke. Thus, the management of blood lipid levels is an area of enormous significance to public health. As an example, Fig. 3.2 shows data from the Framingham Heart Study documenting that elevated high density lipoprotein cholesterol (LDL) and insufficient high density lipoprotein cholesterol (HDL) are independent risk factors for coronary artery disease (CAD). Most public health messages over the past 15 years have focused on the link between LDL and cardiovascular disease, but the medical community is increasingly realizing that both LDL reduction and HDL elevation are critical for reducing the risk of cardiovascular disease.

The importance of HDL to public health is illustrated further by the fact that nearly 40% of males and 15% of females in the United States have HDL values below 40 mg/dL. These data along with the findings that a 6% increase in HDL can translate to 22–29% decreases in coronary events and deaths illustrate the enormous public health significance of identifying a safe and effective therapy to elevate HDL. The importance of blood lipids to public health has led to the development of intensive therapy to modify blood lipids. Table 3.1 provides an overview of agents used

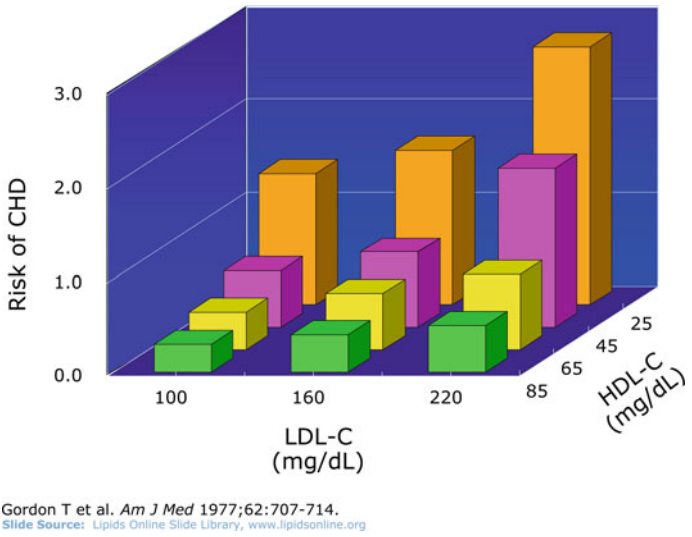

Fig. 3.2 Risk of CHD as a function of blood cholesterol

Table 3.1 Drugs for the management of blood lipids

Drug class	% ↑ in HDL	% ↓ in LDL	% ↓ in TG
Statins	5–6	25–28	11–15
Bile acid sequestrants	3–4	9–18	5–10
Cholesterol absorption inhibitors	1	15–18	5–10
Fibrates	6–18	4–11	21–35
Nicotinic acid	26–32	17–18	27–35

and the range of effects of different agents that have been compiled from many different clinical studies. Nicotinic acid is the oldest drug known to modify serum lipids and it is the most effective drug currently available for elevation of HDL. The statin drugs are effective in reducing LDL but they do not effectively raise HDL. In addition to its ability to raise HDL, nicotinic acid also is effective in reducing cardiovascular risk by lowering serum LDL and triglycerides (TG), which also are a risk factor in CAD. So why is nicotinic acid not the most widely used drug? It is because compliance is poor due to its side effect profile of skin flushing and associated puritis. Furthermore, many individuals with hepatic dysfunction are contraindicated for oral niacin therapy and other oral lipid modification therapies including statin therapy. Standard protocols indicate that nicotinic acid, combined with diet and exercise, should be the first agent used in an attempt to lower LDL, lower TG, and raise HDL. However, the side effect of flushing frequently drives patients with low HDL to seek alternatives.

Currently, there are two sources of nicotinic acid available to elevate HDL levels. First, it can be obtained as over the counter (OTC) formulations in a variety of different forms. The second source is a slow release formulation approved by the US Food and Drug Administration as a once per day dose under the trade name Niaspan. The Niaspan formulation of nicotinic acid decreases vasodilation but still has a very significant side effect profile including skin flushing. Niacin also became available in 2002 as Advicor, a combination therapy that contains a statin, lovastatin, along with Niaspan.

The issue of a side effect profile that limits compliance with therapy is particularly significant for the treatment of a risk factor such as low HDL because most patients on therapy are outwardly healthy and thus an individual who feels fine is reluctant to take a medication that makes one feel uncomfortable. A recently published study (LaRosa and LaRosa 2000) estimates the discontinuation rate of patients placed on oral nicotinic acid therapy at 46%. While the number of patients currently on nicotinic acid therapy is difficult to estimate, it is reasonable to assume that the side effect profile of oral nicotinic acid limits use to far less than 10% of the number of individuals who could benefit from this therapy. We describe below the strategy for development of a new technology for the elevation of HDL and reduction of LDL and TG by transdermal delivery using a nicotinic acid prodrug, lauryl nicotinate. This technology is designed to achieve the stated endpoints without the limiting side effect profile and to provide lipid modulation therapy to those whose hepatic dysfunction contraindicates oral therapies, because transdermal administration greatly reduces the liver first pass effect.

3.3 Niacin's Unique Effects on Blood Lipids

Nicotinic acid is known to inhibit lipolysis in adipose tissue leading to decreases in plasma levels of free fatty acids, decreases in lipoprotein Lp(a), very low-density lipoprotein cholesterol (VLDL or TG) levels, and LDL, while it increases HDL up to 35%. However, it is not completely clear how nicotinic acid increases HDL. A growing interest in the ability of nicotinic acid to inhibit cAMP in adipose tissue due to the Gi-mediated inhibition of adenlyl cyclase (Aktories et al. 1980) and the ensuing hypothesis that nicotinic acid acts as an agonist of a Gi-coupled receptor ultimately resulted in the discovery of the nicotinic acid receptor (Soga et al. 2003; Tunaru et al. 2003; Wise et al. 2003). The potential mechanisms by which nicotinic acid may function through this receptor as noted in the model of Fig. 3.3 include a decrease in TG (VLDL) through decreased cAMP effects on lipolysis (yellow adipocyte, Fig. 3.3), leading to reduced cholesterol ester transfer protein (CETP) mediated exchange of TG and cholesterol esters between Apo-B containing lipoproteins (VLDL, LDL) and HDL, with the end result being increased HDL (grey box, Fig. 3.3) [reviewed in (Offermanns 2006)].

Recent studies demonstrating that nicotinic acid modulates leptin and adiponectin release and down stream signaling as depicted in Fig. 3.3 support alternate mechanisms for affecting HDL metabolism to provide cardioprotective effects. Here, leptin signals via the leptin receptor (LR), a member of the Type I cytokine receptor family that mediates intracellular signaling via the activation of associated

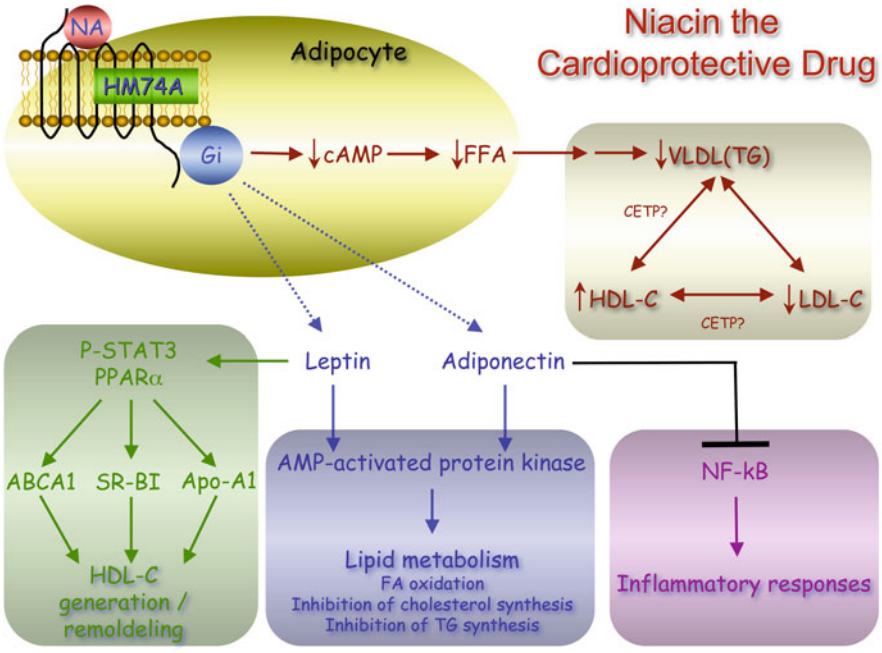

Fig. 3.3 Niacin receptor mediated signaling

Jak family tyrosine kinases (Myers 2004). The LR recruits the signal transducer and activator of transcription 3 (STAT3, green box, Fig. 3.3), which in turn induces important positive effectors of leptin action. The peroxisome proliferator-activated receptor alpha (PPAR-..) has been shown to be a key proximal mediator of the lipopenic action of leptin (Lee et al. 2002), mediating pleiotropic effects such as stimulation of lipid oxidation, alterations in lipoprotein metabolism, and inhibition of vascular inflammation. PPAR-.. binds the hypolipidemic fibrates to increase plasma HDL dependent on expression of apoA-I (Fruchart et al. 1999). Furthermore, PPAR- activation induces cholesterol removal from human macrophage foam cells through stimulation of the ATP binding cassette transporter 1 (ABCA1) pathway (Chinetti-Gbaguidi et al. 2005). Functional interactions between apoA-I and ABCA1 are necessary for the initial lipidation of apoA-I. The expression of hepatic scavenger receptor class BI (SR-BI) is modulated also by leptin (Lundasen et al. 2003). Thus, leptin appears to be important in integrating the transport of cholesterol from extrahepatic tissue to the liver since HDL lipidation, mediated by ABCA1, and delipidation, mediated by SR-BI, are modulated by leptin. Functional SR-BI is critical to the lipid and apolipoprotein composition of HDL and its absence is associated with increased susceptibility to atherosclerosis. It also has been suggested that decreased hepatic uptake and catabolism of HDL-ApoA-I may contribute to increased HDL, however this mechanism is not likely to be mediated via the nicotinic acid receptor as it is not expressed in the liver (Soga et al. 2003; Tunaru et al. 2003; Wise et al. 2003). In addition to modulation of HDL, Fig. 3.3 also shows additional cardioprotective effects mediated by the niacin receptor on NF-kB and the blocking of inflammatory responses known to be very important in CAD (purple box, Fig. 3.3) and effects via leptin and adiponectin illustrated in the blue box of Fig. 3.3. Many studies of the mechanisms that cause diseases of the artery now indicate that inflammation leading to oxidative stress is a major factor in atherosclerosis and that the ability of HDL to prevent oxidative damage to LDL is crucial to the ability of HDL to decrease the risk of arterial diseases (Kontush et al. 2003). HDL is comprised of multiple fractions classified as HDL2 and HDL3, each of which can be further separated into sub fractions designated as HDL3a, b, c, etc.

Many studies now suggest that it is the HDLC3 subfraction that reduces the risk of arterial disease progression and protects LDL from oxidation. Taken together, these observations provide compelling evidence that the goal of therapy to raise HDL should include a focus on the HDL3. Here we overview the evidence for this conclusion. Recent clinical studies have evaluated the relative risk of progression of coronary artery disease to HDL subfractions concluding that risk reduction is associated with the HDL3 and not with HDL2. Yu et al. (2003) examined the risk of incident coronary heart disease in more than 1,700 patients over a 9 year follow up in the Caerphilly coronary heart disease study. The odds ratios demonstrate that HDL3 shows a strong association with risk reduction while HDL2 did not. The study of Syvanne et al. that was derived from the Lopid coronary angiography trial (Syvanne et al. 1998) evaluated the progression of atherosclerosis in 372 subjects. Like the Caerphilly study, this study demonstrated a strong risk reduction associated with HDL3. But in contrast to the Caerphilly study, this study actually detected

an increased risk of disease progression associated with HDL2. Additional evidence supporting the role of HDL in antioxidant functions is derived from recent biochemical studies that have specifically implicated HDL3 as an important protective factor by which HDL achieves risk reduction through prevention of accumulation of oxidized LDL particles. In this case, the small, dense HDL3 particles conferred the greatest protection against LDL oxidation. The order of protection and size from smallest to largest is HDL3c > HDL3b > HDL3a > HDL2a > HDL2b (Yoshikawa et al. 1997) (Kontush et al. 2003). Studies of the fibrate class of drugs provide further evidence implicating HDL3 as being crucial to CAD risk reduction. These drugs reduce the risk of CAD, and they also have been shown to raise HDL3 but not HDL2 (Sasaki et al. 2002). Finally, evidence supporting a role of HDL3 in modulating risk relates to promoting efflux of excess cholesterol from cells in the arterial wall, transporting it back to the liver for elimination, a process known as 'reverse cholesterol transport (RCT)'. Nascent apoA-I containing HDL particles interact with peripheral cells and acquire lipid through a transport process facilitated by ABCA1. RCT has been implicated in atheroprotective role of HDL but its efficacy had not been demonstrated in acute coronary syndromes. However, a recent study has reported a rapid reduction of atherosclerosis with intravenous administration of a recombinant apoA-I Milano (apoA-IM) (Nissen et al. 2003). ApoA-IM is a variant of the naturally occurring apoA-I that was discovered in residents of an Italian village characterized by low rates of cardiovascular disease and longer life spans. ApoA-IM differs from naturally occurring apoA-I in that it has a different amino acid composition. Interestingly, carriers of apoA-IM are characterized by predominant, heterogeneous HDL3 and a marked reduction of HDL2, indicating that apoA-IM has a major effect in HDL particle interconversion and that a shift in HDL subfractions from HDL2 to the smaller, more dense HDL3 confers cardioprotection (Kim et al. 2004).

3.4 Making a Good Old Drug Better

With the goal of enhancing the potential and ameloriating the pitfalls of niacin, we have synthesized, characterized, and screened niacin derivatives as potential prodrugs for transdermal delivery. The objectives in searching for effective transdermal prodrugs were to control the rate of delivery in order to keep niacin concentration below the threshold for vasodilation and to decrease the dose required by avoiding first pass metabolism. An overview of the strategy to deliver nicotinic acid transdermally to the blood circulation is shown in Fig. 3.4. The relative lipophilicity of a number of niacin derivatives was determined using the octanol/water partition coefficients. Partition coefficients were then related to the rates of partitioning of the derivatives from the stratum corneum to the epidermis following topical application using intracellular NAD as a biomarker of delivery. Prodrug candidates from methyl nicotinate through octyl nicotinate failed screening tests because they partitioned at a rate rapid enough to cause vasodilation at the site of application, since following conversion to nicotinic acid they exceeded the threshold for vasodilation. Decyl

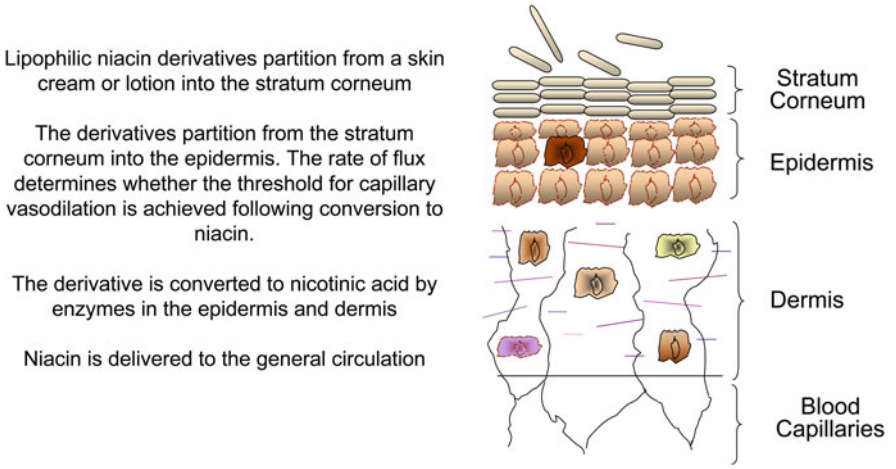

Fig. 3.4 Overview of transdermal delivery strategy using niacin derivatives

nicotinate showed variable effects on vasodilation. Finally, lauryl nicotinate was chosen as the lead candidate because it did not cause vasodilation, yet demonstrated systemic delivery of nicotinic acid in animal models as evidenced by achieving tissue saturation at a skin site distal to the site of application.

The feasibility of modulating blood lipids by transdermal delivery of niacin was then demonstrated in an animal model. Wild type mice are severely limited as an animal model for lipoprotein metabolism as their lipoprotein profile is more than 80% HDL. Transgenic mice carrying human lipoprotein metabolism genes have been used to overcome this limitation for the study of lipoprotein metabolism. For proof of principle studies, transgenic mice carrying the human genes for CETP and apoB 100 were studied. An oral dose of 0.75% nicotinic acid in the drinking water was compared to topical application of lauryl nicotinate doses that corresponded in nicotinic acid equivalents to 12.5 and 25% of the oral dose. Decreases in LDL and TG observed in both topical dosages and in the orally dosed animals were nearly identical, demonstrating that transdermal delivery of nicotinic acid to modify blood lipids is feasible. This led to completion of preclinical toxicology and pharmacology studies whose outcomes supported further drug development.

3.5 Progression to Clinical Trials

A Phase I clinical trial was conducted in a double blind, randomized, placebo controlled dose escalation design. Healthy males ranging in age from 18 to 72 years of age were recruited. Subjects enrolled in the trial were required to have HDL levels below 40 mg/dL, which represent the low end of normal HDL ranges. Each treatment group had nine subjects, six receiving a cream containing 20% lauryl nicotinate, three receiving a placebo control cream. The daily dosages for

topical application were 1.5, 3.0, 6.0, and 9.0 ml of cream that corresponded to approximately 150, 300, 600, and 900 mg of nicotinic acid equivalents. Fasting blood samples were collected at each treatment visit for lipid analysis and safety monitoring. After 1 week of application of placebo cream, dosing was initiated with the lowest dosing group and moved to the higher dose groups based on the lack of any adverse events that would dictate not initiating treatment of the higher dose group. Treatment was for a period of 8 weeks, which is about 50% of the treatment period required to see maximum pharmacodynamic effects on blood lipids when niacin is administered orally.

The safety of lauryl nicotinate was assessed by the reporting of adverse events and by analysis of blood chemistry parameters. There were no instances of systemic skin flushing or puritis in the study. There were no severe adverse events related to drug. Blood chemistry, hematology, and urine parameters also were monitored to assess safety. Oral nicotinic acid therapy has been associated with increased fasting levels of blood glucose, uric acid, and the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In this study, lauryl nicotinate treated subjects did not show a statistically significant elevation in blood glucose, uric acid, AST, or ALT. In addition, there were no statistically significant different values between lauryl nicotinate and placebo subjects in any of the other blood chemistry or hematology parameters that included alkaline phosphatase, total bilirubin, inorganic phosphate, hemoglobin, hematocrit, platelet count, WBC count, and RBC count. In summary, the data indicate that lauryl nicotinate is both safe and well tolerated with no side effects that would limit compliance with the therapy.

The primary endpoint of the Phase I clinical trial was assessment of safety and any side effects not related to safety that might limit compliance with therapy. However, blood lipid profiles were determined to provide preliminary pharmacodynamic information that could prove useful for follow up studies. The statistical power of the pharmacodynamic information obtained in this study was limited by several factors that included (i) lack of information on an optimal dosing schedule, (ii) a limited number of subjects in the study, and (iii) inherent variability of the laboratory determination for HDL. Despite these limitations, useful information was obtained. Blood lipid parameters were determined at baseline and following 4 and 8 weeks of treatment. The data demonstrate a statistically significant increase in both total HDL of approximately 10% and in the ratio of HDL to total cholesterol of approximately 16%. As discussed above, compelling evidence is accumulating that indicates that a desired goal of nicotinic acid therapy is elevation of the HDL3c subfraction. Thus, we Fig. 3.5: HDL subfraction distributions following 8 weeks treatment analyzed HDL subfractions. The data are shown in Fig. 3.5 for the lowest (1.5 ml) dosing group and demonstrate that lauryl nicotinate treatment preferentially increases the HDL3 subfraction. Indeed, the smallest and most potent antioxidant subfraction, HDL3c is elevated an average of 47%, and the next most potent antioxidant subfraction HDL3b is elevated by an average of 19% relative to placebo treatment. The results of lauryl nicotinate treatment reported here are in sharp contrast to results from studies with oral niacin, which increases the HDL2 subfractions (Morgan et al. 2003). An important quantitative note regarding

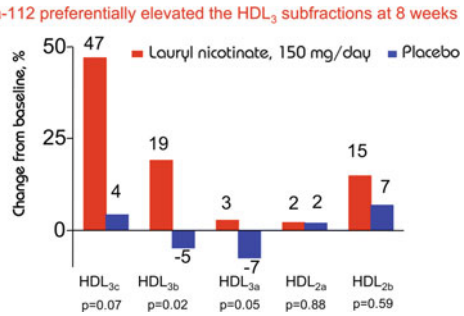

Fig. 3.5 HDL-C subfraction distributions following 8 weeks treatment

percentage changes in cholesterol fractions should be made here. Since HDL is comprised of the smallest particles containing the least cholesterol, it is likely that a 10% increase relative to the total cholesterol pool represents much more than a 10% increase in total number of HDL particles. Reporting changes in percent cholesterol within HDL in this case may represent an under estimate of efficacy, especially if the smaller particles of the HDL fraction are the most efficacious. In summary, this phase 1 clinical trial demonstrated that application of a cream containing lauryl nicotinate was well tolerated and at doses 10% of effective oral doses resulted in statistically significant elevation of HDL and the ratio of HDL to total cholesterol in 8 weeks.

Clearly, the functions of niacin are many (Fig. 3.1). The recent identification of the niacin receptor introduces a new phase of discovery regarding the effects of this vitamin and drug. The potential positive impact on human health of modulating niacin dependent metabolism is immense if its metabolic processes can be further understood to unleash its potential in a selective manner without side effects. Such efforts could have a major effect on both cardiovascular diseases as well as others.

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Chapter 4
Beyond the Antioxidant: The Double Life of Vitamin C

Mario C. De Tullio

Abstract When considering the history of vitamin C, and the names given to this molecule in early days, the Latin proverb nomen est omen suddenly comes to mind. Around 1920, when Casimir Funk introduced the term Vitamin C to indicate the nutritional factor necessary to prevent the pathological state known as scurvy, the nature of the active molecule was still unknown (Davies MB, Austin J, Partridge DA (1991) Vitamin C: Its chemistry and biochemistry. The Royal Society of Chemistry, Cambridge UK). Almost in the same years, Albert Szent-Giörgyi was striving to identify a new 6-carbon sugar he had obtained in crystal form from oranges, lemons, cabbage and adrenal glands. As humorously described by Szent-Giörgyi himself (Szent-Giörgyi A (1963) Lost in the twentieth century. Annu Rev Biochem 36:1–15), he intended to name this yet unknown carbohydrate "ignose". When this name was rejected by Sir Arthur Harden, editor of the Biochemical Journal, he suggested to name it "godnose", meaning that only God could know the real identity of the molecule. Obviously, also this choice was considered inappropriate by Harden, who suggested the plain name "hexuronic acid". Only later, when the structure of "hexuronic acid" had been completely elucidated, and biological tests performed by Swirbely identified this molecule as the anti-scurvy factor vitamin C, Szent-Giörgyi and Walter Norman Haworth decided to eventually name it ascorbic acid (Szent-Giörgyi A (1963) Lost in the twentieth century. Annu Rev Biochem 36:1–15). "Ascorbic" literally means "against scurvy", and scurvy is known to be mainly due to the inactivation of some important dioxygenases involved in the synthesis of a few key molecules, including different collagen forms (De Tullio MC (2004) How does ascorbic acid prevent scurvy? A survey of the nonantioxidant functions of vitamin C. In: Asard H, May J, Smirnoff N (eds) Vitamin C, its functions and biochemistry in animals and plants. Bios Scientific Publishers, Oxford, UK, pp. 159–172). All this has very little to do with the celebrated role of ascorbic acid (ASC) as an antioxidant. So, if the fate of ASC had to be found in its name, its role in the prevention of scurvy (i.e. beyond the antioxidant function) should be considered its main feature. But, in spite of more than 80 years of extensive research (34,424 hits in a PubMed query on January 6 2007), an unprecedented popularity among the general public, an estimated market of several billion dollars (Hancock

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RD, Viola R (2005) Improving the nutritional value of crops through enhancement of l-ascorbic acid (vitamin C) content: Rationale and biotechnological opportunities. J Agr Food Chem 53:5248–5257), we should honestly conclude that the fate of vitamin C is still in the first name it received, many years ago: we still ignore much of its actual relevance in cell metabolism, although we are progressively getting aware of the many facets of this fascinating molecule, and its direct involvement in the regulation of apparently unrelated pathways (Arrigoni O, De Tullio MC (2002) Ascorbic acid, much more than just an antioxidant. Biochim Biophys Acta 1569:1–9; De Tullio MC, Arrigoni O (2004) Hopes, disillusions and more hopes from vitamin C. Cell Mol Life Sci 61:209–219; Duarte TL, Lunec J (2005) When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free Rad Res 39:671–686). Recent data on ASC involvement in cell signalling and gene expression open new perspectives, that will be presented and discussed in this chapter.

Keywords Vitamin C · Ascorbic acid · Scurvy · HIF1-α · Antioxidome

4.1 The Biochemical Role of Vitamin C

The accumulation of mutations in the gene coding for l-guolonolactone oxidase, last enzyme in ASC biosynthetic pathway, made primates (including humans), guinea pigs and a few other animal species unable to synthesize ASC (Nishikimi and Yagi 1991; Nishikimi et al. 1994). The evolutionary reason for this loss has been debated. According to some authors, the loss of ASC synthesis could have increased the rate of mutation and consequent genetic variability, boosting further evolution (Challem and Taylor 1998). More realistically, other authors suggest that dietary intake of vitamin C present in fruit and vegetables was enough to compensate for the lack of synthesis, and that, on the contrary, this event could have been an advantage, since hydrogen peroxide is generated in the reaction catalyzed by l-guolonolactone oxidase (Halliwell 2001). Whatever the reason for the loss of synthesis, humans can be considered as a knock-out experimental model, and scurvy as the most compelling evidence available about actual ASC functions.

Scurvy is a pathological state caused by ASC deficiency. Symptoms of scurvy include lassitude, vision problems, and neurological disorders, but the most evident signs of the disease are caused by alteration of the extracellular matrix in blood vessels, bones, skin, gums and tendons, which result in spots, haemorrhages and bone fragility (Fain 2005). All symptoms can be reversed by ASC administration (Johnston et al. 1985). Many, if not all, symptoms of scurvy can be explained in terms of the inactivation of a few ASC-dependent enzymes, all belonging to the large class of 2-oxoglutarate dependent dioxygenases (2-ODDs), widespread in the cells of all organisms (De Tullio 2004). 2-ODDs catalyze the incorporation of O_2 into an organic substrate. In general, these enzymes share a catalytic mechanism

specifically requiring Fe^{2+}, 2-oxoglutarate and ASC as co-substrates (Tuderman et al. 1977). In some cases, however, differences in co-substrate requirement may occur (Schofield and Zhang 1999; Clifton et al. 2006).

Different 2-ODDs catalyze reactions of hydroxylation, desaturation, oxidative ring-closure or expansion (Fig. 4.1). Such reactions, leading to the incorporation of oxygen into a given substrate, are critical steps in a surprisingly large number of biochemical pathways. It has been suggested that the role of ASC in the reaction is just related to keeping iron in the Fe^{2+} state, but this is contradicted by the fact that many other electron donors cannot replace ASC in maintaining 2-ODDs in their active form. Detailed studies on the catalytic mechanism of peptidyl-prolyl-4-hydroxylase (P4H), the enzyme responsible for post-translational hydroxylation at carbon 4 of proline residues incorporated into polypeptide chains, showed the complexity of the reaction. Molecular oxygen is split by the enzyme and used for both oxidative decarboxylation of 2-oxoglutarate and oxidation of peptidyl proline. The latter is obtained via the generation of an iron-oxygen-atom complex, the ferryl ion, which hydroxylates an appropriate proline residue (Hanauske-Abel and Günzler 1982; Wu et al. 2000). The generation of the ferryl ion can proceed without subsequent hydroxylation in so-called uncoupled reaction cycles (Myllyla et al. 1984). ASC is utilized as a specific alternative acceptor of the ferryl oxygen in these reaction cycles. In the absence of ASC, P4H is rapidly inactivated by self oxidation (Tschank et al. 1994; Kivirikko and Pihlajaniemi 1998). From a chemical point of view, managing the highly reactive ferryl ion is not easy, and highly specialized organization of the enzyme is required (Hoffart et al. 2006). It is very likely that ASC and 2-ODDs underwent molecular co-evolution thus making such complex reaction possible, as can be deduced by the high specificity for ASC in the reactions catalyzed by those enzymes (Majamaa et al. 1986).

Inactivation of P4H by ASC deficiency is the first identified cause of scurvy (Stone and Meister 1962). Hydroxylation of peptidyl-proline is strictly required to ensure proper collagen folding (Pihlajaniemi et al. 1991). Mature collagen is present as chains wound in tight triple helices organized into fibrils of great tensile strength and thermal stability (Holmgren et al. 1998). If ASC is not available, proline

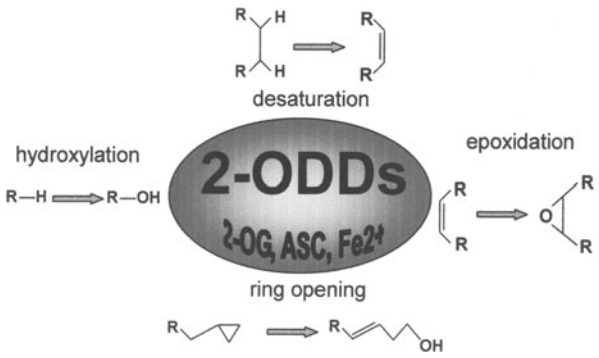

Fig. 4.1 Partial list of reactions catalyzed by 2-oxoglutarate-dependent dioxygenases

residues of collagen are not properly hydroxylated and collagen trimers cannot form. Human unhydroxylated collagen (obtained by heterologous expression in transgenic plants) showed increased flexibility as well as a reduced melting temperature, and this recombinant unhydroxylated collagen did not self-assemble into banded fibrils in physiological conditions (Perrett et al. 2001). In humans, underhydroxylated collagen is degraded in the endoplasmic reticulum (Hosokawa and Nagata 2000).

Also involved in post-translational collagen stabilization is lysyl hydroxylase, another 2-ODD sharing the same reaction mechanism of P4H, but highly specific for lysine residues and present in humans with different development-specific isoforms (Salo et al. 2005). Low expression of lysyl hydroxylase caused marked collagen alterations in a knock-out mouse experimental model (Takaluoma et al. 2006). It is noteworthy that both ASC deficiency (scurvy) and low expression of prolyl hydroxylase due to inherited defects (Prockop and Kivirikko 1995; Myllyharju and Kivirikko 2001) or gene manipulation, converge to the same result.

Many different collagen types have been described, with specific functions in maintaining the integrity of skin, tendons, cartilage, bones, teeth, cornea, muscles, blood vessels and other tissues and organs (Boot-Handford et al. 2003; Jenkins et al. 2005). In some cases, the link between ASC deficiency and altered structure of specific collagen types has been directly investigated. For instance, guinea pigs fed an ASC-free diet showed lower expression of type IV collagen and elastin mRNAs in blood vessels, as well as lower amount of hydroxyproline in the protein. This may contribute to defects observed in blood vessels during scurvy (Mahmoodian and Peterkofsky 1999).

Collagen being the most abundant protein in animals, its hydroxylation conceivably consumes large ASC amounts. This also explains why collagen malfunctioning is the most evident sign of scurvy. However, ASC deficiency also affects other important targets. Both ASC and its oxidized form dehydroascorbate are involved in protein folding in the endoplasmic reticulum (Arrigoni and De Tullio 2002). Recent studies showed that persistent ascorbate deficiency in guinea pigs leads to ER stress, unfolded protein response, and apoptosis in the liver, suggesting that insufficient protein processing participates in the pathology of scurvy (Margittai et al. 2005). In addition, other enzymes sharing ASC dependency with collagen hydroxylases are also affected by ASC deficiency, and their inactivation can lead to less dramatic, but relevant consequences.

Carnitine is an essential metabolite having an indispensable role in energy metabolism, since it enables activated fatty acids to enter the mitochondria, where they are broken down via β-oxidation. Two ASC-dependent 2-ODDs, namely, ε-trimethyl-lysine dioxygenase and γ-butyrobetaine dioxygenase are necessary for the synthesis of carnitine (Vaz and Wanders 2002). ASC deficiency affects carnitine synthesis (Thoma and Henderson 1984).

The basis of neurological disorders observed in scurvy are probably related to low activity of other ASC-dependent enzymes. Indoleamine 2,3-dioxygenase, the first and rate-limiting enzyme in human tryptophan metabolism, has been implicated in the pathogenesis of many diseases (Littlejohn et al. 2000). This enzyme is necessary to form kynurenine (Widner et al. 2000). The kynurenine pathway

regulates the metabolism of tryptophan to neuroactive compounds, and also seems to be a key factor in the communication between the nervous and immune systems (Stone and Darlington 2002). The conversion of tyrosine to 3,4-hydroxyphenyl alanine (DOPA) is catalyzed by tyrosine hydroxylase. In turn, DOPA is further modified by dopamine-β-hydroxylase, yielding the neurotransmitter noradrenalin (Diliberto et al. 1991). DOPA and noradrenalin metabolism is apparently correlated with depression (Racagni and Brunello 1999).

Synthesis of vitamin A requires the activity of β,β-carotene-15,15′-oxygenase, an ASC-dependent enzyme catalyzing the cleavage of β-carotene into two molecules of retinal (von Lintig and Vogt 2000; von Lintig et al. 2005).

Peptidylglycine R-amidating monooxygenase (PAM) is responsible for the activation of many peptide hormones and neuropeptides that require amidation of their C terminus for biological activity. PAM uses separate enzymatic domains to catalyze this amidation reaction in two steps: (i) hydroxylation of the CR of a C-terminal glycine and (ii) disproportionation of the R-hydroxyglycine. The first step is catalyzed by the peptidylglycine R-hydroxylating monooxygenase (PHM), a two-copper, ascorbate-dependent enzyme (Crespo et al. 2006).

Recently, a new group of ASC-dependent dioxygenases has been identified, with the unique property of catalyzing the repair of methylated bases (1-methyladenine and 3-methylcytosine) in DNA sequences (Duncan et al. 2002). E. coli AlkB and human ABH1-3 proteins catalyze oxidation of the relevant methyl groups and release them as formaldehyde (Koivisto et al. 2004).

The biological role of the many ASC-dependent plant dioxygenases involved in the synthesis of hormones such as gibberellins and ethylene and other signalling molecules was discussed elsewhere (Arrigoni and De Tullio 2000).

All those findings not only prove that the general reaction mechanism of dioxygenases is common to many different pathways and functions, but also that novel dioxygenases with surprising functions are likely to be discovered in the future.

4.2 ASC in HIF Hydroxylation Signaling

The reaction catalyzed by dioxygenases obviously cannot take place in the absence of oxygen. Therefore, a complex and elegant mechanism evolved in animal cells, coupling the capability of dioxygenases to sense oxygen with the orchestration of cellular defenses against oxygen deprivation (hypoxia). In 2001, two independent groups discovered that hydroxylation of two proline residues present in a specific sequence of the transcription factor hypoxia-inducible factor 1α (HIF1α) underlies the mechanism of oxygen sensing in mammalian cells (Ivan et al. 2001; Jaakkola et al. 2001; Epstein et al. 2001). HIF1, the first characterized member of the HIF family, transcriptionally activates hundreds of genes associated with angiogenesis, energy metabolism, nutrient transport; and cell migration (Chun et al. 2002; Greijer et al. 2005). It is a heterodimer, comprised of α and β subunits. In human cells, 2 proline residues (Pro402 and Pro564) of HIF1α are hydroxylated by 3 different prolyl

hydroxylases (HIF-P4H) which are not directly related to collagen hydroxylases mentioned above, although sharing the same catalytic mechanism (Tuckerman et al. 2004). HIF-P4H are in the cytosol, whereas collagen hydroxylases are typically in the endoplasmic reticulum. In HIF-P4H, the Km values for O_2 are slightly above its atmospheric concentration, indicating that the HIF-P4Hs are effective oxygen sensors (Hirsila et al. 2003). The 3 HIF-P4H have different roles in the hydroxylation of the 2 proline residues of HIF1α̃ (Berra et al. 2003; Appelhoff et al. 2004).

When oxygen is available (normoxic conditions), proline residues of HIF1α are hydroxylated (Fig. 4.2). Hydroxylation of HIF 1α is necessary for the binding of a multiprotein complex containing the von Hippel-Lindau tumor suppression protein (pVHL). In turn, the formation of this multiprotein complex leads to rapid targeting of HIF1α to proteasome-mediated degradation (Schofield and Ratcliffe 2004). When oxygen availability lowers (hypoxia) or no oxygen at all is available (anoxia), hydroxylation cannot take place, HIF1α cannot bind pVHL, is not degraded, and migrates to the nucleus, where it binds HIF1β. The dimer binds a specific sequence (the Hypoxia Responsive Element) in the promoter of an array of hypoxia-induced genes, including those for the synthesis of erythropoietin, transferrin, ceruloplasmin, vascular endothelial growth factor, nitric oxide synthase, carbonic anhydrase9, insulin-like growth factor, glyceraldehyde-3-phosphate dehydrogenase, and many more (Schofield and Ratcliffe 2005). The activation of these genes is of paramount importance in many important processes, including angiogenesis in cancer (Harris 2002; Hickey and Simon 2006) and ischemia (Kido et al. 2005). The potential

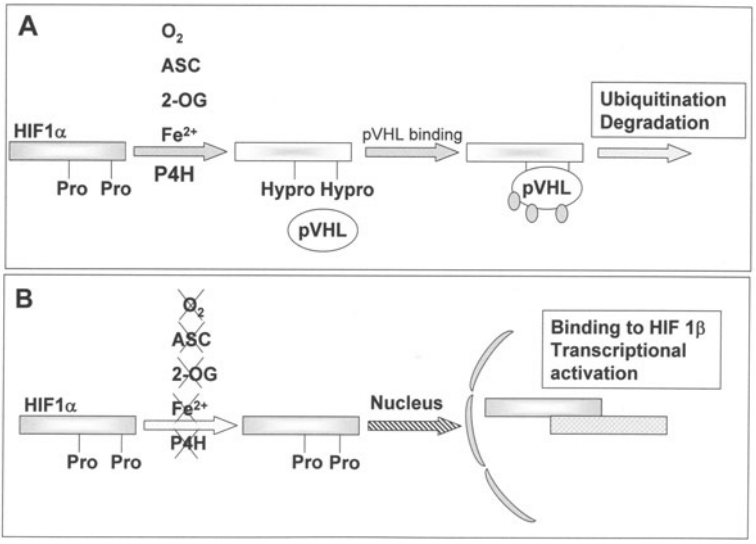

Fig. 4.2 Different fate of HIF1 in the presence (**a**) or in the absence (**b**) of the co-substrates required for its hydroxylation. (See text for details)

therapeutic fallout of those findings is currently under investigation (Hewitson and Schofield 2004).

Beside proline hydroxylation, HIF signaling is also regulated by hydroxylation of an asparagine residue (Asn803 in the human HIF1α) by asparaginyl hydroxylase, an enzyme similar to P4H, but whose ASC dependency is still uncertain (Knowles et al. 2003; Schofield and Ratcliffe 2005). Hydroxylation of this residue prevents binding of HIF1α to a co-activator (p300) required for transcriptional activation (Schofield and Ratcliffe 2004).

When the mechanism of oxygen sensing and activation of hypoxic response by hydroxylases was identified, the role of ASC in the regulation of this process was underestimated. Only later, a few studies directly addressed the role of ASC in controlling the expression and activation of HIF1α expression. Knowles et al. (2003) reported that, under normoxic and mild hypoxic conditions, ASC addition in physiological concentrations decreased the amount of HIF1 protein, by increasing the rate of its degradation. This means that lower ASC content could activate molecular responses to hypoxia, thus being beneficial in case of ischemia. This could possibly explain the contradictory results obtained when attempting to correlate plasma ASC concentrations with the risk of myocardial infarction (Padayatty and Levine 2000a).

Kinetic analyses of recombinant HIF hydroxylases have given apparent Km values of 140–180 μM, a value in a similar range to those cited for procollagen prolyl hydroxylase (Hirsila et al. 2003). Under certain conditions, ASC availability may be limiting for hydroxylase activity. ASC addition has been shown to reduce the normoxic accumulation of HIF-α that is observed in certain rapidly growing tissue culture cells (Knowles et al. 2003; Lu et al. 2005). Such effects were not seen in cells treated with 2-oxoglutarate analogues that block HIF hydroxylase activity, and are not seen in VHL deficient cells, supporting the interpretation that they arise from the promotion of hydroxylase activity by ASC, and not by its generic "antioxidant function". In another study, ASC was shown to promote HIF hydroxylation in cells deficient in the expression of junD, a member of the AP-1 family of transcription factors (Gerald et al. 2004).

A recent study providing a computational model of oxygen sensing by HIF1α (Qutub and Popel 2006) shows that low ASC concentration markedly affects the rate of hydroxylation of HIF1α protein, slowing down its degradation. According to the model, ASC has a dual role in the reaction: reactivating the enzyme by reducing the accumulating Fe^{3+} and binding independently to the saturable enzyme complex formed by the binding of the hydroxylase with Fe^{2+}, 2-oxoglutarate and O_2 (Majamaa et al. 1986). A third function of ASC in the hydroxylation reaction, i.e. its role as an acceptor of the ferryl ion in uncoupled decarboxylation (Myllyla et al. 1984), was not considered in the model calculated by Qutub and Popel (2006), but is likely to significantly affect the activity of HIF-hydroxylating enzymes.

Additional studies on HIF signalling have demonstrated that HIF1α not only accumulates in hypoxic conditions, but also in response to other nonhypoxic stimuli, comprising the Krebs cycle intermediates pyruvate and oxaloacetate. ASC proved a major controller of such nonhypoxic activation of HIF signalling (Lu et al.

2005). Enhancement of HIF-1 by glucose metabolites has been proposed as a novel feed-forward signalling mechanism involved in malignant progression (Lu et al. 2005). This leads to conclusion that hydroxylase-mediated signalling not only acts in oxygen sensing, but functions in a broader context than previously appreciated, and that ASC has a key role in its regulation.

A fierce debate on ASC in cancer treatment has been going on over more than 3 decades, following the high hopes raised by the controversial work started in 1971 by Linus Pauling and Ewan Cameron (Pauling and Cameron 1979). However, contrasting results have been obtained on this important issue (Moertel et al. 1985; Riordan et al. 1995; Padayatty and Levine 2000b). Angiogenesis, a process stimulated by HIF-1α, has a fundamental role in cancer development (Harris 2002) and HIF-1α is constitutively highly expressed in cancer cells (Zhong et al. 1998). ASC deficiency has been observed in cancer patients (Mayland et al. 2005). All in all, it seems now possible to find a rationale explanation for the use of ASC in cancer therapy. Jones et al. (2006) successfully explored this possibility by combining the use of monoclonal antibodies against transferrin (resulting in the inhibition of tumor cell proliferation, but in the induction of angiogenetic responses via HIF signalling) with the use of physiologic concentration of ASC (which causes HIF down-regulation).

The involvement of HIF1 in immune reactions has been recently identified (Hellwig-Burgel et al. 2005). Also in this case, this could explain the postulated, but never really proved, function of ASC in improving immune defences (Hemila 1997). There is little doubt that those very promising fields of research shall be further explored in the next years.

The discovery of the role of peptidyl-proline hydroxylation in the control of the stability of HIF1α and in oxygen sensing induced more scientists to investigate whether the same mechanism could be involved in the control of the turnover of other proteins. Kuznetsova et al. (2003) reported that ubiquitination and degradation of the large subunit of RNA polymerase II follows a mechanism requiring hydroxylation of proline residues in the protein, followed by binding of pVHL. In this case, degradation is regulated by UV radiation and not by oxygen availability. A striking difference between the mechanism of ubiquitination of HIF1α and that of RNA polymerase II is that in the former, degradation is the default process occurring under normal conditions (normoxia), whereas in the latter, hydroxylation, binding of pVHL and ubiquitination occur under stress conditions (UV). Hydroxylation of proline residues represents therefore a dynamic and efficient tool for the control of protein turnover. A similar system has recently been invoked for the degradation of the Iron Regulatory Protein 2 (IRP2), a posttranscriptional regulator of iron metabolism (Wang and Pantopoulos 2005). It can be easily predicted that in the near future more proteins sharing this regulative mechanism will be identified.

Notably, the activities of other ASC-dependent 2-ODDs, besides P4H, are apparently involved in important signal transduction pathways: this is the case of tyrosine hydroxylase (Paulding et al. 2002) and aspartyl hydroxylase (Maeda et al. 2003). ASC is also required for the hydroxylation, by aspartyl-β-hydroxylase (AAH), of aspartyl and asparaginyl residues in epidermal growth factor (EGF)-like domains

(Gronke et al. 1990) present in different signaling proteins, involved in protein-protein interaction (Stenflo et al. 2000). Some of these target proteins (Notch and Notch homologs) have roles in cell migration and development (Selkoe and Kopan 2003; Lubman et al. 2007).

4.3 ASC and Gene Expression

A large wealth of literature demonstrates ASC involvement in the transcription of many different genes.

Early observations suggested that vitamin C could be involved in gene transcription (Price 1966). Many recent reports further substantiate this hypothesis. In some cases, vitamin C seems to stabilize specific mRNAs (Arrigoni and De Tullio 2002 and references therein). For example, ASC-dependent stabilization of collagen transcript and destabilization of elastin transcript in smooth muscle cells and skin fibroblasts was observed (Davidson et al. 1997). Transcription of the gene coding for tyrosine hydroxylase is enhanced by ASC (Seitz et al. 1998).

A special role of ASC in inducing differentiation of several mesenchymal cell types was observed (Duarte and Lunec 2005, and references therein). Differentiation of chondrocytes (Dozin et al. 1992), cardiomyocytes (Sato et al. 2006) and osteoblasts (Harada et al. 1991) is apparently induced by ASC via collagen synthesis. ASC is specifically required for the expression of osteocalcin, a calcium-binding protein made by osteoblasts. ASC treatment increases osteocalcin gene transcription by way of a complex mechanism that is blocked by the inhibition of collagen hydroxylation, and that requires a specific sequence present in the promoter of the osteocalcin gene (Xiao et al. 1997). ASC was also successfully used to induce myelination in a mouse model of Charcot Marie Tooth syndrome, a severe neuropathy (Passage et al. 2004), although the underlying biochemical mechanism is not clear.

It has been suggested that, due to its effects in cell differentiation, ASC might be useful in the in vitro production of specialized cells/tissues from embryonic stem cells for future clinical treatment (Duarte and Lunec 2005).

The number of microarray studies investigating ASC-dependent gene expression is increasing. Catani et al. (2001) demonstrated that ASC administration induces transcription of the fra-1 gene, which codes for a transcription factor of the Fos family, and down-regulates the activator protein-1 (AP-1)-target genes. Expression profiles of human mesenchymal stem cells during the mineralization process were analyzed in the presence of ASC and other osteogenic supplements. ASC and other agents were necessary for the mineralization process and for transcriptional stimulation of 55 genes and repression of 82 genes among more than 20,000 examined (Doi et al. 2002). In an attempt to identify the mechanism of ASC-induced differentiation of embryonic stem cells into mineralised osteoblasts (Harada et al. 1991), Carinci et al. (2005) analysed the trascriptome of pre-osteoblasts treated with ASC, and identified 97 up-regulated and 373 down-regulated genes involved in cell growth, metabolism, morphogenesis, cell death, and cell communication.

The picture emerging from these data suggests that ASC controls gene expression by still unclear mechanisms. ASC seems to operate in transcription; RNA stabilization; post-translational modification of proteins; hydroxylation, epoxidation and desaturation of many different substrates. Although the complete picture is far from being complete, this is enough to get an idea of the many still ignored functions of ASC.

4.4 Antioxidant vs. Non-antioxidant Functions of ASC: A False Problem?

The free radical theory of aging and disease is one of the most fortunate scientific hypotheses of the last century (Halliwell and Gutteridge 1984). The possibility to explain in a single, unifying theory the etiology of almost all pathological conditions (including ageing!) in all organisms was, and still is, very seductive (Marx 1985). In addition, at least in its early formulation, the theory was based on a simple dichotomy between "bad" oxidants and "good" antioxidants (Azzi et al. 2004). This simple, schematic concept rapidly became very popular also among non-scientists.

The antioxidative properties of already known and newly discovered molecules became the focus of much research. Provided that all in vitro antioxidants actually have biological activities (Azzi et al. 2004), different organisms have apparently evolved a different blend of antioxidant molecules and enzymes (introducing a new term this could be called "antioxidome"), which is in part genetically determined, and in part responsive to environmental clues. The great relevance of ASC in the antioxidome of both plants and animals has been underlined in much scientific literature, due to its abundant presence in almost all biological systems (Frei et al. 1989; Rose and Bode 1993; Smirnoff 2005).

There is an increasing number of reports also pointing at "non-antioxidant" functions of vitamin E (Zingg and Azzi 2004) and vitamin C (De Tullio and Arrigoni 2004; Duarte and Lunec 2005), but a contraposition between "oxidant" and "non-antioxidant" activities runs the risk of becoming a new, worthless scientific dispute. It is imperative to find a reasonable explanation accounting for this "double life" of vitamin C.

The involvement of ASC in cellular responses to nickel toxicity (Salnikow et al. 2004; Salnikow and Kasprzak 2005; Karaczyn et al. 2006) possibly opens new perspectives to our understanding of actual ASC function in biological systems.

Exposure of cells to carcinogenic compounds of nickel and cobalt caused hydroxylase-dependent activation of the HIF-1 transcription factor and of its transcriptional activity under normoxic conditions. As mentioned above, hydroxylation of HIF1α cannot take place if any of the co-substrates in the reaction catalyzed by prolyl hydroxylase (2-oxoglutarate, Fe^{2+}, oxygen, and ASC) is missing (Fig. 4.2). Nickel or cobalt did not affect the level of intracellular iron, but greatly depleted intracellular ASC. Co-exposure of cells to metals and ASC reversed both metal-induced stabilization of HIF-1α and HIF-1-dependent gene transcription

(Salnikow et al. 2004). Studying the expression of carbonic anhydrase IX (CA IX) and NDRG1/Cap43, both known as intrinsic hypoxia markers and cancer-associated genes, Karaczyn et al. (2006) observed a clear ASC-dependent regulation via HIF signalling in fibroblasts exposed to nickel or grown to high cell density without medium change, but with adequate oxygen supply. The loss of intracellular ASC triggered the induction of both tumour markers. The involvement of an ASC-dependent, but HIF-independent signalling pathway was also observed, suggesting that other transcription factors beside HIF are ASC-regulated. Karaczyn et al. (2006) concluded that, in addition to low oxygenation, insufficient ASC supply can contribute to the induction of hypoxia-associated proteins via both HIF-dependent and independent mechanisms.

Information so far presented and discussed in this chapter can be summarized in three sentences:

(i) ASC acts as an antioxidant
(ii) ASC is specifically required for the activity of dioxygenases (non-antioxidant role)
(iii) ASC affects gene expression

A model of ASC-dependent gene regulation was recently proposed (Duarte and Lunec 2005), suggesting that ASC can influence gene expression by means of a "redox regulation" mechanism. However, as in many other papers, this redox regulation is ill-defined, and largely remains a big black box in which hiding anything that cannot be explained in terms of molecules and mechanisms (Azzi et al. 2004).

Are both the antioxidant and the non-antioxidant role of ASC just the two sides of the same coin? Do they co-operate in the regulation of gene expression? The identification of ASC as an important regulator of HIF signalling induces to hypothesize that HIF is not just an oxygen sensor, as initially considered, but a general mechanism to perceive and integrate different stimuli, directly connecting them to an array of different cellular response via immediate transcription of "first aid" genes. This can eventually explain why such complex mechanism evolved for the activation of the HIF (and possibly other) transcription factor: why going through a double round of hydroxylation? Probably because all co-substrates of the hydroxylation reaction (oxygen, 2-oxoglutarate, iron and ASC) can bring useful information about cellular health conditions. So the role of HIF signalling is not only oxygen sensing: the Krebs cycle intermediate 2-oxoglutarate can act as an indicator of cellular metabolism (hence the down-regulation of HIF by other Krebs cycle intermediates; Lu et al. 2005). In this context, ASC would have a key role in perceiving oxidative conditions (antioxidant role) and in transducing this signal via the activation of hydroxylases (non-antoxidant role) in a bona fide two-component signalling system operated by the same molecule (Fig. 4.3). This model explains both the prominent role of ASC in the antioxidome of almost all organisms, and its molecular co-evolution with 2-ODDs in their complex catalytic mechanism.

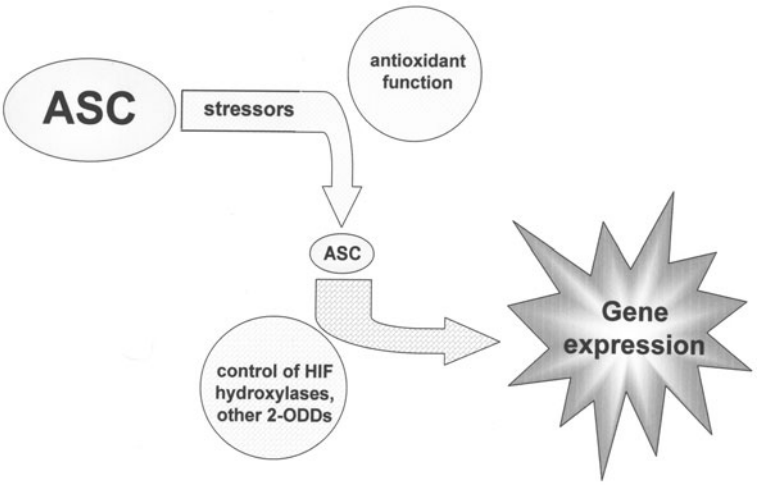

Fig. 4.3 A model of ASC-dependent gene regulation mediated by oxidative stress and dioxygenase activity

4.5 Conclusions

Is it time to change once more the name of vitamin C? Should we call it "ignose" again? Maybe this is not necessary. We are now learning more and more about this tricky molecule and its decisive role in cellular processes, and hopefully next years' research will allow us to overcome present limitations. On the other hand, it is important to avoid sterile discussion on the relative importance of the antioxidant and the non-antioxidant roles, as they are likely to be the Yin and Yang of ASC reactivity. Time (and hard bench work!) will tell.

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Chapter 5
Vitamin C in Sepsis

John X. Wilson and F. Wu

Abstract Bacterial bloodstream infection causes septic syndromes that range from systemic inflammatory response syndrome (SIRS) and encephalopathy to severe sepsis and septic shock. Microvascular dysfunction, comprising impaired capillary blood flow and arteriolar responsiveness, precedes multiple organ failure. Vitamin C (ascorbate) levels are low in critically ill patients. The impact of ascorbate administered orally is moderate because of its limited bioavailability. However, intravenous injection of ascorbate raises plasma and tissue concentrations of the vitamin and may decrease morbidity. In animal models of polymicrobial sepsis, intravenous ascorbate injection restores microvascular function and increases survival. The protection of capillary blood flow and arteriolar responsiveness by ascorbate may be mediated by inhibition of oxidative stress, modulation of intracellular signaling pathways, and maintenance of homeostatic levels of nitric oxide. Ascorbate scavenges reactive oxygen species (ROS) and also inhibits the NADPH oxidase that synthesizes superoxide in microvascular endothelial cells. The resulting changes in redox-sensitive signaling pathways may diminish endothelial expression of inducible nitric oxide synthase (iNOS), tissue factor and adhesion molecules. Ascorbate also regulates nitric oxide concentration by releasing nitric oxide from adducts and by acting through tetrahydrobiopterin (BH4) to stimulate endothelial nitric oxide synthase (eNOS). Therefore, it may be possible to improve microvascular function in sepsis by using intravenous vitamin C as an adjunct therapy.

Keywords Adjuvant therapy · Ascorbic acid · Bacteremia · Blood pressure · Brain · Capillary blood flow · Critical illness · Dehydroascorbic acid · Endothelial cells · Endotoxin · Infection · Inflammation · Microvascular function · Sepsis · Vitamin C

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5.1 Causes of Morbidity and Mortality in Sepsis

Sepsis is defined as suspected or proven infection plus systemic inflammatory response syndrome (SIRS; e.g., fever, tachycardia, tachypnea, leukocytosis). Severe sepsis is sepsis with organ dysfunction (hypotension, hypoxemia, oliguria, metabolic acidosis, thrombocytopenia, or obtundation). The mainstay of emergency management is goal-directed therapy plus ventilation, antibiotics and possibly activated protein C (Rivers et al. 2001; Russell 2006). Nevertheless, despite best management, mortality remains high. One third to half of all patients with severe sepsis die before leaving the hospital (Rice and Bernard 2005).

Bacterial bloodstream infection, trauma and burn injury are common causes of SIRS. But the pathophysiological progression of septic syndromes has been studied most extensively in the case of Gram-negative organisms. All clinically significant aerobic Gram-negative bacteria contain a macromolecule, endotoxin (e.g. Escherichia coli lipopolysaccharide, LPS), embedded in the outer layer of their cell walls. In patients, LPS unleashes a proinflammatory cascade involving production of cytokines (e.g., interferon-gamma, IFNgamma). This cascade induces septic syndromes that range from SIRS and encephalopathy to severe sepsis and septic shock (Russell 2006; Sharshar et al. 2005; Wilson and Young 2003).

Sepsis is associated with maldistribution of blood flow within organs and loss of microvascular control of tissue oxygenation, as well as with arteriolar hyporesponsiveness to vasoconstrictors and vasodilators (Sakr et al. 2004; Spronk et al. 2002; Tyml et al. 2005) (Fig. 5.1). Red blood cell flow in the microcirculation is altered by sepsis even after fluid resuscitation, as evidenced by the decreased density of perfused capillaries and increased proportion of non-perfused capillaries observed in fluid-resuscitated septic patients with adequate arterial blood oxygenation and cardiac output (Sakr et al. 2004). This increased heterogeneity of capillary blood flow leads to tissue hypoxia because the diffusion distance for oxygen from perfused capillaries to parenchymal cells becomes longer (Goldman et al. 2004). Tissue hypoxia may precipitate organ failure. Indeed, microvascular dysfunction is a strong predictor of death and one-third of severe sepsis patients die of organ failure even when shock is prevented (Rivers et al. 2001, Sakr et al. 2004). Capillary blood flow improves in survivors but fails to improve in non-survivors (Rivers et al. 2001, Sakr et al. 2004). Therefore, septic patients may benefit from adjunctive therapy that targets microvascular dysfunction acutely.

Sepsis and tissue hypoxia are also associated with increased production of reactive oxygen species (ROS) and peroxynitrite that deplete antioxidant molecules and cause oxidative stress (Wilson and Gelb 2002; Wilson and Young 2003). The latter involves oxidization of the body's proteins, lipids and nucleic acids. Furthermore, ROS modulate redox-sensitive intracellular signaling pathways that control the expression of genes critical to the initiation and perpetuation of sepsis; these genes encode proteins that synthesize inflammatory cytokines, increase blood coagulation, and alter endothelial cells' regulation of blood pressure and capillary blood flow (Fig. 5.1). Therefore, patients with sepsis may benefit from adjuvant therapy that counters ROS and peroxynitrite by increasing antioxidant levels acutely.

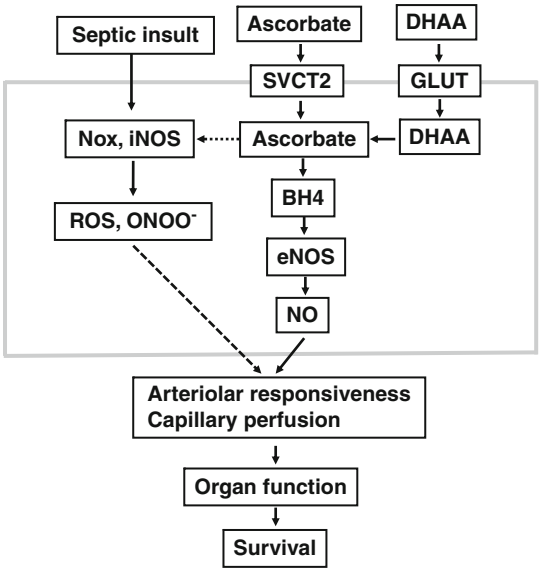

Fig. 5.1 Septic insult and vitamin C alter microvascular function. The *largest rectangle* represents a microvascular endothelial cell. *Arrows with solid lines* indicate stimulation and those with *dotted lines* indicate inhibition. Septic insult increases NADPH oxidase (Nox) and inducible nitric oxide synthase (iNOS) activities, which elevate reactive oxygen species (ROS) and peroxynitrite (ONOO-) production. The ROS and ONOO- impair capillary blood flow and arteriolar responsiveness to vasoconstrictors and vasodilators. Septic insult may also inhibit transport of ascorbate into cells by SVCT2. Dehydroascorbic acid (DHAA) enters the cells through GLUT and then is reduced to ascorbate. Intracellular ascorbate scavenges ROS and ONOO-, while preventing induction of iNOS and Nox enzymes. Ascorbate also acts through tetrahydrobiopterin (BH4), endothelial nitric oxide synthase (eNOS) and nitric oxide adducts, to increase the local concentration of nitric oxide (NO). Therefore, vitamin C may maintain ROS and NO at homeostatic levels that promote normal organ function and survival

5.2 Vitamin C Deficiency in Sepsis

Ascorbate (the predominant form of reduced vitamin C at physiological pH) is an antioxidant that defends against excess ROS. In so doing, it becomes oxidized to ascorbyl free radical (AFR) and dehydroascorbic acid (DHAA). Ascorbate concentrations are subnormal in plasma and leukocytes, whereas plasma AFR concentration in plasma is elevated, in critically ill patients (Borrelli et al. 1996; Galley et al. 1996; Long et al. 2003; Metnitz et al. 1999; Rumelin et al. 2005; Schorah et al. 1996). Ascorbate deficiency may have serious consequences, since prior depletion of ascorbate decreases survival in mice injected with pathogenic bacteria (Gaut et al. 2006).

In most cell types that have been studied, the uptake and reduction of DHAA to ascorbate is not impaired by LPS. DHAA is carried into cells through facilitative

hexose transporters such as GLUT1 (Wilson and Dragan 2005) (Fig. 5.1). LPS stimulates DHAA uptake through the 55 kDa isoform of GLUT1 in macrophages (May et al. 2005) and also upregulates this transporter in endothelial cells (Spolarics et al. 1996). Exposure to LPS in vivo accelerates DHAA reduction in mouse liver (Kuo et al. 2005). Similarly, preincubation with LPS and IFNgamma (LPS+IFNgamma) increases DHAA reduction in astrocytes permeabilized with saponin, although this septic insult decreases the activity of the 45 kDa isoform of GLUT1 in astrocytes with intact plasma membrane (i.e., absent saponin; Wilson and Dragan 2005). Taken together, these results suggest that septic insult may accelerate the rate at which extracellular DHAA is taken up and reduced to ascorbate in cells that express 55 kDa GLUT1. Indeed, injection of a sublethal dose of LPS increases ascorbate concentration in the liver, kidney, adrenal gland, and heart of mice (Kuo et al. 2005).

One cause of ascorbate deficiency in patients may be hospital food that contains too little vitamin C (McErlain et al. 2001). Even when patients are nourished intravenously, the parenteral nutrition solutions may generate hydrogen peroxide that degrades ascorbate (Dupertuis et al. 2005; Knafo et al. 2005; Laborie et al. 2002). A second cause of vitamin C deficiency in critically ill patients is an increased requirement for ascorbate (Baines and Shenkin 2002). The basis for this increased requirement may be oxidation of ascorbate by excess ROS. However, changes in cellular mechanisms of vitamin C transport and metabolism may also be important.

Inflammation may decrease the transport of ascorbate into cells. The underlying mechanism involves the ascorbate-specific transporters, SVCT1 and SVCT2, through which ascorbate enters most cell types (Wilson and Dragan 2005) (Fig. 5.1). Inflammatory cytokines (tumor necrosis factor-alpha, interleukin-1beta) inhibit ascorbate uptake in human endothelial cells that spontaneously express SVCT2 (Seno et al. 2004). Moreover, incubation with LPS+IFNgamma inhibits SVCT2-mediated ascorbate uptake in cultured cerebral astrocytes, thereby decreasing the rate of intracellular ascorbate accumulation from extracellular vitamin C in this abundant brain cell type (Korcok et al. 2002).

5.3 Adjuvant Therapy with Intravenous Ascorbate

Ingestion of ascorbic acid, ascorbate or its reversibly oxidized metabolite, DHAA, raises the plasma concentration of ascorbate in normal human subjects. The impact of oral vitamin C on plasma ascorbate concentration in these subjects is moderate, however, because of its limited bioavailability (Wilson 2005). In contrast, intravenous ascorbate can raise plasma ascorbate concentration markedly (Padayatty et al. 2004). Clinical consequences arise from the limited bioavailability of oral ascorbate. For example, when oral and intravenous routes of ascorbate administration (500 mg/day for 30 days) were compared in sedentary men, only intravenous ascorbate improved endothelial function as indicated by flow-mediated vasodilatation (Eskurza et al. 2004).

There is evidence that ascorbate may improve outcome in critically ill patients, who either are or are at risk of becoming septic. In a randomized, prospective, double-blind, placebo-controlled trial with 216 critically ill patients, 28-day mortality was decreased in the patients who received combined ascorbate and vitamin E by enteral feeding compared to those who did not (Crimi et al. 2004). A randomized, prospective clinical trial of 595 critically ill surgical patients found that a treatment combining ascorbate (1,000 mg q8h by intravenous injection) and vitamin E (1,000 IU q8h by naso- or orogastric tube) decreased relative risk of pulmonary morbidity and multiple organ failure and also shortened the duration of mechanical ventilation and intensive care unit stay (Nathens et al. 2002). Yet another randomized, prospective study reported decreased morbidity for severely burned patients who were infused with high-dose ascorbate (Tanaka et al. 2000).

A great deal has been learned about ascorbate therapy in animal and cell culture models of septic syndromes. Ascorbate prevents hypotension and lung injury in LPS-injected animals (Dwenger et al. 1994; Shen et al. 2005); it prolongs survival in experimental bacteremia (Gaut et al. 2006); and it improves capillary blood flow, arteriolar responsiveness, arterial blood pressure and survival in polymicrobial sepsis models (Armour et al. 2001; Tyml et al. 2005; Wu et al. 2003, 2004).

Some of the most clinically relevant models of polymicrobial sepsis involve cecal ligation and puncture (CLP). Similar to the changes observed in septic patients, CLP increases oxidative stress markers and decreases ascorbate concentration in plasma and tissue (Armour et al. 2001; Tyml et al. 2005; Wu et al. 2003). Injection of ascorbate bolus (200 mg/kg i.v.) increases survival in CLP mice (Wu et al. 2004). Survival rates at 24 h post-CLP are 9 and 65%, respectively, in vehicle-injected and ascorbate-injected mice. The protective effect of the vitamin does not appear to be due to inhibition of bacterial replication at the site of infection, because the number of bacterial colony forming units in peritoneal lavage fluid at 6 h post-CLP does not differ between vehicle- and ascorbate-injected mice (Wu et al. 2004).

5.4 Effects of Vitamin C on Capillary Blood Flow

CLP causes tissue hypoxia due to microvascular dysfunction (Goldman et al. 2004; Miyaji et al. 2003). Similar to what was reported for septic patients, capillary blood flow is altered by CLP even after volume resuscitation, as evidenced by the decreased density of perfused capillaries and increased proportion of nonperfused capillaries in skeletal muscle (Armour et al. 2001; Tyml et al. 2005). Maldistribution of capillary blood flow at 24 h post-CLP is prevented by ascorbate even when intravenous injection of the vitamin is delayed until 6 h post-CLP. Moreover, the increased proportion of nonperfused capillaries at 48 h post-CLP is prevented even when the ascorbate injection is delayed until 24 h post-CLP (Tyml et al. 2005). These results show that ascorbate can reverse microvascular dysfunction after the onset of sepsis.

The restoration of capillary blood flow by a single intravenous ascorbate bolus (76 mg/kg) lasts at least 18–24 h in CLP rats (Tyml et al. 2005). Microvascular endothelial cells in culture can accumulate a high level of intracellular ascorbate (e.g., 4–16 mmol/L) that persists longer than does extracellular ascorbate (Wilson et al. 1996; Wu et al. 2007). Taken together, these findings suggests that prophylactic, intravenous injection of vitamin C may elevate ascorbate concentration within endothelial cells long after plasma ascorbate concentration has returned to pre-injection levels.

High concentrations of intracellular ascorbate affect nitric oxide through several mechanisms. First, millimolar concentrations of ascorbate prevent consumption of nitric oxide by superoxide (Jackson et al. 1998) (Fig. 5.2). Secondly, ascorbate releases nitric oxide from adducts (e.g., by ascorbate's reduction of S-nitrosothiols; May 2000). Thirdly, ascorbate in endothelial cells acts through (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) to stimulate nitric oxide synthesis by nitric oxide synthases (NOS) (Kim et al. 2006; Smith et al. 2002) (Fig. 5.1). This

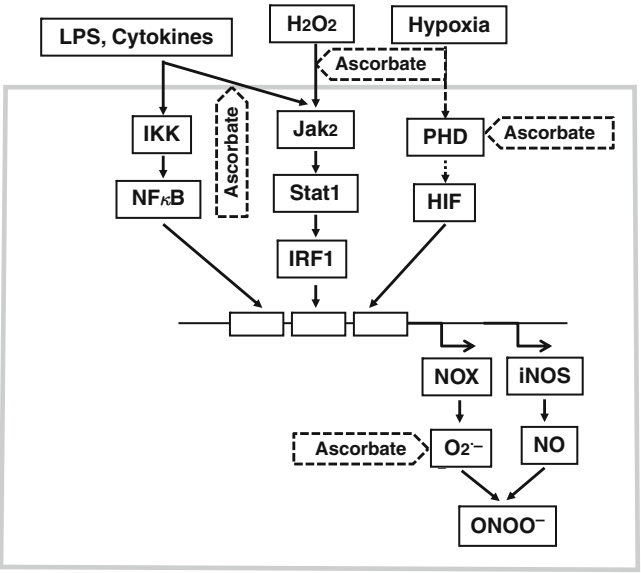

Fig. 5.2 Redox-sensitive signaling pathways by which septic insult and ascorbate may alter inflammatory responses in microvascular endothelial cells. The *largest rectangle* represents an endothelial cell. *Arrows with solid lines* indicate stimulation and those with *dotted lines* indicate inhibition. Lipopolysaccharide (LPS), cytokines and hydrogen peroxide (H_2O_2) stimulate activation of signaling pathways that trigger expression of inducible nitric oxide synthase (iNOS), NADPH oxidase (Nox) and other inflammatory mediators. Activation of the Jak2-Stat1-IRF1 pathway is inhibited by ascorbate. Hypoxia increases hypoxia inducible factor 1 (HIF-1), by inhibiting HIF-1 proly-hydroxylase (PHD), and thereby induces expression of sepsis-associated genes. Ascorbate increases the activity of PHD. Further, ascorbate acts as a scavenger of reactive oxygen species (e.g. superoxide, O_2.-) to decrease peroxynitrite (ONOO-) concentration and regulate local nitric oxide (NO) at homeostatic levels

action distinguishes ascorbate from other low molecular weight antioxidants such as N-acetylcysteine (Schneider et al. 2005). As described later in this section, homeostatic levels of nitric oxide prevent platelet and leukocyte adhesion to the microvascular endothelium, and thereby improve capillary blood flow in sepsis.

Septic insult increases the activity and expression levels of NADPH oxidases in vascular cells, as has been shown in blood vessels of CLP animals and in endothelial cell cultures (Wu et al. 2005, 2007; Yu et al. 2006). Endothelial NADPH oxidases are composed of several combinations of regulatory subunits (p22phox, p40phox, p47phox, p67phox) and catalytic subunits (Nox1, Nox2, Nox4) that synthesize intracellular superoxide (Li and Shah 2004).

Endothelial NADPH oxidases synthesize intracellular superoxide, which reacts to form other ROS (e.g., dismutation of superoxide produces hydrogen peroxide) that induce prolonged redox signaling effects (Ardanaz and Pagano 2006; Cai 2005; Griendling et al. 2000). Increases in intracellular ROS may stimulate expression of NADPH oxidase subunits that subsequently assemble and produce more ROS in endothelial cells (Jacobi et al. 2005) (Fig. 5.2). By scavenging the signal ROS that are produced in microvascular endothelial cells exposed to septic insult (Wu et al. 2002), intracellular ascorbate may interrupt this feed-forward mechanism and thereby prevent expression of NADPH oxidase subunits. Indeed, Wu et al. (2007) observed that preincubation of microvascular endothelial cells with high concentrations of ascorbate (500 and 1,000 umol/L) prevented the induction by LPS+IFNgamma or hydrogen peroxide of endothelial NADPH oxidase activity and p47phox expression. The effect on p47phox expression was mediated by the Jak2/Stat1/IRF1 signaling pathway; ascorbate prevented activation of this pathway by LPS+IFNgamma and hydrogen peroxide (Wu et al. 2007) (Fig. 5.2).

Endothelial NADPH oxidase-derived ROS increase the expression of inducible nitric oxide synthase (iNOS) in microvascular endothelial cells exposed to septic insult (Wu et al. 2001, 2002, 2005). Ascorbate acts within endothelial cells to prevent induction of iNOS by CLP, bacterial endotoxin and inflammatory cytokines (Wu et al. 2002, 2003, 2004). A preliminary report suggests that ascorbate's suppression of NADPH oxidase activity mediates this inhibition of iNOS expression (Wu et al. 2005). Ascorbate injection also prevents induction by CLP of iNOS in liver and this effect may not be confined to endothelial cells (Kim and Lee 2004).

Clearly, ascorbate modifies inflammatory responses. The way this is accomplished, in addition to the mechanisms described above, may involve suppression of gene expression that is dependent on the transcription factor, hypoxia inducible factor-1 (HIF-1) (Fig. 5.2). HIF-1 is an alpha-beta heterodimer that stimulates expression of iNOS, GLUT1 and other inflammatory mediators (Wenger 2002; Vissers et al. 2007). Normally the HIF-1alpha subunit has a short half-life because it is covalently modified by HIF-1 prolyl-hydroxlase (PHD), which is a ferrous iron (Fe^{2+})-dependent enzyme that targets HIF-1alpha for proteolysis by the ubiquitin-proteosome system. NADPH oxidase-derived ROS inhibit PHD activity (Goyal et al. 2004). Inhibition by ascorbate of endothelial NADPH oxidase (Wu et al. 2007) may thereby preserve PHD activity. Further, experiments in cells exposed to oxidative stress indicate that intracellular ascorbate reduces ferric iron (Fe^{3+}) to

Fe^{2+}, which then activates PHD to modify HIF-1alpha for proteolysis (Knowles et al. 2003). In various cell types, including human umbilical vein endothelial cells, ascorbate has been shown to decrease HIF-1 levels and inhibit HIF-1alpha–dependent expression of inflammatory mediators (Lu et al. 2005; Vissers et al. 2007).

NADPH oxidase-derived superoxide avidly interacts with NOS-derived nitric oxide to form peroxynitrite. Further, peroxynitrite oxidizes the essential NOS cofactor. BH4. The lack of BH4 makes each NOS isoform a superoxide-producing (i.e., uncoupled) enzyme. Therefore, septic activation of NADPH oxidase may lead to a local deficiency of nitric oxide and excesses of peroxynitrite and ROS in endothelial cells. These intracellular changes may cause plugging of microvessels that subsequently slows capillary blood flow, as is described below.

The plugging of microvessels may be an important factor in septic impairment of capillary flow, because adhesion of leukocytes, platelets and red blood cells to the endothelium is elevated in experimental sepsis (Cerwinka et al. 2003; Merx et al. 2005; Ondiveeran and Fox-Robichaud 2004; Secor et al. 2010; Singer et al. 2006; Sundrani et al. 2000). Local application of exogenous nitric oxide prevents adhesion of blood cells to the venular endothelium during experimental sepsis (Cerwinka et al. 2002). Similarly, ascorbate prevents the increase in leukocyte adherence that is induced by LPS in mice (De la Fuente and Victor 2001). The mechanism underlying this effect on cell adhesion may involve ascorbate inhibiting NADPH oxidase activity, p47phox expression and iNOS expression, thereby preventing the intracellular scarcity of nitric oxide and excess of ROS and peroxynitrite.

The constitutive NOS isoform in endothelial cells is endothelial nitric oxide synthase (eNOS). During ascorbate therapy in sepsis, this enzyme may play a role in rapid restoration of capillary blood flow, which may precede de novo synthesis of p47phox and iNOS (Secor et al. 2010). Moreover, ascorbate increases synthesis of nitric oxide by eNOS in endothelial cells and the underlying mechanism, at least in part, appears to involve enhancing BH4 levels to prevent eNOS uncoupling (Forstermann and Munzel 2006; Kim et al. 2006; Mittermayer et al. 2005).

Superoxide stimulates expression of cell surface intercellular adhesion molecule 1 (ICAM-1) in microvascular endothelial cells (Fan et al. 2002; Li et al. 2005). ICAM-1 mediates adhesion of leukocytes to the endothelium and may thereby impair the microcirculation. Since ascorbate inhibits superoxide production in microvascular endothelial cells exposed to septic insult (Wu et al. 2002, 2007), further research is required to determine if this action suppresses ICAM-1 expression and leukocyte plugging of venules.

Sepsis decreases the deformability of red blood cells (Bateman et al. 2001) and this may contribute to plugging of microvessels. There is evidence that antioxidants (e.g., tirilazad) can maintain red blood cell deformability in stressed animals (Chmiel et al. 2001). Further, it has been shown that aminoguanidine treatment, which probably lowers the concentration of nitric oxide metabolites (e.g., peroxynitrite), prevents the decrease in red blood cell deformability caused by CLP (Bateman et al. 2001). Ascorbate prevents overproduction of nitric oxide metabolites in vivo (Wu et al. 2003, 2004). Thus, it is plausible that ascorbate may

maintain red blood cell deformability, although further research is necessary to test this hypothesis.

Coagulability is another potential cause of capillary blood flow impairment that may be modulated by ascorbate therapy. ROS stimulate induction of tissue factor in endothelial cells (Salvemini and Cuzzocrea 2002). Tissue factor (formerly known as thromboplastin) is a transmembrane glycoprotein that functions as the primary cellular initiator of blood coagulation in vivo (Steffel et al. 2006). Because endothelial cells and monocytes do not express tissue factor under physiological conditions, there is no appreciable contact of cellular tissue factor with the circulating blood. However, these cells do express tissue factor when exposed to inflammatory cytokines and this contributes to coagulopathy in sepsis (Steffel et al. 2006). Coagulation is activated rapidly after septic insult and results in diffuse microvascular clot formation that may disrupt blood flow in capillary beds (Laudes et al. 2002; Salvemini and Cuzzocrea 2002). Similar to the effect observed after injection of ascorbate (Wu et al. 2004), injection of tissue factor pathway inhibitor increased survival in a CLP model of sepsis (Opal et al. 2001).

In critically ill patients, vasodilators transiently increase the proportion of perfused capillaries (Spronk et al. 2002). Although ascorbate enhances arteriolar vasodilatation in some patient populations (Schneider et al. 2005; Tousoulis et al. 2005), whether vasodilatation is a direct cause of ascorbate's restoration of capillary blood flow in sepsis is unknown.

5.5 Effects of Vitamin C on Arteriolar Responsiveness and Hypotension

Infusion of ascorbate reverses arteriolar hyporesponsiveness to vasoconstrictors (norepinephrine, angiotensin, vasopressin) in patients who have inflammatory disease or have been injected with LPS (Ferlitsch et al. 2005; Pleiner et al. 2003). Infusion of ascorbate or BH4 also prevents inhibition by LPS of endothelium-dependent vasodilatation responses (assessed as changes in forearm blood flow) to acetylcholine in human subjects, and this effect of ascorbate is associated with a 32% increase in plasma BH4 concentration (Mittermayer et al. 2005). It is likely ascorbate raises BH4 levels within the endothelial cells of arterioles and thereby enhances acetylcholine-stimulated synthesis by eNOS of the vasodilator, nitric oxide.

Bacterial bloodstream infection can lead to septic shock in patients (Stoclet et al. 1999). Similarly, in animals made septic by CLP, increased heterogeneity of capillary blood flow is followed by the development of arterial hypotension (Tyml et al. 2005). The hypotension may be caused by impairment of myocardial function (Piper et al. 1999) and arteriolar responsiveness to vasoconstrictors (Wu et al. 2003, 2004). Arteriolar responsiveness and arterial blood pressure are higher in CLP rats injected intravenously with ascorbate, compared to those injected with vehicle, when those parameters are measured at 18–24 h post-injection (Armour et al. 2001,

Tyml et al. 2005). However, ascorbate improves only arteriolar responsiveness and not hypotension in CLP mice at 3 h post-injection, indicating that the initial target of ascorbate protection is arteriolar rather than myocardial function (Wu et al. 2004). Arteriolar vasoconstriction and arterial blood pressure pressor responses to norepinephrine and angiotensin II are inhibited in mice at 6 h post-CLP (Wu et al. 2003, 2004). Intravenous ascorbate and iNOS gene deficiency ($iNOS^{-/-}$ mice) are equally effective at preventing this CLP-induced impairment of arteriolar responsiveness. iNOS synthesizes abundant superoxide (when uncoupled) or nitric oxide (when coupled) that reacts with superoxide to form the strong oxidant, peroxynitrite. This process may lead to impairment of vascular function. For example, iNOS expression in omental arteries of septic patients is associated with a subnormal vasoconstrictor response to norepinephrine that is normalized by the NOS inhibitor, N(G)-methyl-l-arginine (L-NMMA) (Stoclet et al. 1999). A phase III clinical trial of L-NMMA in patients with septic shock was terminated early because of increased mortality, but post hoc analysis indicated an overall survival benefit for low-dose L-NMMA (Lopez et al. 2004). The adverse effect of high-dose L-NMMA may be due to inhibition of endothelial cells' eNOS that is necessary for vascular homeostasis (Wang et al. 2004).

5.6 Vitamin C in Sepsis-Associated Encephalopathy

Most hospital patients with bacteremia show neurologic symptoms ranging from lethargy to coma (Wilson and Young 2003). Microcirculatory dysfunction in the brain may contribute to sepsis-associated encephalopathy, since impairment of cerebral activation flow coupling precedes changes in evoked potentials in LPS-injected rats (Rosengarten et al. 2006). Arteriolar responsiveness is depressed in the cerebral circulation of patients with severe sepsis (Terborg et al. 2001). Therefore, the hypothesis that the effects of ascorbate on capillaries and arterioles (reviewed above) may ameliorate encephalopathy in sepsis is plausible although untested.

Sepsis is also associated with ascorbate depletion, excessive inflammation (i.e., NADPH oxidase and NOS activation), and glutamatergic excitoxicity in brain parenchymal cells (Sharshar et al. 2005; Wilson and Young 2003; Wilson and Dragan 2005). The depletion of ascorbate may contribute to the inflammation and excitoxicity, since lowering intracellular ascorbate concentration in cerebral astrocytes increases iNOS expression and decreases glutamate clearance by these cells (Korcok et al. 2002).

Whereas normal neurotransmission requires high ascorbate and low DHAA concentrations in brain extracellular fluid, there is evidence that septic insult inhibits the clearance of extracellular DHAA and export of ascorbate by astrocytes (Wilson and Dragan 2005). Normally, glutamate released from active neurons stimulates astrocytes to export ascorbate, which the astrocytes synthesize by taking up and reducing DHAA (Wilson 2005). The ascorbate that enters the extracellular fluid is then accumulated by neurons and may protect them from the toxic effects of

excess glutamate. Additionally, the ascorbate released from glutamate-stimulated astrocytes may potentiate nitric oxide-dependent vasodilatation and contribute to activation flow coupling. In other words, a demand-driven component of ascorbate export from astrocytes may occur just in time and in the right place to reach active neurons and also redirect blood flow to those neurons. However, cell culture experiments have shown that septic insult inhibits the replacement of extracellular DHAA with ascorbate by astrocytes (Wilson and Dragan 2005). The underlying mechanism involves decreased activities of astrocytes' high-affinity glutamate transporters and 45-kDa GLUT1 transporter (Wilson and Dragan 2005); the latter transporter mediates DHAA uptake in astrocytes and is distinct from the 55-kDa isoform that is common in other cell types (Korcok et al. 2003). The decrease in extracellular ascorbate concentration in septic brain may result from this derangement of astrocyte function. Therefore, interventions that restore the ability of astrocytes to replace extracellular DHAA with ascorbate should be investigated as adjunctive treatments to the current antimicrobial and supportive therapy of sepsis.

5.7 Other Issues Concerning Vitamin C in Sepsis

There have been no studies published that identified which redox state of injectable vitamin C (i.e., ascorbate or DHAA) is most effective for treating sepsis. The maximal transport capacity for DHAA uptake exceeds that for ascorbate uptake in most mammalian cell types. Therefore, when present in the extracellular fluid at identical concentrations and under physiological conditions, DHAA increases intracellular ascorbate concentration more rapidly than does ascorbate (Wilson and Dragan 2005). It is not known if intravenous DHAA is superior to ascorbate for raising intracellular ascorbate in sepsis, but it is in experimental ischemia-reperfusion of heart and liver (De Tata et al. 2005; Guaiquil et al. 2004). Furthermore, injection of DHAA improves neurologic outcome in mice subjected to cerebral ischemia-reperfusion, whereas equal doses of ascorbate do not (Huang et al. 2001). Intravenous injection of DHAA increases cerebral ascorbate concentration (Mack et al. 2006) and the underlying mechanism likely involves uptake and reduction of DHAA to ascorbate by brain cells.

Maintenance of neutrophils' antimicrobial function is an important consideration during infection. It appears that ascorbate does not impair the respiratory burst in neutrophils. Bolus intravenous injection of 2 g ascorbate in human subjects does not alter superoxide production by neutrophils (Ellis et al. 2000). Similarly, incubation of neutrophils with either ascorbate or DHAA, to raise intracellular ascorbate concentration, does not inhibit the production of extracellular ROS elicited when these cells are subsequently challenged by stimuli such as E. coli (Carr and Frei 2002; Sharma et al. 2004). The preservation of neutrophils' ability to produce ROS during exposure to high extracellular ascorbate levels may allow these cells to destroy pathological microorganisms and thus defend against infection.

Finally, the safety of high-dose vitamin C therapy is controversial. For vitamin C administered orally, doses up to 2,000 mg/day appear to be safe for the general adult population (Hathcock et al. 2005). But less is known about the safety of vitamin C injections. Pharmacokinetic modeling indicates that bolus intravenous injection of 10 g ascorbate raises plasma ascorbate to a peak of 5,000 umol/L and keeps it above 2,000 umol/L for 2 h in human subjects (Padayatty et al. 2004). In vitro experiments showed that these concentrations of ascorbate are not lethal to normal human cells (Chen et al. 2005). Similarly, repeated intravenous injections of 750 mg/day or 7,500 mg/day of ascorbate for 6 days in healthy volunteers do not induce a pro-oxidant change in plasma markers of oxidative stress (Muhlhofer et al. 2004). However, injection of ascorbate 2 h before major surgery increases oxidative modification of plasma lipids in venous blood during the ischemic phase of surgery, and the consequences of this lipid modification are unknown (Bailey et al. 2006).

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Chapter 6
Vitamin C Transport and Its Role in the Central Nervous System

James M. May

Abstract Vitamin C, or ascorbic acid, is important as an antioxidant and participates in numerous cellular functions. Although it circulates in plasma in micromolar concentrations, it reaches millimolar concentrations in most tissues. These high ascorbate cellular concentrations are thought to be generated and maintained by the SVCT2 (Slc23a2), a specific transporter for ascorbate. The vitamin is also readily recycled from its oxidized forms inside cells. Neurons in the central nervous system (CNS) contain some of the highest ascorbic acid concentrations of mammalian tissues. Intracellular ascorbate serves several functions in the CNS, including antioxidant protection, peptide amidation, myelin formation, synaptic potentiation, and protection against glutamate toxicity. The importance of the SVCT2 for CNS function is supported by the finding that its targeted deletion in mice causes widespread cerebral hemorrhage and death on post-natal day 1. Neuronal ascorbate content as maintained by this protein also has relevance for human disease, since ascorbate supplements decrease infarct size in ischemia-reperfusion injury models of stroke, and since ascorbate may protect neurons from the oxidant damage associated with neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's. The aim of this review is to assess the role of the SVCT2 in regulating neuronal ascorbate homeostasis and the extent to which ascorbate affects brain function and antioxidant defenses in the CNS.

Keywords Brain · SVCT2 · Ascorbate transport · Dehydroascorbate · Neurons · Glutamate

6.1 Ascorbic Acid Physiology and Recycling

Ascorbic acid is a vitamin because humans, higher primates, and a few other vertebrates such as guinea pigs have lost the ability to synthesize it from glucose. These species lack the enzyme L-gulono-1,4-lactone oxidase (EC 1.1.3.8), which

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carries out the last step in ascorbate biosynthesis (Chatterjee et al. 1960). Species that cannot synthesize ascorbate must therefore have efficient mechanisms for both absorption of the vitamin and for its recycling. Absorption of vitamin C occurs largely in the distal ileum of humans and guinea pigs, where uptake is mediated by a saturable, sodium- and energy-dependent transporter (Stevenson and Brush 1969; Bianchi et al. 1986) that is located in the apical brush border membrane (Bianchi et al. 1986; Rose 1988). This transporter has been cloned (Tsukaguchi et al. 1999) and is termed SVCT1 (sodium-dependent vitamin C transporter-1, Slc23a1). The SVCT1 helps to generate plasma ascorbate concentrations of 40–60 μM in humans (Table 6.1), which can double with typical levels of dietary ascorbate supplementation (e.g., 250–1,000 mg daily) (Levine et al. 1996). In contrast to plasma ascorbate concentrations, tissue concentrations are typically 1 mM or higher, as exemplified by leukocytes and platelets in human blood (Table 6.1). High intracellular ascorbate concentrations are achieved by two mechanisms. The most important is probably unidirectional transport of the vitamin into cells on a transporter very closely related to the SVCT1, termed the SVCT2 (Slc23a2) (Tsukaguchi et al. 1999). These transporters share 65% sequence homology, substrate saturability, high affinity for ascorbate (20–60 μM), as well as energy- and sodium-dependence. Intracellular ascorbate tends to exit cells only slowly (May et al. 1998a), probably because of its hydrophilic structure and negative charge at physiologic pH. The second route by which ascorbate can enter cells is by uptake of its two-electron-oxidized form, dehydroascorbic acid (DHA, Fig. 6.1). DHA is taken up on the ubiquitous

GLUT-type glucose transporters (Vera et al. 1993). Although DHA, a triketone with a strained lactone ring, has little resemblance to glucose, it forms a hydrated bicyclic hemiketal (Pastore et al. 2001) for which these transporters have affinity (Bigley and Stankova 1974). Since the GLUTs mediate facilitated diffusion, transport of DHA by this mechanism is bi-directional. This also means that any DHA formed within cells by ascorbate oxidation will rapidly efflux and be lost. This is prevented by efficient cellular mechanisms of DHA reduction or recycling to ascorbate.

Table 6.1 Ascorbate concentrations in plasma and blood cells from normal humans

Tissue/fluid	Ages; # of subjects	Diet (mg/day)	Concentration (mM)	References
Plasma	>60 years; 226	< 120	0.057 ± 0.003	Jacques et al. (1995)
Plasma	73 years; 161	257	0.072 ± 0.027	Taylor et al. (1997)
Aqueous humor	73 years; 161	257	1.64 ± 0.58	Taylor et al. (1997)
Lens	73 years; 161	257	3.74 ± 1.4	Taylor et al. (1997)
Neutrophils	19–27 years; 13	100	1.17 ± 0.17	Levine et al. (2001)
Monocytes	17–55 years; 41	Unknown	3.8	Evans et al. (1982)
Platelets	17–55 years; 41	Unknown	1.9	Evans et al. (1982)
Erythrocytes	17–55 years; 41	Unknown	0.043	Evans et al. (1982)
Plasma	17–55 years; 41	Unknown	0.045	Evans et al. (1982)

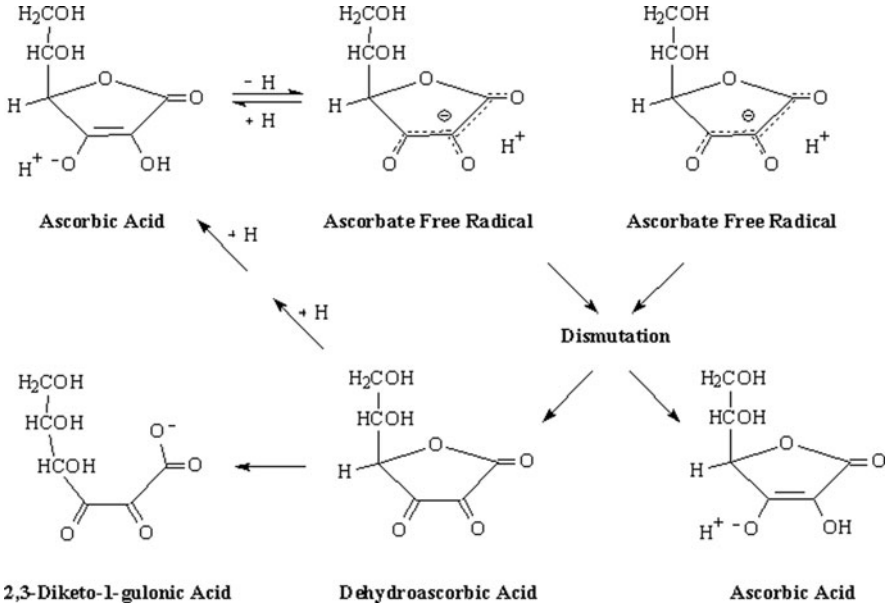

Fig. 6.1 Ascorbate oxidation and recycling

Ascorbate recycling from both of its oxidized forms occurs largely within cells, although cell-surface reduction of its radical one electron-oxidized form has been described (May et al. 2000, VanDuijn et al. 2000). As shown in Fig. 6.1, ascorbate is oxidized when it donates a hydrogen atom (proton + electron) to enzyme reactions or to scavenge free radicals. The one-electron oxidized form of ascorbate, commonly termed the ascorbate free radical (AFR) or monodehydroascorbate, is surprisingly stable and can be detected at 10 nM concentrations in biological fluids by EPR (Mehlhorn 1991, Coassin et al. 1991, Buettner and Jurkiewicz 1993). Although the AFR has a lower mid-point reduction potential than ascorbate (Buettner 1993), it is not very reactive. Instead of donation of another hydrogen and electron, the preferred reaction shown in Fig. 6.1 is for two molecules of the AFR to dismutate, forming ascorbate and dehydroascorbate (Bielski et al. 1981). DHA is unstable at physiologic pH, with a half-life of about 6 min (Drake et al. 1942; Winkler 1987) (Tolbert and Ward 1982). Hydrolysis of the lactone ring irreversibly converts DHA to 2,3-diketo-1-gulonic acid (Fig. 6.1) (Chatterjee 1970; Bode et al. 1990). Ascorbate loss by DHA decomposition is obviously wasteful of the vitamin, and DHA reduction to ascorbate is rapid and efficient within cells.

Mechanisms of GSH-dependent DHA reduction include direct chemical reduction (Winkler et al. 1994) and enzyme-dependent reduction with GSH serving as electron donor (Maellaro et al. 1994; Winkler et al. 1994; Washburn and Wells 1999). DHA is also reduced to ascorbate by NADPH-dependent enzymes, including thioredoxin reductase (May et al. 1997) and a liver 3α-hydroxysteroid

dehydrogenase (Del Bello et al. 1994). The AFR is also reduced to ascorbate with high affinity by both NADH-dependent reductases (Nazemi and Staudinger 1968; Ito et al. 1981; Villalba et al. 1993) and by the NADPH-dependent enzyme thioredoxin reductase (May et al. 1998a). As reviewed previously (May and Asard 2004), these cellular systems provide redundant and efficient mechanisms for recycling of ascorbate from its oxidized forms.

6.2 Functions of Ascorbate

As noted above, ascorbate provides electrons for crucial enzyme reactions in cells and acts as a primary antioxidant capable of scavenging radicals generated within cells or plasma. The chapter by Dr. deTulio details the non-antioxidant functions of ascorbate, which are crucial in preventing scurvy and for maintaining cell integrity and health. The main antioxidant function of the vitamin involves direct scavenging of radical species before they can damage DNA, proteins, or lipids. Another mechanism by which ascorbate functions as an antioxidant is to recycle other antioxidants. For example, ascorbate can reduce the α-tocopheroxyl radical at the surface of biological membranes, thus contributing to the ability of α-tocopherol to break the chain of lipid peroxidation in lipid bilayers (Buettner 1993). Another example relates to the finding that ascorbate spares tetrahydrobiopterin in cultured endothelial cells, thus allowing continued action of endothelial nitric oxide synthase (Heller et al. 2001; Baker et al. 2001). As with α-tocopherol, the mechanism of this effect also appears to be due to reduction of the tetrahydrobiopterin radical by ascorbate (Patel et al. 2002). Consideration of both non-antioxidant and antioxidant functions of the vitamin shows that it has multiple sites of action in cells. Although it may not be absolutely required for any specific action in a cell, ascorbate is clearly necessary for proper function of many organs. Perhaps most important is its requirement in the brain and central nervous system (CNS), as discussed next.

6.3 Importance of Vitamin C in the CNS

Ascorbic acid is an essential micronutrient in the CNS. Whereas whole brain ascorbate concentrations are one to two mM, intracellular neuronal concentrations have been calculated to be much higher (Rice and Russo-Menna 1998; Rice 2000), as discussed below. Neurons are also especially sensitive to ascorbate deficiency, perhaps because they have 10-fold higher rates of oxidative metabolism than supporting glia (Wilson 1997; Hediger 2002). This heightened neuronal sensitivity is most apparent in states of ascorbate deficiency and in conditions in which there is excess oxidant stress. A neuroprotective role for ascorbate is also suggested by the existence of homeostatic mechanisms that maintain high concentrations of ascorbate in cerebrospinal fluid (CSF) and in neurons. A key feature in this regard is the ability to sustain steep ascorbate concentration gradients: (a) from plasma to the CSF across

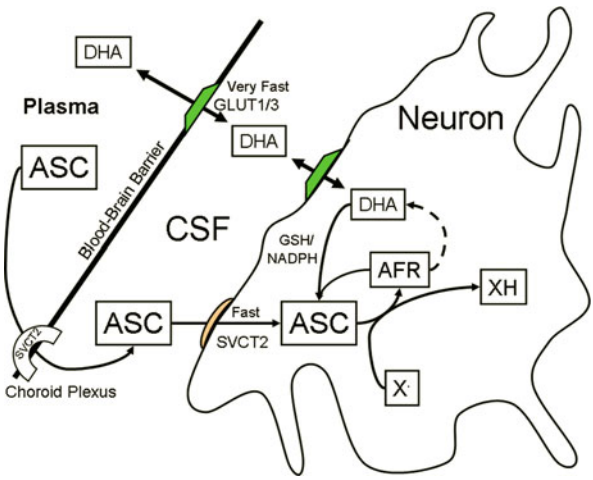

Fig. 6.2 Ascorbate uptake and metabolism in the CNS. ASC, ascorbate; AFR, ascorbate free radical; DHA, dehydroascorbate; X● oxidizing free radical species. AFR generated by X● dismutates to form ascorbate and dehydroascorbate. Both the AFR and DHA are recycled back to ascorbate by cellular metabolism

the choroid plexus and (b) from the CSF and interstitium into neurons (Fig. 6.2). In both instances, this gradient is generated for the most part by the SVCT2 protein. As discussed next, intracellular ascorbate and thus the SVCT2 protects the CNS in syndromes of oxidant stress.

Scurvy causes severe lassitude and asthenia in humans. Although the disease has been associated with paraparesis in humans, death appears to be due more to complications of systemic collagen dysfunction and not to a distinct neurologic syndrome (Hirschmann and Raugi 1999). This likely relates to the fact that ascorbate is avidly retained by the CNS during ascorbate deficiency (Hornig 1975). For example, 14 days after removal of ascorbate from their diet, guinea pig brains still had 24% of their original ascorbate content, compared to only 4% for adrenal gland and 3% for spleen (Hughes et al. 1971). Whereas this could indicate that decreases in CNS ascorbate do not play a major role in generalized scurvy, the argument can also be made that the strong retention of ascorbate in the CNS reflects its importance to neuronal function. This hypothesis is supported by our own studies in guinea pigs with moderate vitamin E deficiency in which an acute deficiency of ascorbate was superimposed (Hill et al. 2003). After 2 weeks of vitamin E deprivation in male guinea pigs, plasma and brain α-tocopherol concentrations were modestly decreased by 65% and 32%, respectively. These animals gained weight and appeared completely normal without evidence of abnormal gait or ataxia (signs of vitamin E deficiency). However, within 5–6 days of removing vitamin C from their diet, the animals typically developed a progressive ascending paralysis and died within 24 h. No neurologic signs were apparent in animals with single deficiencies of either vitamin C or E alone. Death occurred in the doubly deficient animals despite only 37% and 67% decreases in brain and spinal cord ascorbate concentrations, respectively. Despite over 95% decreases in plasma and liver ascorbate in the animals on the vitamin C-deficient diet, neither the C-deficient nor the doubly deficient animals

had signs of scurvy (no skin or hair changes, no hemarthroses). The doubly deficient animals had only small increases in liver and brain F2-isoprostanes (a marker of oxidant stress). Standard hematoxylin and eosin staining of the brains and spinal cords showed no specific abnormalities. However, subsequent studies using Nissl and silver degeneration stains revealed widespread neuronal loss and degeneration in the pons and long tracts of the spinal cord (Burk et al. 2006). Thus, even a modest decrease in CNS ascorbate accelerated signs of vitamin E deficiency in this model, indicative of a crucial role for ascorbate in protecting the brain against oxidant stress.

If the SVCT2 transporter is responsible for maintaining high CSF and neuronal ascorbate concentrations, what happens when the protein is knocked out in the mouse? Targeted deletion of the SVCT2 protein results in homozygotes that die shortly after birth: although they appear to develop normally in utero, they never take a breath and have diffuse cerebral hemorrhage (Sotiriou et al. 2002). Whereas the latter likely reflects increased capillary fragility due to defects in basement membrane type IV collagen (Gould et al. 2005), the animals do not show hemorrhage elsewhere and lack signs of generalized scurvy. Further, hydroxyproline levels in skin are normal (Sotiriou et al. 2002). The cause of death is unknown. Catecholamine synthesis is decreased, but this is apparently not the cause of death (Bornstein et al. 2003). Although the lungs fail to expand, their architecture is normal, as are surfactant levels (Sotiriou et al. 2002). Brain ascorbate concentrations are decreased 30–50% in the heterozygotes, and are very low to undetectable in the homozygous mice (Sotiriou et al. 2002). It seems likely that diffuse cortical capillary hemorrhage along with neuronal dysfunction contributed significantly to the death of these mice, although further studies are needed to assess the role of the lung and metabolic defects.

Perhaps the most dramatic acute oxidant stress in the CNS is the ischemia-reperfusion injury that occurs with stroke. It is generally accepted that ischemia depletes intracellular GSH and ascorbate in brain, and that subsequent reperfusion generates reactive oxygen species (ROS) that further deplete these two antioxidants and extend tissue damage to areas with decreased oxidant defenses (Rice et al. 1995). Ascorbate supplements decrease infarct size in animal studies. For example, in monkeys given 1 g/day of ascorbate parenterally for 6 days before middle cerebral artery occlusion, brain ascorbate was increased by 50%. This indicates that ascorbate had access to the CNS. Further, infarct size was decreased by 50% in the ascorbate-treated group compared to the control group (Ranjan et al. 1993). Similarly, in rats with middle cerebral artery occlusion, high dose dehydroascorbate given by intraperitoneal injection just before, 15 min after, or three h after occlusion markedly decreased infarct volume, mortality, and neurological deficits in mice (Huang et al. 2001). In contrast, ascorbate itself showed no protective effect in these studies. As shown in Fig. 6.2, dehydroascorbate is rapidly transported across the blood-brain barrier by glucose transporters (Agus et al. 1997; Huang et al. 2001; Mack et al. 2006), taken up by glucose transporters in neurons, and reduced to ascorbate (May and Asard 2004). The inability of acute treatment with ascorbate to prevent neuronal damage in the rat model is supported by other evidence that ascorbate does not cross the blood-brain barrier in its fully reduced form (Agus et al.

1997; Hosoya et al. 2004). This appears to be due to lack of SVCT2 in endothelial cells of the blood-brain barrier, as shown by in situ hybridization (Berger et al. 2003; García et al. 2005) and more recently by immunostaining for the SVCT2 protein (Mun et al. 2006).

The ability of ascorbate to decrease infarct size following ischemia-reperfusion likely relates to scavenging of ROS, both within and around cells. Ascorbate at the concentrations present in CSF and neurons in vivo (i.e., >100 μM) will effectively scavenge superoxide (Jackson et al. 1998) and spare or recycl-tocopherol in the lipid bilayer (Niki et al. 1995; May et al. 1998b). For example, an increase in intracellular (but not extracellular) ascorbate content of (rat) brain slices decreased the swelling induced by oxidant stress (Brahma et al. 2000). We reported that ascorbate prevented loss of α-tocopherol and lipid peroxidation induced by culture in oxygenated medium (Li et al. 2003). Finally, in our recent studies, primary cultures of hippocampal neurons prepared from homozygous SVCT2 knockout mice showed increased susceptibility to cell death induced by N-methyl-D-aspartate (NMDA) and H_2O_2 compared to cultures from wild-type mice (Qiu et al. 2007). The improved survival of neurons containing the SVCT2 was likely due to small amounts of endogenous ascorbate (0.1–0.2 mM) retained by the cells after 2 weeks in culture, since the culture medium did not contain ascorbate.

Regarding extracellular ascorbate, it has long been known that ischemia releases large amounts of ascorbate from brain cells (Hillered et al. 1988). Since this release was associated with glutamate uptake by neurons and glia (Miele et al. 1994; Rice 2000), these two processes were thought to be linked in mechanism involving heteroexchange across one or more transporters (Grünewald 1993; Rebec and Pierce 1994; Yusa 2001). Removal of extracellular glutamate by such heteroexchange would decrease excitotoxicity caused by activation of cell surface and synaptic glutamate receptors (Grünewald 1993; Rebec and Pierce 1994; Yusa 2001). In addition, intracellular ascorbate would be expected to scavenge ROS generated during to glutamate-induced excitotoxicity. In this regard, ascorbate added to the culture medium was found to prevent glutamate-induced cell damage and death in cultured cerebellar granule cells (Ciani et al. 1996; Atlante et al. 1997). Ascorbate also has been shown to protect neurons from excitotoxicity induced by activation of the NMDA receptor (MacGregor et al. 1996; Majewska et al. 1990; Majewska and Bell 1990). Whereas intracellular ascorbate protects cells against glutamate toxicity, recent evidence suggests that it does not do so via a heteroexchange mechanism. Wilson and colleagues confirmed that glutamate increases ascorbate efflux from cerebral astrocytes, but found no effect of intracellular ascorbate on glutamate uptake (Wilson et al. 2000). The release of ascorbate was attributed to glutamate-induced cell swelling followed by opening of organic ion channels through which ascorbate can pass (Wilson et al. 2000). We found the analogous results in SH-SY5Y neuroblastoma cells: glutamate caused release of intracellular ascorbate, but neither intra- nor extracellular ascorbate affected glutamate uptake (May et al. 2006). Together, the results of these studies strongly support a protective role for ascorbate and possibly for the SVCT2 in brain during acute ROS generation.

6.4 Ascorbate Function in the CNS

Whereas ascorbate is well known to be involved in neuronal biochemistry (e.g., peptide amidation, myelination, and catecholamine synthesis), evidence for a direct role in neuronal maturation and function has only recently been forthcoming. Culture of embryonic cortical precursor cells in medium containing ascorbate was first shown to induce their maturation into neurons and astrocytes by Lee and colleagues (Lee et al. 2003). We confirmed and extended these findings in primary cultures of hippocampal neurons (Qiu et al. 2007). Key to these experiments was the use of cells prepared from late-stage embryos (gestational day 18–19) that either expressed or completely lacked the SVCT2. After 10–14 days in culture in serum-free medium in the absence of ascorbate, no ascorbate was present in cells prepared from Slc23a2 (–/–) mice, whereas the ascorbate content of cells from wild-type littermates was 0.1–0.2 mM. The SVCT2 was not detected by immunostaining in cells from the Slc23a2 (–/–) mice, whereas it was clearly present in cells from wild-type mice. Although the SVCT2 was previously reported to be restricted to neuronal cell bodies in sections prepared from adult rat brain (Mun et al. 2006), we found that it was present in punctate pattern almost exclusively in neuronal axons and not in dendrites or cell bodies. The presence of the SVCT2 enhanced neuronal maturation in culture. Compared to neurons from wild-type mice, neurons from Slc23a2 (–/–) mice showed slower neuronal growth, as indicated by fewer dendritic branches and reduced total dendritic length. In addition, the numbers of α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptor subunit GluR1clusters were also significantly reduced in cells lacking the SVCT2. The mechanism for reduced GluR1 clustering was not established, but it may be due to ascorbate-dependent abnormalities in neuronal activity, since neuronal activity can induce neurotransmitter receptor clustering (Rao et al. 2000). Perhaps related to the abnormalities in AMPA receptor subunit clustering, cells lacking the SVCT2 also had decreased neuronal activity. In patch-clamp experiments, cells from Slc23a2 (–/–) mice showed both decreased amplitude and frequency of miniature excitatory post-synaptic currents. It seems likely that these findings are due to the ability of cells containing the SVCT2 to retain intracellular ascorbate in culture, rather than to the presence of the SVCT2 per se.

6.5 Ascorbate Accumulation and Maintenance in the CNS: Role of SVCT2

As shown in Fig. 6.2, there are two mechanisms by which ascorbate can enter neurons and glia: transport of ascorbate on the SVCT2 and uptake and reduction of dehydroascorbate. The SVCT2 mediates transport of ascorbate both from plasma across the choroid plexus to the CSF and across the neuronal cell plasma membrane. The plasma-CSF ascorbate gradient is about 4-fold in humans (Reiber et al. 1993; Lönnrot et al. 1996) and animals (Spector and Lorenzo 1973; Spector 1977),

resulting in CSF ascorbate concentrations of about 200 μM, compared to plasma concentrations of 50 μM or less (Table 6.1). Although DHA enters the CNS more rapidly than ascorbate, the latter does readily penetrate the central nervous system after oral administration (Kontush et al. 2001). For example, supplements of one g vitamin C a day to volunteers already replete with vitamin C increased plasma levels from 50 to 75 μM, and increased CSF ascorbate from 200 to 250 μM (Lönnrot et al. 1996). Neuronal ascorbate uptake further accentuates the ascorbate concentration gradient from plasma to neurons, and brain neurons are said to have the highest ascorbate concentrations in the body (Rose 1988). In this regard, neuronal ascorbate concentrations in young rats have been calculated to be as high as 10 mM, whereas glial ascorbate concentrations are only 0.9 mM (Rice and Russo-Menna 1998; Rice 2000). Such high ascorbate concentrations in young rats agree with the finding that SVCT2 mRNA is increased in fetal mouse brain compared to brain from adult animals (Castro et al. 2001).

It also appears that much of the ascorbate in brain is in neurons. This was first shown over four decades ago in histochemical studies (Shimizu et al. 1960). More recent investigations have localized high ascorbate concentrations to neuron-rich areas of the hippocampus and neocortex in normal human brain, where the ascorbate content is as much as two-fold higher in than in other brain regions (Mefford et al. 1981; Milby et al. 1982). The higher ascorbate contents of neurons in comparison to glia, as alluded to above, may well account for varying ascorbate contents of different brain regions. In post-natal rat brain, for example, areas rich in glia have ascorbate levels only about 10–20% those of areas with high neuronal density (Rice and Russo-Menna 1998). Indeed, whereas GSH is the major low molecular weight cytosolic reducing agent in most cells (e.g., in glia the ascorbate/GSH ratio is 0.24), in neurons ascorbate may take that role, since neuron-rich brain areas have an ascorbate/GSH ratio of 4.0 (Rice and Russo-Menna 1998). This differential in ascorbate content is probably due to the SVCT2, since in situ hybridization studies in rat brain show that SVCT2 mRNA is present only in neurons and not in astrocytes (Castro et al. 2001; Berger et al. 2003). More recent studies show that SVCT2 mRNA is expressed in certain glial elements in the hypothalamus (García et al. 2005).

Although astrocytes may not express the SVCT2 in vivo, oxidant stress can induce SVCT2 expression in astrocytes and increase expression in neurons. For example, ischemia-reperfusion injury following middle cerebral artery occlusion in rats increased SVCT2 mRNA over several hours in the peri-infarct penumbra, both in neurons and in glia (Berger et al. 2003). Also, astrocytes in culture develop high affinity ascorbate transport and SVCT2 mRNA (Wilson et al. 1990), an effect that has been attributed to exposure to high oxygen concentrations in culture (Berger et al. 2003).

As noted previously, the second route by which ascorbate can enter the CNS is via transport of dehydroascorbate across the blood-brain barrier on the ubiquitous GLUT1 glucose transporter. Any DHA generated in the brain interstitium would also be taken up by GLUT1 and GLUT3 in glia and neurons (Fig. 6.2). Since dehydroascorbate concentrations in plasma are usually only 0–2% of ascorbate

concentrations (Dhariwal et al. 1991), this route of entry across the blood-brain barrier may not contribute substantially to total brain ascorbate concentrations under normal conditions. However, during oxidant stress in the CNS, ascorbate in the CSF and interstitial space will be oxidized to the AFR, which will then dismutate to form ascorbate and DHA (Fig. 6.1). The latter can then enter both glia and neurons on glucose transporters in substantial amounts, where it will be rapidly reduced to ascorbate. This likely serves as a back-up mechanism to recover oxidized ascorbate before it is irretrievably lost with degradation of DHA. A similar mechanism in cultured HL-60 cells has recently been termed the "bystander" effect (Nualart et al. 2003).

The SVCT2 thus appears to be crucial in maintaining ascorbate homeostasis in the CNS and in neurons. However, little known about SVCT2 structure as it relates to function or about specific mechanisms by which SVCT2 expression or function are regulated.

6.6 Structure-Function and Regulation of the SVCT2 in Neurons

The SVCT2 transporter was one of two SVCT-type transporters cloned in 1999 by Hediger's group (Tsukaguchi et al. 1999), both of which turned out to be members of the solute carrier (Slc) family of transporters. As noted previously, the two SVCT proteins are quite homologous, with only minor differences between species (Takanaga et al. 2004). There has been confusion in the literature regarding official designations of the ascorbate transporter genes, but currently accepted usage is that the Slc23a1 gene encodes the SVCT1 protein, and that Slc23a2 encodes the SVCT2 protein (Takanaga et al. 2004). The distributions of the two SVCT proteins differ in that mRNA for the SVCT1 is found in epithelial tissues involved in ascorbate absorption (intestine) and re-absorption (kidney). The SVCT2 (Fig. 6.3) is the major isoform in most other tissues in the mouse, with highest expression in brain and in areas of high neuronal density in particular (Tsukaguchi et al. 1999).

Neuronal and most other cells have long been known to accumulate ascorbate against a concentration gradient via a saturable, sodium-dependent mechanism (Wilson 2004). With the cloning of the protein, it became apparent that this sodium- and energy-dependent transport is mediated largely by SVCT2 (Tsukaguchi et al. 1999). The SVCT2 has a high affinity for ascorbate (Km = 20–40 μM) that corresponds well to plasma ascorbate concentrations (30–50 μM). In neuronal cells the apparent Km for ascorbate appears to be somewhat higher (113 μM (May et al. 2006)), but even with this modest decrease in affinity, the SVCT2 will still be nearly saturated at the ascorbate concentrations present in CSF (200–400 μM). If the SVCT2 is saturated, then acute regulation of its function by changes in substrate affinity will be minimal in contrast to changes in transporter Vmax or transporter expression in the membrane. It seems likely that transporter number and ability to function rather than affinity are the major mechanisms involved in regulation of the SVCT2.

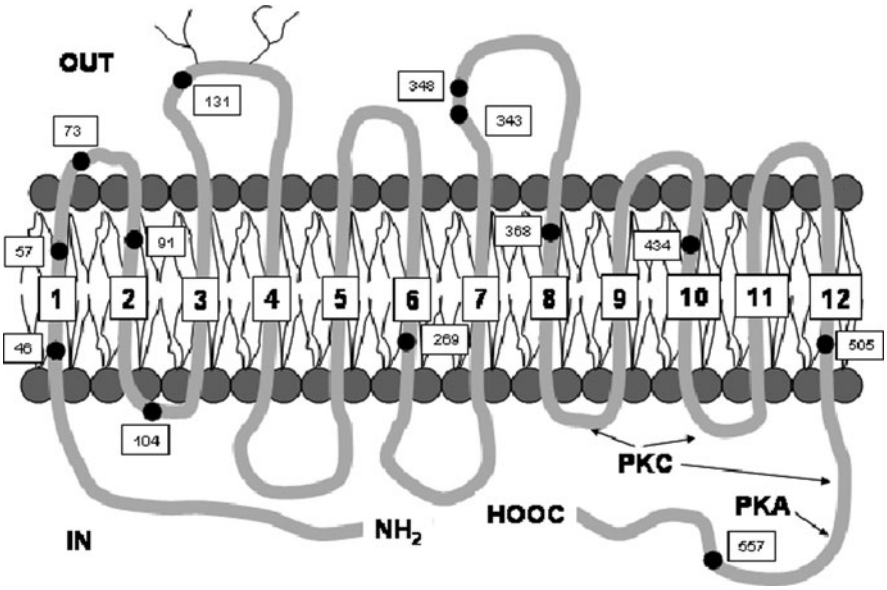

Fig. 6.3 Trans-membrane structure of rat brain SVCT2 (Tsukaguchi et al. 1999). *Numbered dots* show the location of the 13 cysteines in the sequence

Rat brain SVCT2 cDNA codes for a 647-amino acid protein that hydropathy analysis shows to cross the cell membrane 12 times, with both the N- and C-termini in the cytoplasm (Fig. 6.3) (Tsukaguchi et al. 1999). Assuming an average amino acid molecular weight of 121, this predicts that it will migrate as a 78 kDa protein on electrophoretic gels. This electrophoretic migration on Western blotting has been reported by two laboratories (Lutsenko et al. 2004; García et al. 2005). Although there have been reports of the SVCT2 from various tissues migrating in the 50–65 kDa region on Western blots using detection by polyclonal antibodies (Li et al. 2003; Wu et al. 2003; Jin et al. 2005; Mun et al. 2006), these results could be explained by proteolysis during sample preparation, by expression of short forms of the transporter (Lutsenko et al. 2004), or by variable glycosylation of the protein. Regarding the latter, the SVCT2 does have potential N-glycosylation sites on an exofacial loop between trans-membrane segments three and four (Tsukaguchi et al. 1999). Confirmation that the protein is glycosylated was provided by Liang and co-workers (Liang et al. 2002), who showed that endoglycosidase digestion of extracted protein decreased its apparent migration on electrophoretic gels from about 80 to 65 kDa. This finding adds support to the trans-membrane loop topology originally projected by hydropathy analysis (Tsukaguchi et al. 1999). Moreover, the expression of non-glycosylated transporter precursors could explain the presence of bands migrating at lower apparent molecular weights on Western blots in the studies noted above. Shortening of the protein will also generate lower molecular weight bands. Lutsenko et al. (2004) showed that a truncated form of the SVCT2 was

present in cultured cells by Western blotting and in normal human brain by PCR. They hypothesized that this truncated SVCT2 corresponds to a non-functional variant that is missing 115 amino acids from domains five and six (Fig. 6.3). Whereas a 75–80 kDa protein found on Western blots likely represents the SVCT2, shorter forms may also be present as well.

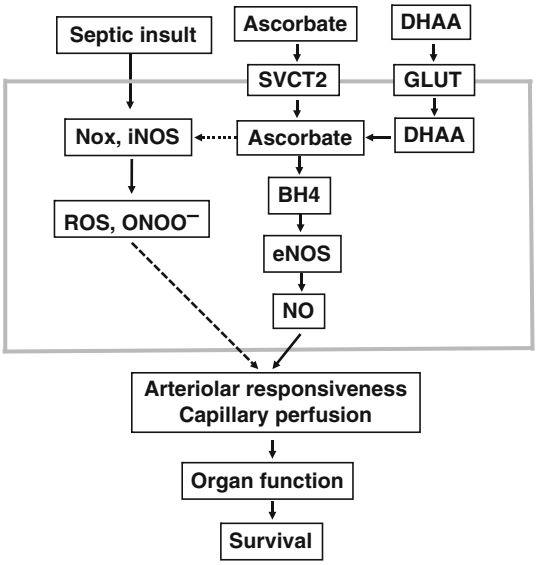

Fig. 6.4 Ascorbate preservation of nitric oxide sustains capillary perfusion

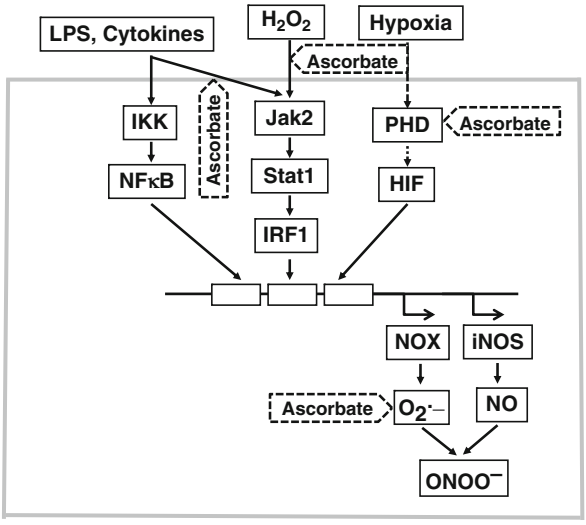

Fig. 6.5 Sites of cellular protection by ascorbate against oxidative stress

There are several plausible mechanisms for regulation of SVCT2 function. First, the SVCT2 protein has putative sites for protein kinase-A and protein kinase-C regulation on the cytoplasmic portions (Tsukaguchi et al. 1999) (Fig. 6.3) Studies in cultured cells support the notion that these are functional in acute regulation of the protein, since ascorbate transport Vmax is decreased by both dibutyryl cyclic AMP (Siushansian and Wilson 1995; Korcok et al. 2000) and by protein kinase-C agonists (Daruwala et al. 1999; Liang et al. 2002) (Figs. 6.4 and 6.5). Intracellular ascorbate may also regulate SVCT2 expression, based on the findings of Wilson et al., that there is a time-dependent up-regulation of ascorbate transport in astrocytes depleted of the vitamin in culture (Wilson et al. 1990). A recent study (Seno et al. 2004) has also shown that function of the SVCT2 expressed in human umbilical vein endothelial cells is inhibited by tumor necrosis factor-α and by interleukin-1β. Since these inhibitor effects were maximal after 5 or more hours of exposure in culture, it seems likely that they were due to changes in protein expression rather than affinity. If so, these studies bring up the notion that the SVCT2 may be regulated by inflammatory cytokines and thus possibly by the level of oxidant stress experienced by the cells.

In addition to regulating SVCT2 transporter expression, oxidant stress could directly modify transporter function by oxidizing key amino acids in the protein. This would most likely involve formation of mixed disulfides or oxidation of cysteine sulfhydryls or methionine, thus causing changes in cell surface or protein redox status. For example, we found in cultured endothelial cells (May and Qu 2004) that rates of ascorbate transport on the SVCT2 are very sensitive to sulfhydryl reagents. Transport was inhibited by agents restricted to the extracellular space, suggesting that one or more of the four cysteines on the exofacial transporter (Fig. 6.2) are involved in the transport mechanism. The presence of one or more exofacial sulfhydryls on the SVCT2 mirrors the situation with the GLUT1 glucose transporter. On that protein, modification of a single sulfhydryl (Cys-429) decreased affected transporter affinity for glucose (May 1988; May et al. 1990), although cysteine-scanning mutagenesis showed that its substitution with serine did not affect transporter function in Xenopus oocytes (Due et al. 1995). SVCT2 expression or function may also be regulated by changes in intracellular redox stress. For example, ascorbate transport in cultured endothelial cells is decreased in parallel with intracellular GSH concentrations in cultured endothelial cells (May and Qu 2004), which suggests that it is susceptible to redox regulation.

The presence of a non-functional truncated SVCT2 may also modify transporter function. In cultured cells truncated SVCT2 was present on the cell surface and expressed inversely in proportion to rates of ascorbate transport (Lutsenko et al. 2004). Further, transfection of the cells with truncated SVCT2 cDNA inhibited ascorbate transport. This dominant-negative inhibition was thought due to protein-protein interaction between functional and non-functional transporters. decreased ascorbate transport (Lutsenko et al. 2004).

Whether any of these potential mechanisms for SVCT2 regulation are important for the CNS protein remains to be determined. Other considerations regarding how neuronal ascorbate concentrations are regulated are the extent to which entry of ascorbate as DHA on the GLUT transporters supplies neuronal ascorbate, whether

neurons and glia interact to maintain CNS ascorbate, and the extent to which glutamate-induced ascorbate efflux from neurons modulates intracellular ascorbate concentrations.

6.7 Conclusion

Lethality of the SVCT2 knockout on day 1 of life could reflect the need for ascorbate in the lung or the CNS. Indeed, any damage to the CNS in utero may simply show that ascorbate is required for neuronal maturation, not for maintenance and function of mature neurons. Nonetheless, differential retention of brain ascorbate in scurvy and the novel two-step concentrating mechanism used by the CNS to get ascorbate into neurons suggests that the importance of the vitamin for neuronal repair, myelination, and function may have been underestimated.

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Chapter 7
Genetic Aspects of Folate Metabolism

Anne M. Molloy

Abstract The vitamin folate functions within the cell as a carrier of one-carbon units. The requirement for one-carbon transfers is ubiquitous and all mammalian cells carry out folate dependent reactions. In recent years, low folate status has been linked to risk of numerous adverse health conditions throughout life from birth defects and complications of pregnancy to cardiovascular disease, cancer and cognitive dysfunction in the elderly. In many instances inadequate intake of folate seems to be the primary contributor but there is also evidence that an underlying genetic susceptibility can play a modest role by causing subtle alterations in the availability, metabolism or distribution of intermediates in folate related pathways. Folate linked one-carbon units are essential for DNA synthesis and repair and as a source of methyl groups for biological methylation reactions. The notion of common genetic variants being linked to risk of disease was relatively novel in 1995 when the first functional folate-related polymorphism was discovered. Numerous polymorphisms have now been identified in folate related genes and have been tested for functionality either as a modifier of folate status or as being associated with risk of disease. Moreover, there is increasing research into the importance of folate-derived one-carbon units for DNA and histone methylation reactions, which exert crucial epigenetic control over cellular protein synthesis. It is thus becoming clear that genetic aspects of folate metabolism are wide-ranging and may touch on events as disparate as prenatal imprinting to cancer susceptibility. This chapter will review the current knowledge in this area.

Keywords Folate · Vitamin B12 · Homocysteine · Methylenetetrahydrofolate reductase (MTHFR)

7.1 Introduction

The availability of one-carbon units is critical to cell proliferation and metabolism. The vitamin folate acts as a transitional molecule in the transfer of one-carbon units from donor molecules to various intermediates in the synthesis of DNA and in

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biological methylation reactions, including the methylation of DNA, proteins, phospholipids, neurotransmitters, etc. Accordingly, maintenance of folate homeostasis is essential to growth and development. The effects of clinical folate deficiency on rapidly proliferating tissues such as those of the erythropoietic system are long known and well described (Lindenbaum and Allen 1995). More recent studies go beyond this knowledge by implicating a mild to moderately disrupted folate homeostasis in increased risk of numerous adverse health conditions throughout an individual's lifetime, including birth defects and other unfavorable developmental outcomes, cardiovascular disease (CVD), some cancers and cognitive dysfunction (Choi and Mason 2002; Herrmann and Knapp 2002; Mangoni and Jackson 2002; Molloy et al. 2008; Rampersaud et al. 2003; Ravaglia et al. 2005; Ueland et al. 2000; Vollset et al. 2000; Xu and Chen 2009). These diseases had long been known to have complex etiologies, involving genetic and environmental components.

The close association of plasma total homocysteine (tHcy) with folate status has generated more research interest than any other aspect of folate metabolism and has been a key analysis in determining the functionality of newly discovered polymorphisms in folate related genes. The first suggestions that common genetic diversity in folate related genes might influence risk of disease came from work by Kang et al. (1988a) who studied tHcy concentrations in subjects with CVD and identified a subset of patients with mild hyperhomocysteinemia who appeared to have a thermolabile form of the folate metabolizing enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). Around the same time, a growing body of evidence culminated in clinical trials that conclusively showed that periconceptional supplementation with folic acid could prevent the majority of neural tube defects (NTDs) (Czeizel and Dudas 1992; MRC 1991). Women who had given birth to children with NTDs were not clinically deficient in folate but they did tend to have lower blood folate concentrations (Daly et al. 1995), suggesting an interaction of folate nutrition and genetic factors that was negatively affecting some aspect of folate related pathways at the time of neural tube closure. Current knowledge of the genetic aspects of folate metabolism has been driven primarily by the search for polymorphisms in candidate genes conferring NTD risk (Molloy et al. 2009) but this has expanded logically to examine functionality in other respects by searching for alternative clinical or biochemical phenotypes such as an association of such polymorphisms with CVD or cancer and an effect on blood indices of folate status, mainly serum folate, red cell folate and tHcy. Numerous polymorphisms have now been identified in folate pathway genes and have been examined for such functionality. Perhaps not surprisingly, apart from the 677C→T polymorphism in the MTHFR gene (discussed in more detail later), the outcomes of these association studies have been inconsistent and often confusing, due largely to the fact that such common genetic diversity has a low penetrance in relation to disease and biochemical phenotype. Because of this low penetrance, studies are often limited by factors such as low sample size and inadequate power to detect small differences between groups; inappropriate control selection for such type of study; variability of genotype prevalence in different ethnic groups and complex interactions between nutrient status and genotype prevalence in different populations (Amorim et al. 2007; Gueant-Rodriguez et al. 2006; Mitchell et al. 2004).

An alternative line of research has focused on the links between folate and metabolic processes that control gene expression. These studies address the important question of whether low folate status can alter the quantity and pattern of methylated DNA or methylated histone in specific tissues, including DNA and histone methylations that may influence early carcinogenesis, or methylations in utero that may form part of the fetal programming of genes for long-term expression and silencing (Antony 2007; Duthie et al. 2004; Esteller 2008; Lillycrop et al. 2008; Sinclair et al. 2007; Xu and Chen 2009). Although the technology to carry out this type of research with adequate sensitivity is relatively new and is still being developed, impressive evidence is already accumulating to confirm a fundamental role of folate dependent one-carbon metabolism in epigenetic processes and a susceptibility of these processes to changes in folate status.

7.2 Folate Cofactor Interactions

The biochemical role of the folate cofactors in one-carbon metabolism has been documented in numerous reviews (Bailey and Gregory 1999; Mason 2003; Scott and Weir 1998; Selhub 2002; Stover 2004). Tetrahydrofolate (THF) is the biologically active parent molecule and consists of a fully reduced pteridine ring linked to a para-aminobenzoyl unit and thence to a glutamic acid residue (Fig. 7.1). Within the cell, up to eight additional glutamic acid residues are added to the molecule in order to ensure its retention in the cell and increase its affinity for folate dependent enzymes (Shane 1989). THF carries one-carbon units as methyl (CH3), formyl (CHO), methylene (CH2), methenyl (CH=) or formimino (CHNH) derivatives on carbon 5, or carbon 10 of the pteridine ring, or as a 5,10 adduct of the pteroyl-para-aminobenzoyl moiety.

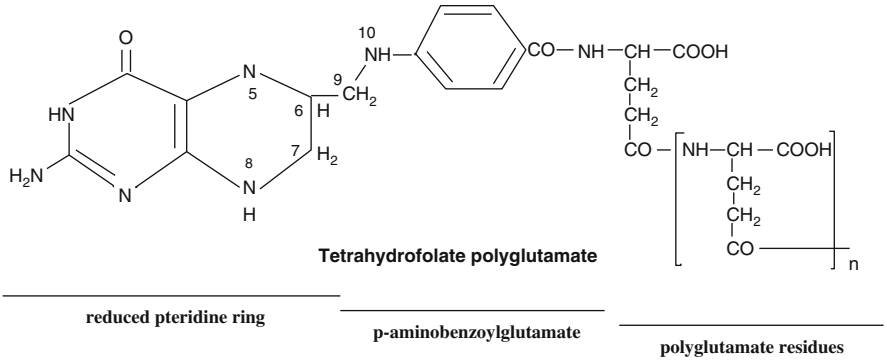

Fig. 7.1 Tetrahydrofolate (THF) polyglutamate, showing the reduction of the pteridine ring at positions 5,6,7 and 8 of the pteridine ring; the attachment of the pteridine to p-aminobenzoylglutamate at the C9–N10 bond; and the presence of a number of polyglutamate residues. One-carbon units are added to this molecule at positions 5 and 10 or as a 5,10 adduct of the pteroyl-para-aminobenzoyl moiety

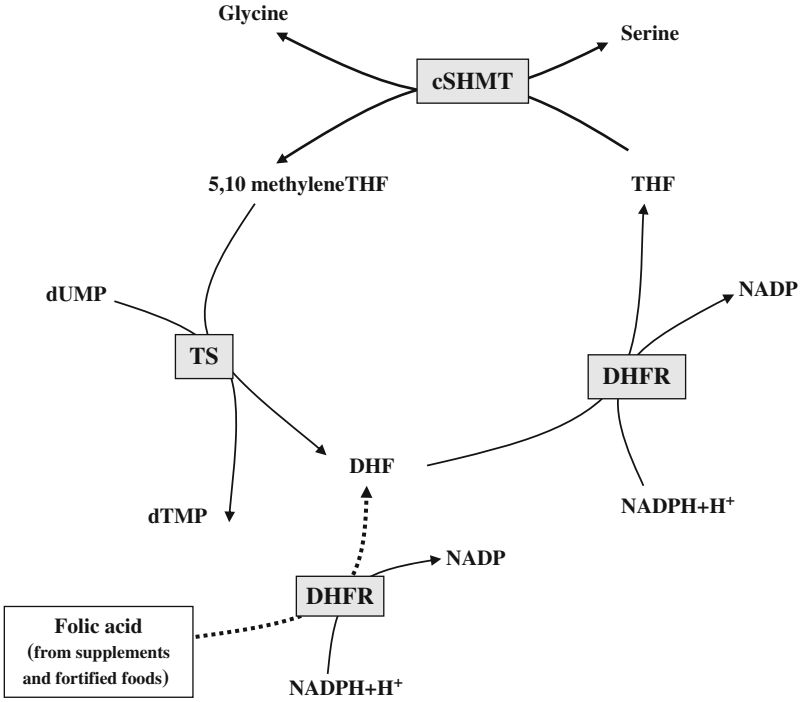

Fig. 7.2 Folate dependent thymidylate synthesis: the enzymes in this pathway are thymidylate synthase (TS); dihydrofolate reductase (DHFR); and cytosolic serine hydroxymethyltransferase (cSHMT). The metabolites are deoxyuridine monophosphate (dUMP); deoxythymidine monophosphate (dTMP); dihydrofolate (DHF); tetrahydrofolate (THF). Synthesis of thymidylate occurs in the nuclear fraction of the cell

One-carbon units are obtained from several sources, most importantly from mitochondrially generated formate through the trifunctional C1-synthase enzyme encoded by the MTHFD1 gene and from serine, through cytosolic and mitochondrial isoforms of serine hydroxymethyltransferase (SHMT). As folate derivatives, these one-carbon units are transferred to specific intermediates in the de-novo synthesis of purines and thymidylate and in the synthesis of methionine from homocysteine. The biological importance of folate lies in the crucial downstream functions of these molecules, as summarized below.

7.3 Folate in DNA Synthesis

Two metabolic systems in the folate network transfer folate cofactors to intermediates in DNA synthesis. In one system, deoxyuridine monophosphate (dUMP) is converted to deoxythymidine monophosphate (dTMP) by the addition of a methyl group from 5,10-methylene THF. Thymidylate synthase (TS) catalyzes

this reaction and the co-product dihydrofolate (DHF) is then converted to THF via dihydrofolate reductase (DHFR). The cycle is completed with THF accepting another one-carbon unit and regenerating 5,10 methylene THF (principally via cSHMT). Recent work demonstrates that this thymidylate synthesis system is under a high degree of metabolic control and the three enzymes involved in de-novo thymidylate synthesis become localised to the nucleus or the nuclear periphery for activity (Anderson et al. 2007). A common non-synonymous single nucleotide polymorphism (SNP):1420C→T (L474F), in the cSHMT gene has been shown to weaken the interaction of SHMT with other components in this process (Woeller et al. 2007). Apart from the obvious importance that the thymidylate synthesis system has in relation to cell proliferation, there has been considerable recent research on its role in the ongoing maintenance and repair of DNA. Impaired folate status

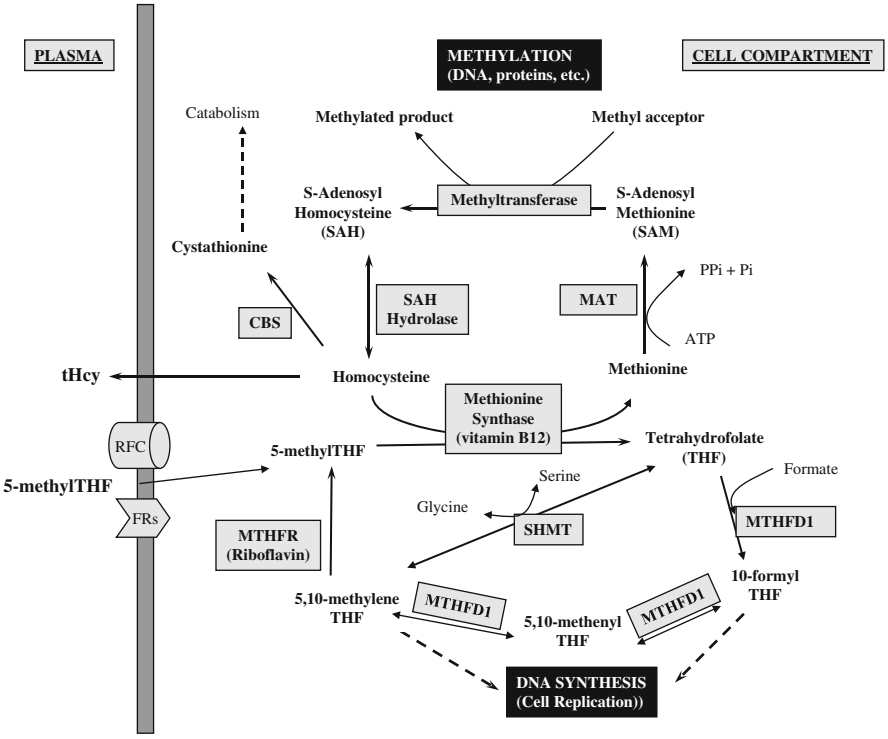

Fig. 7.3 The flux of one-carbon units through folate cofactors: the enzymes annotated in this figure are 5,10-methylenetetrahydrofolate reductase (MTHFR); methionine adenosyltransferase (MAT); S-adenosylhomocysteine hydrolase (SAH hydrolase); cystathionine-β-synthase (CBS); serine hydroxymethyltransferase (SHMT); the trifunctional C1-synthase enzyme incorporating the activities of formyl-THF synthetase, cyclohydrolase and dehydrogenase activities (MTHFD1, the gene encoding these enzyme activities). The transport of reduced folates into the cell is facilitated primarily by the reduced folate carrier (RFC). Folate receptors (FRs) also transport folates into cells. Plasma total homocysteine is denoted by tHcy

in the microenvironment of a specific tissue can result in abnormalities of DNA synthesis, such as uracil misincorporation into DNA, leading to replication abnormalities, strand breaks, increased micronucleus formation and genomic instability (Duthie et al. 2004; Fenech 2001). It is now accepted that these effects occur at suboptimal folate concentrations above the clinically deficient cut-off level. Persistent uracil misincorporation into DNA has been one of the primary hypotheses for explaining the role of folate in the development of certain cancers (Choi and Mason 2002; Xu and Chen 2009).

Several studies have examined the SHMT 1420C→T polymorphism for association with disease risk. No significant association with NTD was observed (Heil et al. 2001; Relton et al. 2004) but a positive link was found between the occurrence of CVD in persons with the T allele, particularly in those who also carried the MTHFR 677 TT genotype (Lim et al. 2005). Common polymorphisms in TS and DHFR have also been studied, in particular a polymorphic 28 bp double or triple repeat in the TS promoter enhancer region (TSER) and a 19 bp insertion/deletion polymorphism in DHFR. In general, reported association studies of these polymorphisms with NTDs and with certain forms of cancer have been inconsistent (Kalmbach et al. 2008; Parle-McDermott et al. 2007; Skibola et al. 2002; Ulrich et al. 2005; Volcik et al. 2003) although it appears that an interaction between low dietary folate status and the TSER polymorphism may provide a decreased risk of some forms of cancer (Skibola et al. 2002; Ulrich et al. 2005). Interestingly, recent work suggests that the DHFR 19 bp polymorphism might influence an individual's ability to convert folic acid, the folate precursor found in vitamin supplements and fortified foods, to biologically active THF (Kalmbach et al. 2008). This is a biologically plausible effect since reduction to DHF and thence to THF via the enzyme DHFR is the mechanism whereby folic acid enters the cellular folate pool.

In the second DNA synthesis system, one-carbon units from mitochondrially-generated formate enter the tri-functional C1-synthase complex as 10-formyl THF via the enzyme's 10-formylTHF synthetase domain. These folate cofactors then participate in the formation of intermediates in de-novo purine synthesis (Anguera et al. 2006; Christensen and MacKenzie 2006; Fowler et al. 1997) or undergo rearrangement to 5,10 methyleneTHF for use in thymidylate synthesis or for channelling through MTHFR to 5-methylTHF. A polymorphism in the synthetase domain of MTHFD1 (R653Q) has been associated with NTDs and other pregnancy complications (Brody et al. 2002; De Marco et al. 2006; Parle-McDermott et al. 2005a, b). Consistent with the deleterious nature of the R653Q polymorphism, mice lacking this synthetase domain have been found not to survive the embryonic period (MacFarlane et al. 2009), but little else is known of the phenotype.

7.4 Folate in Methylation Reactions

The third fate of folate derived one-carbon units is in the remethylation of homocysteine to methionine, catalyzed by the vitamin B12 (cobalamin) dependent enzyme, methionine synthase. This reaction is crucial to maintain a supply of methyl

groups through S-adenosylmethionine (SAM) for the methylation of DNA, proteins, neurotransmitters, etc. Methionine synthase, encoded by the MTR gene, is one of only two enzymes in mammalian systems that are known to require vitamin B12 as a cofactor, in this case as methylcobalamin. This cofactor is prone to oxidation and the enzyme therefore requires a reductive methylation system involving the flavoprotein methionine synthase reductase and SAM as the methyl donor to re-prime the enzyme every 200–1,000 turnovers (Bandarian et al. 2003; Yamada et al. 2006). Because of this, adequate functioning of both methionine synthase and methionine synthase reductase is required to maintain the methylation of homocysteine and the provision of methyl groups to SAM mediated methyltransferase reactions. Genetic mutations in these two enzymes are responsible for two complementation classes of vitamin B12 deficiency, CblG and CblE (Leclerc et al. 1996; Watkins et al. 2002; Wilson et al. 1999a). Complete ablation of the MTR gene in mice is embryonically lethal and mice lacking a functional MTRR gene (that encodes methionine synthase reductase) also show evidence of reproductive failure (Deng et al. 2008; Elmore et al. 2007). Common polymorphisms have been described in the genes for both enzymes. The most widely studied of these are the 2756A→G (D919G) variant in MTR, and the 66A→G (I22M) variant in MTRR. These polymorphisms have been evaluated in numerous studies for metabolic and disease risk effects. Modest impacts on vitamin B12 status, plasma tHcy concentration and on birth defect, CVD and cancer risk have been reported but the results are not consistent between studies and several large highly-powered negative studies have tended to cast doubt on the reliability of smaller positive studies (Bosco et al. 2003; Chen et al. 1997:1998, 2001; Christensen et al. 1999; Dekou et al. 2001; Klerk et al. 2003; Tsai et al. 1999a; Ulrich et al. 2005; Ulvik et al. 2004; Wilson et al. 1999b; Yang et al. 2008).

As indicated above, SAM is the universal donor of methyl groups for almost all biological methyltransferase reactions, involving in excess of 40 known enzymes in mammalian systems. SAM is synthesized from methionine and ATP via methionine adenosyltransferase (MAT). Liver expresses a MATIA isoform, which can produce high SAM concentrations as the intracellular methionine level increases (Finkelstein 2007; Molloy et al. 1990). Most other tissues express the MAT II isoform, which is inhibited by high tissue levels of SAM, ensuring that utilization and synthesis of SAM are tightly balanced (Kotb and Kredich 1990; LeGros et al. 2001). Severe genetic mutations in the MATIA gene lead to hyper-methioninemia without hyperhomocysteinemia and severe deficiency can lead to neurological symptoms similar to those seen in deficiency of MTHFR but little is known of genetic mutations in the MATII gene or of disease risk or biochemical effects associated with common polymorphisms in these genes. (Chamberlin et al. 2000; Couce et al. 2008; Perez Mato et al. 2001; Surtees et al. 1991; Ubagai et al. 1995).

The simultaneous conversion of SAM to S-adenosylhomocysteine (SAH) in methyltransferase reactions, followed by the hydrolysis of the SAH adenosyl moiety via SAH hydrolase, regenerates homocysteine and completes this methylation cycle whereby methyl groups from the folate one-carbon pool are incorporated into a multitude of biological compounds. Along with folate, homocysteine is an essential carrier of methyl groups in this process since folate derived methyl groups

are provided to the methionine cycle via the constant recycling of homocysteine to form methionine. Methylation is a crucial regulatory modification that affects a wide spectrum of housekeeping functions within the cell. A decrease in availability of methyl groups from the folate cofactor pool leads to impaired recycling of homocysteine, and inhibition of methylation reactions, including those that regulate gene expression and other epigenetic or post-translational processes. The inhibition of methyltransferase reactions is, to a large extent, due to the equilibrium of the SAH hydrolase reaction, which favours synthesis of SAH rather than hydrolysis and leads to a build-up of intracellular SAH, a potent competitive inhibitor of all methyltransferase reactions (Caudill et al. 2001; Finkelstein 2007; McKeever et al. 1995; Molloy et al. 1992; Yi et al. 2000). Individuals with genetic mutations in SAH hydrolase show markedly elevated plasma SAH concentrations (Baric et al. 2004; Buist et al. 2006).

The influence of folate status on the methylation cycle has been difficult to demonstrate although many current studies are addressing this issue by examining the effect of folate deficiency on total and site specific genome DNA methylation patterns (Basten et al. 2006; Jacob et al. 1998; Shelnutt et al. 2004; Wasson et al. 2006; Yi et al. 2000). Histone methylation, which provides the next layer of epigenetic control (Esteller 2008; Lee and Mahadevan 2009; Mathers and McKay 2009) is also potentially susceptible to folate status. It is now thought that in conjunction with uracil misincorporation into DNA, as noted earlier, changes in DNA or histone methylation, resulting from persistent marginally low folate status, may be an important early mechanism predisposing to cancer development (Duthie et al. 2002; Fenech 2001; Friso and Choi 2002; Jang et al. 2005; Mason 2003). Furthermore, a line of current research is exploring the notion that maternal prenatal folate status may, through its effects on embryonic methylation reactions, influence the expression and repression of developmental genes and may culminate in permanent modifications to the expression of certain genes in the offspring (Burdge et al. 2009; Lillycrop et al. 2008; Sinclair et al. 2007; Zeisel 2009). Such changes may provide mechanistic support to the theory that there are developmental origins to diseases in later life (Mathers and McKay 2009; Zeisel 2009).

7.5 Homocysteine

Unlike folate, homocysteine is a highly reactive and toxic metabolite whose level must be carefully balanced within the cell. The ongoing requirement for methylation of DNA, proteins, lipids, neurotransmittors and other biological intermediates within the cell generates a persistent supply of homocysteine that either must be recycled to methionine or catabolized via cystathionine β-synthase, a vitamin B6 dependent enzyme that is only expressed at high levels in liver and kidney (Finkelstein 2000). The gene encoding cystathionine β-synthase has been well described and a number of severe genetic mutations in this gene have been shown to cause the classical clinical condition of homocystinuria (Kraus 1998; Linnebank

et al. 2004; Miles and Kraus 2004; Vyletal et al. 2007). A relatively common 68 bp insertion polymorphism (844ins68) has been studied for its effect on plasma tHcy concentrations (Tsai et al. 1996, 1999b). Some studies suggest that this polymorphism interacts with polymorphisms in MTHFR and MTRR to modulate the plasma tHcy concentration, but the phenotypic effect is small (Dekou et al. 2001; Kluijtmans et al. 2003; Silaste et al. 2001; Summers et al. 2008; Tsai et al. 1999b).

Liver and kidney also contain high levels of a second enzyme, betaine-homocysteine methyltransferase (BHMT) that is capable of remethylating homocysteine independently of folate (Finkelstein 2007; Sunden et al. 1997; Ueland et al. 2005). The activity of this enzyme has an important sparing effect on folate in these tissues and the substrate, betaine, that provides the methyl group in the reaction, is useful as an alternative clinical therapy for lowering plasma tHcy in individuals with abnormally high tHcy concentrations (Holm et al. 2007; Melse-Boonstra et al. 2005; Schwab et al. 2006; Schwahn et al. 2007; Ueland et al. 2005). A common polymorphism in the BHMT gene (742G→A; R239Q) has been studied for disease or functional associations but no strong or consistent trends have been reported to date (Ananth et al. 2007; Boyles et al. 2006; da Costa et al. 2006; Fredriksen et al. 2007; Weisberg et al. 2003; Zhu et al. 2005).

7.6 Methylenetetrahydrofolate Reductase

One enzyme in particular, MTHFR, exerts central control over the partitioning of folate cofactors between methylation and DNA synthesis reactions. MTHFR catalyzes the essentially irreversible conversion of 5,10-methyleneTHF to 5-methylTHF. This enzyme therefore channels one-carbon units away from purine and pyrimidine synthesis and into the provision of methyl groups for SAM mediated methylation reactions. MTHFR is a flavoprotein containing loosely bound flavin adenine dinucleotide (FAD) that accepts reducing equivalents from NADP(H). The enzyme exhibits strong allosteric inhibition by SAM, the downstream product of the MTHFR-methionine synthase metabolic locus (Daubner and Matthews 1982; Kutzbach and Stokstad 1971). In mammals, the enzyme exists as a homo-dimer of about 77 kd, containing a catalytic domain and a SAM regulatory domain (Matthews et al. 1984). Several features are remarkable about the MTHFR-methionine synthase locus. Firstly, the reduction of 5,10-methyleneTHF via MTHFR is the only biological process known to generate 5-methylTHF. Secondly, newly absorbed dietary folates that are not retained within the enteric or hepatic folate pools are released into the circulation as 5-methylTHF. This therefore is the predominant form of the vitamin found in the circulation and presented to the cell. 5-methylTHF must be de-methylated through methionine synthase in order to be incorporated into the intracellular folate pool, therefore optimal activities of both MTHFR and methionine synthase (along with adequate availability of three vitamins; folate, vitamin B12 and riboflavin) are required to remethylate homocysteine and ensure constant functioning of methylation processes and also to incorporate circulating folate into the

cell. Recent research has shown that MTHFR essentially competes with cSHMT for available 5,10-methyleneTHF (Herbig et al. 2002; Oppenheim et al. 2001; Woeller et al. 2007) indicating that there are several layers of control in maintaining the balance of folate cofactors between DNA synthesis and methylation reactions.

7.7 The MTHFR677C→T Polymorphism

Genetic inborn errors in MTHFR that give rise to less efficient function and animal models that diminish MTHFR activity reduce the supply of methyl groups to the methylation cycle. These conditions are characterised by abnormally high plasma tHcy concentrations and display a variety of neurological and vascular symptoms (Baumgartner et al. 1985; Chwatko et al. 2007; Erbe 1979; Goyette et al. 1996; Li et al. 2008; Rosenblatt 1995; Schwahn et al. 2007; Sibani et al. 2003). In the 1980s, Kang and co-workers (Kang et al. 1988a, b) discovered a 'thermolabile' version of MTHFR in patients with coronary artery disease, which they predicted was due to a genetic mutation in MTHFR. When the cDNA for the human MTHFR gene was isolated, (Goyette et al. 1994) a common polymorphism was discovered (677C→T) that resulted in an amino acid change in the protein from alanine to valine (A222V) (Frosst et al. 1995). The variant enzyme was thermolabile in vitro; i.e. lymphocyte cultures from homozygote individuals displayed reduced total MTHFR activity and significantly lower residual activity after heating to 42°C (Frosst et al. 1995). The observation that the polymorphism was also a cause of mild hyper-homocysteinemia helped to convince researchers that this was the mutation predicted by Kang and co-workers.

The polymorphism has been studied in great detail in the past decade and is somewhat unique among folate polymorphisms as having known functionality in terms of molecular mechanism, biochemical phenotype, effect modification by nutrient status and impact on risk of disease. Studies in vitro using E. coli constructs (the homologous mutant being A177V) (Guenther et al. 1999; Pejchal et al. 2006) or recombinant human enzyme (Yamada et al. 2001) demonstrated weaker binding affinity for the flavin cofactor when the protein contained valine instead of alanine at this position. These workers showed that in vitro the valine containing enzyme easily loses its flavin cofactor in conditions of low riboflavin or low folate, causing the biologically active tetrameric form of the enzyme to break down into inactive monomers, but that the presence of high folate concentrations could stabilize FAD binding and preserve enzyme functionality. Moreover, these effects could be modulated by SAM, indicating that riboflavin, folate, and methionine would all be important for maintenance of MTHFR activity in individuals with the variant enzyme (Yamada et al. 2001). These conclusions have now been abundantly supported by cell culture and human studies. Individuals homozygous for the TT genotype tend to have higher plasma tHcy (Frosst et al. 1995; Harmon et al. 1996; Hustad et al. 2007), and lower plasma and red cell folate concentrations, (Kluijtmans et al. 2003; Molloy et al. 1997; Yang et al. 2008) suggesting an

increased requirement for folate. There is an altered distribution of folate cofactors within the cell (Bagley and Selhub 1998; Smulders et al. 2007) and riboflavin status appears to be critical to maintaining adequate enzyme function in TT homozygotes (Hustad et al. 2007; Jacques et al. 2002; Lathrop Stern et al. 2003; McNulty et al. 2002:2006). Several studies have shown that increased folate and riboflavin intake can lower the plasma tHcy in TT individuals (Ashfield-Watt et al. 2002; Chuang et al. 2006; de Bree et al. 2003; Jacques et al. 2002; McNulty et al. 2006; Yang et al. 2008). Recently, it was suggested that low vitamin B12 status might also modulate the effect of the TT genotype on plasma tHcy (Bailey et al. 2002; D'Angelo et al. 2000; Hustad et al. 2007).

The distribution of the polymorphism varies considerably worldwide. Mexican and Southern European populations have the highest frequency with some studies reporting more than 30% of individuals homozygous for the TT genotype. This contrasts with a TT homozygous frequency of about 12% in Northern Europeans and less than 1% among African groups (Botto and Yang 2000; Esfahani et al. 2003; Gueant-Rodriguez et al. 2006; Kirke et al. 2004; Mutchinick et al. 1999; Pepe et al. 1998; Wilcken et al. 2003). Such heterogeneity in prevalence has contributed to the difficulty in establishing the polymorphism as a risk factor for disease even though many studies on this topic have been reported (Ueland et al. 2001). To date, there is good evidence that the MTHFR 677C→T polymorphism confers increased risk of neural tube defects (Botto and Yang 2000; de Franchis et al. 2002; Kirke et al. 2004; Shields et al. 1999) and it may also be associated with other congenital defects (Botto and Yang 2000; Brouns et al. 2008; Mills et al. 2008). Many research groups have examined the prevalence of TT versus CC homozygotes among individuals with various forms of CVD, because of the association of the polymorphism with elevated plasma tHcy concentrations, an established risk factor for CVD. Two important meta-analyses of these studies (Klerk et al. 2002; Wald et al. 2002) confirmed a higher risk of vascular disease among TT individuals, although wide variations in risk were observed between populations, highlighting the difficulties in carrying out disease risk studies for genetic variants that can be modified by diet or environment. The polymorphism has also been studied as a risk factor for several forms of cancer (Boccia et al. 2008; Chen et al. 1998; Choi and Mason 2002; Ma et al. 1997; Robien and Ulrich 2003; Ryan et al. 2001; Sharp and Little 2004; Suzuki et al. 2008; Ulvik et al. 2004). Again there is considerable disagreement between studies, but in general, there are trends towards an interaction between folate status and risk, suggesting that when folate status is above average, TT status may be associated with decreased risk. Such an effect is consistent with a proportionally higher intracellular content of folate cofactors in the form available for maintenance and repair of DNA.

In summary of the above, human cross-sectional and intervention studies on the 677C→T polymorphism have led researchers to conclude that MTHFR is at the centre of a 'functional metabolic network' that may have important effects on nutritional status and disease risk (Hustad et al. 2007; Yang et al. 2008). It is important to note that a second polymorphism in the MTHFR gene:1298A→C, has also been described and tested for association with risk of several disease conditions

and pregnancy complications. This SNP is in strong linkage disequilibrium with the 677C-T variant (Stegmann et al. 1999). Most studies find no discernable effect of the 1298C variant when considered independently of the 677C→T polymorphism, and in those studies that have found effects, the results tend to conflict (Devlin et al. 2006; Fredriksen et al. 2007; Nurk et al. 2004; Parle-McDermott et al. 2003, 2006; Stegmann et al. 1999; Ulvik et al. 2007).

7.8 Folate Absorption and Uptake into Cells

Because folate is not synthesized in mammalian tissues, the bioavailability of ingested dietary folate and the function of enzymes and transporters that move folates across intestinal and tissue membranes are an important aspect in the regulation of folate homeostasis (Gregory 1995; Hamid et al. 2009; McNulty and Pentieva 2004; Molloy 2002). All of the monoglutamate folate forms are extremely hydrophilic and have a low capacity to diffuse across biological membranes, therefore several carrier-mediated systems are responsible for the absorption of folate from the intestinal lumen and its transport across other cell membranes. These include the reduced folate carrier (RFC), folate receptors (FRs) and a recently discovered proton coupled folate transporter (PCFT). Several excellent reviews describe the different functions of these transporters (Hamid et al. 2009; Low and Antony 2004; Matherly and Goldman 2003; Zhao et al. 2009). Genetic factors that may affect these systems are of special interest and have been studied in some depth, including with the use of animal gene-knock-out models (Ma et al. 2005; Piedrahita et al. 1999) In particular, studies have examined the possibility that genetic variability in these binding proteins and enzymes can influence an individual's achievable folate status, since such an effect might have implications for risk of NTDs and other chronic disease states. Some of these are described below.

Before being absorbed, dietary folate polyglutamates within the food matrix must be hydrolysed to the monoglutamate form (Hannon-Fletcher et al. 2004; McKillop et al. 2006). This is catalysed by a brush border folylpolyglutamate hydrolase, encoded by the glutamate carboxypeptidase II (GCPII) gene (Halsted et al. 1998). A 1561C→T polymorphism in the GCPII gene was initially thought to be associated with lower folate status (Devlin et al. 2000) but this was not confirmed (Devlin et al. 2006; Vargas-Martinez et al. 2002) and other studies found a small but significant association of this polymorphism with higher plasma folate and lower tHcy concentrations (Afman et al. 2003; Fodinger et al. 2003; Halsted et al. 2007; Lievers et al. 2002; Winkelmayer et al. 2003). Studies to date have not shown an association of this polymorphism with risk of NTD (Afman et al. 2003; Morin et al. 2003).

Several of the folate transporters have also been examined for effect on status and risk of NTD. The RFC gene is a member of the SLC19 family of transporters and is ubiquitously expressed in normal tissues. A common polymorphism (80G→A) in this gene has been examined for association with blood folate levels and disease risk including NTDs, orofacial clefts and vascular disease (Devlin et al. 2006;

Fredriksen et al. 2007; O'Leary et al. 2006; Vieira et al. 2005; Winkelmayer et al. 2003) but no strong associations have been observed. Folate receptors (FRs) internalize folate molecules via endocytosis. Four isoforms of FRs are now known to exist (α,β,γ, and δ) (Low and Antony 2004; Matherly and Goldman 2003; Walker 2007; Yamaguchi et al. 2007) The high concentration of some of these receptor isoforms in placental tissue and fetal cells makes genetic variation in the genes for these receptors attractive candidates for conferring risk of NTD and other birth defects. Embryonic lethality or severe embryonic defects are an important feature in mouse studies where the genes for the α, or β FRs are inactivated, and in some instances these effects can be modulated or prevented by folic acid supplementation (Piedrahita et al. 1999; Spiegelstein et al. 2004; Tang and Finnell 2003; Taparia et al. 2007; Zhu et al. 2007). Nevertheless, human studies to date have not found significant associations between polymorphisms in folate transport genes and risk of NTDs (Barber et al. 1998:2000; Finnell et al. 2008; O'Leary et al. 2003). One reason for this may be the conserved nature of these genes. Genetic analysis of the gene for the α-FR in humans found that polymorphic nucleotides were extremely rare (Barber et al. 1998), but recent work suggests that the promoter region might have a higher level of genetic variability (Finnell et al. 2008) Finally, mutations in the recently discovered PCFT have been identified as the causative factor in congenital folate malabsorption (Min et al. 2008; Qiu et al. 2006; Zhao et al. 2007). All of these receptors remain an important focus for future study.

7.9 Summary

Folate cofactor metabolism involves an intricate network of at least 50 genes encoding products such as receptors, binding proteins and enzymes (Molloy 2004). When downstream factors such as methylation reactions that rely on folate derived metabolites are added to that list, the interplay of folate with genetic and epigenetic factors becomes very complex indeed. Although the phenotypic consequences of individual factors may be very small, the combined effect of small alterations in the function of many proteins may have an important influence on the availability, flux and final distribution of one-carbon units, leading to innate differential risk of disease between individuals. Therefore the impact of genetic variation needs to be considered in aiming to establish optimal protection against risk of diseases that are linked to folate status. In the past few years, epigenetic analysis techniques have become more sophisticated and high-throughput multiplex genotyping systems are widely available. The strategy for studying genetic variation has evolved to the extent where micro-satellite analysis of all the genes in a metabolic axis can be scanned for association with disease or phenotype and where genome-wide association studies are emerging for many diseases and biochemical phenotypes. This new strategy is being applied to the folate metabolic network and such analyses are beginning to emerge (Meyer et al. 2004; Molloy et al. 2009; Shaw et al. 2009; Souto et al. 2005; Tanaka et al. 2009; Vermeulen et al. 2006). Future studies on large cohorts will provide a clearer insight into the genetic influences involved at this important nutrient locus.

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Chapter 8
Enzymatic and Non-enzymatic Antioxidative Effects of Folic Acid and Its Reduced Derivates

Olaf Stanger and Willibald Wonisch

Abstract A great part of the population appears to have insufficient folate intake, especially subgroups with higher demand, as determined through more sensitive methods and parameters currently in use. As the role of folate deficiency in congenital defects, e.g. in cardiovascular and neurodegenerative diseases, and in carcinogenesis has become better understood, folate has been recognized as having great potential to prevent these many disorders through folate supplementation or fortification for the general population. Folates are essential cofactors in the transfer and utilization of one-carbon groups in the process of DNA-biosynthesis with implications for genomic repair and stability. Folate acts indirectly to lower homocysteine levels and insures optimal functioning of the methylation cycle. Homocysteine was shown to be an independent risk factor for neurodegenerative and cardiovascular disease, which includes peripheral vascular disease, coronary artery disease, cerebrovascular disease and venous thrombosis. In fact, it was long believed that the beneficial effects of folate on vascular function and disease are related directly to the mechanism of homocysteine-diminution. Recent investigations have, however, demonstrated beneficial effects of folates unrelated to homocysteine-diminution, suggesting independent properties. One such mechanism could be free radical scavenging and antioxidant activity, as it is now recognized that free radicals play an important role in the oxidative stress leading to many diseases. It was found that folic acid and, in particular, its reduced derivates act both directly and indirectly to produce antioxidant effects. Folates interact with the endothelial enzyme NO synthase (eNOS) and, exert effects on the cofactor bioavailability of NO and thus, on peroxynitrite formation. Folate metabolism provides an interesting example of gene-environmental interaction.

Keywords Endothelial NO synthase (eNOS) · Folic acid · Glutathione peroxidase · Peroxynitrite · Superoxide anion

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Abbreviations

Ang	angiotensin
BH4	tetrahydrobiopterin
CAD	coronary artery disease
CβS	cystathionine-β-synthase
DDPH	2,2-diphenyl-1-picrylhydrazyl
DHF	dihydrofolate
DHFR	dihydrofolate reductase
ED	endothelial dysfunction
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelial derived relaxing factor
eNOS	Endothelial NO synthase
FRAP	ferric reducing ability of plasma
GPx	glutathione peroxidase
Hcy	homocysteine
HHcy	hyperhomocysteinemia
H_2O_2	hydrogen peroxide
LDL	low density lipoprotein
LPO	lipid peroxidation
MDA	malondialdehyde
MS	methionine synthase
MTHFR	methylenetetrahydrofolate reductase
5-MTHF	5-methyl-tetrahydrofolate
NO	nitric oxide
O_2^-	superoxide anion
$^{\bullet}OH$	hydroxyl radical
$ONOO^-$	peroxynitrite
PG	prostaglandin
RF	relaxing factor
RFC-1	reduced folate carrier-1
ROS	radical oxygen species
SAM	S-adenosylmethionine
SOD	superoxide dismutase
TAC	total antioxidant capacity
TAS	total antioxidant status
TBARS	2-thiobarbituric acid-reactive substances
TEAC	trolox equivalent antioxidant capacity
THF	tetrahydrofolate
TNFα	tumor necrosis factor α
TXB2	thromboxane B2

8.1 Introduction

Folates are essential cofactors in the transfer and utilization of one-carbon-groups and play a key role in the remethylation of methionine, thus providing essential methyl groups for numerous biological reactions. Furthermore, folates donate one-carbon units in the process of DNA -biosynthesis with implications for the regulation of gene expression, transcription, chromatine structure, genomic repair and genomic stability (Stanger 2002).

Tetrahydrofolate (THF) polyglutamates function in cells as a family of coenzymes that activate and carry single carbons on the N5 and/or N10 position. THF-activated carbons are carried at three different oxidation states, e.g. formates as 10-formyl-THF, 5-formyl-THF, or 5,10-methenyl-THF. In addition, THF transports activated formaldehyde as 5,10-methylene-THF and activated methanol as 5-methyl-THF (5-MTHF), the predominant form of folate circulating in the blood-stream and available for cellular uptake.

As folates function as acceptors and donors of one-carbon moieties in reactions involving nucleotide and amino acid metabolism, 5-MTHF is an essential cofactor for the Zn^{2+}- and cobalamin (vitamin B12)-dependent remethylation of homocysteine (Hcy) to methionine catalyzed by methionine synthase (MS). Loss of MS enzyme activity, e.g. through cobalamin deficiency, inhibits nucleotide biosynthesis due to the accumulation of cytoplasmic folate cofactors such as 5-MTHF. This effect is referred to as the "folate or methyl group trap" (Scott and Weir 1981); this elevates Hcy, because remethylation is impaired and Hcy accumulates. Thus folate deficiency consequently elevates homocysteine (hyperhomocysteinemia; HHcy). Although this chapter will not focus on the folate-cobalamin interrelationship in particular, the situation illustrates the difficulty of discriminating between cobalamin and/or folate deficiciency as the cause for HHcy.

Low folate status and mild plasma HHcy (>12[15] μmol/L) are both associated with a significantly increased cardiovascular risk (Stanger et al. 2003, 2009). Various epidemiological studies support a link between folate status and atherosclerotic vascular disease (Pancharuniti et al. 1994; Verhoef et al. 1996; Schwartz et al. 1997; Robinson et al. 1998; Rimm et al. 1998; Bunout et al. 2000). There is evidence for a dose-response relationship between Hcy levels and risk without threshold, even within the normal range (Boushey et al. 1995). Due to the close and inverse relationship between folate bioavailability and Hcy, the individual damaging or protective effects may be difficult to separate, in particular under clinical conditions.

Patients with homocystinuria (see below) represent a "natural" condition with normal folate but extremely elevated Hcy concentrations in plasma that is associated with greatly enhanced cardiovascular risk (Mudd et al. 1985; Wilcken and Wilcken 1997; Yap et al. 2000). Besides the generally accepted toxic effect on vascular structures, HHcy is thought to exert its effects through mechanisms involving radical oxygen species (ROS) and oxidative damage (Loscalzo 1996). Treatment with high-dose vitamins (folate and B-vitamins) dramatically reduces cardiovascular risk but rarely lowers Hcy to normal levels (Wilcken and Wilcken 1997; Yap et al. 2001). In parallel, folates were found to improve endothelial dysfunction (ED)

independent of Hc-diminution, thus bringing into question the causative role of Hcy and suggesting that folates have independent antioxidative effects.

8.2 Structure and Metabolism of Folates

8.2.1 Structure

The term "folic acid" usually describes a group of compounds derived from 5,6,7,8-tetrahydropteroyl-γ-glutamate, more commonly referred to as tetrahydrofolate (THF). THF is made up of a pteridine core ring system (2-amino-4-hydroxy-pteridine) that is linked to p-aminobenzoic acid (that is, pteroic acid) by a methylene bridge from the C-6 position of the pyrazine ring, thus forming tetrahydropteroic acid (Fig. 8.1). An additional link attaches glutamate to the p-aminobenzoic acid (with 1–9 residues). The pyrazine ring is fully reduced at the 5,6,7 and 8 positions and forms the biologically active form, referred to as 5,6,7,8-tetrahydrofolate (THF). Dihydrofolate (DHF) is formed through stepwise reduction of the 7 and 8 positions, using two molecules of NADPH. Several other biologically active compounds derive from this basic structure.

Mammals may synthesize the pteridine ring, but are unable to link it with the other compounds. Thus humans depend on dietary intake and synthesis of biologically active folate.

Vital processes in folate disposition include conversion of dietary folylpolyglutamates to monoglutamates, intestinal resorption, receptor and carrier-mediated transport across cell membranes, cellular metabolism and export. Both receptor-mediated and carrier-mediated transport mechanisms are variably expressed in different tissues, thus partially explaining the differential effects of impaired metabolism. Multiple reactions are required to convert dietary folic acid into metabolically active folates. The greater part is attached to enzymes, indicating tight metabolic regulation and limited availability of folates (Donnelly 2001).

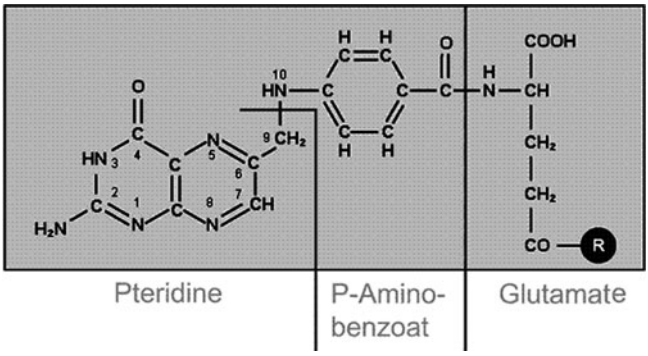

Fig. 8.1 Structure of folic acid

8.2.2 Uptake and Metabolism

Absorbed folate is cleared from the circulation within minutes and is taken up by various tissues and the liver, which is the most important storage organ. Hepatocytes store 10–20% of the folates; the greater part is released after metabolism, mainly into the bile. There, the folates participate in the enterohepatic circulation, with a daily turnover rate of approx. 90 μg (Steinberg et al. 1979). The folate concentration in bile is about the tenfold of serum levels. This means that bile is a readily available endogenous folate source of monoglutamate compounds, which can balance short-term deficiencies, e.g. during fasting. The serum levels of folate and bilirubin are inversely correlated.

THF is enzymatically converted and is mainly available as 5-MTHF, 5,10-methylene-THF and 10-formyl-THF. Alternatively, 5- and 10-formyl-THF derivates can also be converted to 5,10-methenyl-THF, 5,10-methylene-THF and, in an irreversible step, to 5-MTHF (Fig. 8.2).

5-MTHF makes up approx. 40–50% of the total folate polyglutamates in red blood cells. In serum, 5-MTHF is mainly present as monoglutamate, and intracellularly as polyglutamate. Polyglutamates are transported across cellular membranes, ensuring their cellular retention; they are the preferred form in one-carbon metabolism (Shane 1989). 5-MTHF serves as substrate for the cobalamin-dependent

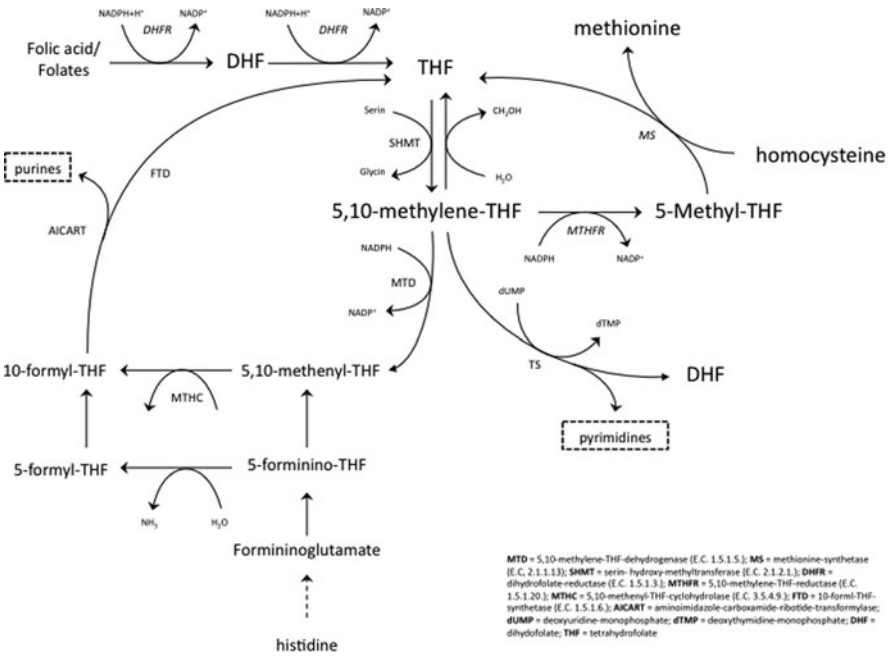

Fig. 8.2 Metabolism of folates

MS (E.C. 2.1.1.13) in the remethylation cycle, passing the methyl group on to Hcy, thus generating methionine and recycling THF.

Receptors and specific carriers are active in transmembranous folate transport. Membrane-located receptors have a high affinity for both folic acid and reduced folates (THF) (Anthony 1996). Three isoforms of such receptors are currently known, one of which is found only in the placenta and most likely plays an important role in folate supply to the embryo through the mother (Prasad et al. 1994). These receptors with high affinity for 5-MTHF are also found in hematopoetic cells and tubular cells of the kidneys (Luhrs and Slomiany 1989). The various carrier mechanisms are characterized by the different affinities for folate compounds and also by specific sensitivity for temperature and pH (Sirotnak and Tolner 1999). There is a carrier system with low affinity but great capacity for reduced folate (RFC-1; reduced folate carrier) in several tissues, but mostly in the brain (Said et al. 1996). The coding gene was demonstrated in luminal epithelial cells of the small intestine (Chiao et al. 1997) and in murine epidermis (Sprecher et al. 1998). Presence and distribution of the RFC-1 gene vary greatly in different tissues (Sirotnak and Tolner 1999). Tissue-specific differences in gene expression could explain local folate deficiency, leading to impairment of local resistance and susceptibility to infections due to dysfunction of immune-competent cells (Sirotnak and Tolner 1999).

8.2.3 Tissue Distribution

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-MTHF from 5,10-methylene-THF, which is used for Hcy remethylation to methionine, the precursor of S-adenosylmethionine (SAM). Impaired MTHFR activity will increase Hcy levels and compromise SAM-dependent methylation reactions.

Mild MTHFR deficiency is common in many populations due to a polymorphism at bp 677. The genotype determines the distribution of folates in various tissues (Ghandour et al. 2004) and increased enzyme activity accelerates folate turnover rates, depletes cellular folate concentrations, and may account in part for tissue-specific differences in folate accumulation (Anguera et al. 2003).

To assess how impaired MTHFR activity affects folate metabolism in various tissues in vivo, electrochemical HPLC detection was used to analyze the distribution of folates in plasma, liver, and brain of MTHFR-deficient mice. The most pronounced difference in total folate was observed in plasma (Ghandour et al. 2004). In MTHFR$^{-/-}$ mice, plasma total folate levels were approximately 25% of those in wild-type (MTHFR$^{+/+}$) mice. Only 40% of plasma folate in MTHFR$^{-/-}$ mice was comprised of 5-MTHF, compared to at least 80% in the other 2 genotype groups.

In liver and brain, there were no differences in total folate. However, the proportion of 5-MTHF in both tissues was again markedly reduced in mice with the MTHFR$^{-/-}$ genotype. In this genotype group, 5-MTHF is likely derived from the diet. The study demonstrated reduced total circulatory folate and altered distribution of folate derivatives in liver and brain as a consequence of MTHFR deficiency. Decreased methylfolates and increased nonmethylfolates would affect the flux of

one-carbon units between methylation reactions and nucleotide synthesis. This altered flux has implications for several common disorders, including cancer and vascular disease.

Another study sought to determine the effects of depleting cytoplasmic 5-formyl-THF on cellular folate concentrations and folate turnover rates in cell cultures by expressing the human methenyltetrahydrofolate synthetase cDNA in human MCF-7 cells and SH-SY5Y neuroblastoma. Cells with increased methenyltetrahydrofolate synthetase activity exhibited: (1) increased rates of folate turnover, (2) elevated generation of p-aminobenzoylglutamate in culture medium, (3) depressed cellular folate concentrations independent of folic acid concentrations in the medium, and (4) increased average polyglutamate chain lengths of folate cofactors (Anguera et al. 2003). These data indicate that folate catabolism and folate polyglutamylation are competitive reactions that influence cellular folate concentrations, and that increased methenyltetrahydrofolate synthetase activity accelerates folate turnover rates, depletes cellular folate concentrations, and may account in part for tissue-specific differences in folate accumulation.

In conclusion, cellular folate concentrations and tissue distribution are influenced by folate availability, cellular folate transport efficiency, folate polyglutamylation, enzyme activities and folate turnover specifically through degradation.

8.2.4 Oxidative Degradation of Folates

In subjects with HHcy there is usually an inverse relationship to folic acid levels, and supplementation with folic acid will lower Hcy concentrations. The pathogenesis of most – if not all – diseases that are accompanied by moderate HHcy involves cellular immune activation and these patients therefore very often also show a positive correlation between Hcy concentrations and the degree of immune activation, as indicated, e.g. by increased neopterin concentrations. Since neopterin concentrations also serve as an estimate of oxidative stress emerging from immune system activation, this association suggests that cellular immune activation and oxidative stress could be involved in the development of HHcy.

In vitro experiments have shown that superoxide anions (O_2^-) may induce folate cleavage (Shaw et al. 1989). Because THF is very susceptible to oxidation, increased oxidative degradation of THFs may become relevant under oxidative stress conditions. Such increased folate catabolism may lead to enhanced folate requirements in patients who are exposed to increased oxidative stress, such as hypercholesterolemic and diabetic patients. Increased oxidative stress may also lead to increased consumption of folate, because of its role as an antioxidant as well as in the regeneration of tetrahydrobiopterin (BH4). In this way, folate deficiency may develop despite adequate dietary intake. HHcy may thus be considered as an indirect consequence of hyperconsumption of antioxidant vitamins (e.g. folates) during prolonged states of immune activation (Widner et al. 2002).

Observations of oxidative degradation of THF and DHF were published very early (Blair 1957; Chippel and Scrimgeour 1970). Indeed, folate cofactors are highly

susceptible to oxidative degradation in vitro with the exception of 5-formyl-THF, which may be a storage form of folate. THF, like other reduced pteridines, is readily oxidized (Heales et al. 1988; Odin et al. 1998). Oxidation eliminates the side chain of the pteridine structure, yielding xanthopterin derivates (Murr et al. 1996). The oxidative loss of the side chain is irreversible, and THFs once oxidized in this way can never be recovered by any enzymatic reaction in any organism. Thus, oxidative stress could represent an endogenous reason for THF deficiency even when dietary intake of the vitamin is within the recommended range (Widner et al. 2001; Fuchs et al. 2001).

One of the important examples of the oxidative N-dealkylation of amines is the C9-N10 bond cleavage of folic acid leading to the formation of the amine 2- and 6-substituted pterins (Patro et al. 2005). For this, participation of the superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), via a hydrogen atom transfer mechanism or a ferryl-hydroxo complex (Fe^{4+}–OH) have been reported (Patro et al. 2005). Given the relatively high cellular concentrations of free iron and hydrogen peroxide (H_2O_2), the biotoxic hydroxyl radicals may play a major role in oxidative degradation of folic acid in the cellular systems.

The same oxidative degradation was observed with 5-MTHF, the main extracellular folate and the predominant form of the vitamin found in food and blood, yielding a pteridine residue and P-ABG, the latter step resulting in irreversible loss of vitamin activity (Lulock et al. 1995).

Ascorbic acid is efficacious in the facile salvage of 5-MTHF. In serum of folate-saturated healthy men, folate concentrations were measured before and up to 8 h after administration of placebo, 5-MTHF (343 μg + 290 mg ascorbic acid), and 5-MTHF (343 μg + 973 mg ascorbic acid). When 5-MTHF was concurrently administered with either dose of ascorbic acid, the total serum folate response, calculated as area under the curve (AUC), was significantly improved (Verlinde et al. 2008). Thus, administration of a physiological dose of 5-MTHF with ascorbic acid significantly improved the measured folate response in saturated healthy subjects.

Under conditions of increased oxidative stress, compensatory amplification of the redox-sensitive transsulfuration pathway will consume Hcy to generate the endogenous antioxidant glutathione (Tchantchou 2006). Data from clinical conditions in which oxidative stress induces depletion of B vitamins or antioxidants, e.g. prolonged states of inflammation or immune activation (Fuchs et al. 2001) or Alzheimer`s disease (McCaddon et al. 2002), further support the hypothesis that HHcy may be an epiphenomenon (albeit with own tissue-damaging effects) of a more complex pathogenic background. Hcy may be an accurate predictor of folate deficiency under otherwise normal conditions but may not always reflect folate deficiency in the presence of underlying risk factors known to promote increased oxidative stress (Rogers et al. 2007).

As illustrated above, Hcy is elevated in folate deficiency. Both hyperhomocysteinemia and hyperhomocysteinuria have been shown to be independent risk factors for atherosclerotic vascular disease. Considering their close metabolic relationship, clinical studies may not be feasable for distinguishing between HHcy and low folate as a primary etiological factor in cardiovascular disease. Each compound deserves individual attention.

8.3 Homocysteine

8.3.1 Homocystinuria

Homocystinuria may serve as a (natural) model experiment to address the harmful effects of Hcy, and also to question the therapeutic potential of folic acid, betaine and other vitamins associated with Hcy metabolism. Patients with this rare (∼1:200.000) recessively inherited disorder of methionine metabolism exhibit extremely elevated Hcy concentrations in plasma (>300 μmol/L) that are associated with greatly enhanced cardiovascular risk (Yap et al. 2000). Patients typically develop occlusive vascular disease in early adulthood or even in childhood. A worldwide review of 627 untreated cases found that a vascular event had occurred in ∼50% before the age of 30 (Mudd et al. 1985). This was attributed to both the toxic and vessel damaging effects of homocysteine.

8.3.2 Oxidative Stress in Homocystinuria

The extreme Hcy concentrations observed in homocystinuria are associated with marked oxidative stress indices. In patients homozygous for cystathionine-β-synthase (CβS)-deficiency urinary 8-iso-PGF2α excretion is significantly higher, and urinary 11-dehydro-TCB2 (index of platelet activation) excretion is enhanced as compared to healthy subjects (Davi et al. 2001). It is noteworthy that platelet-active 8-iso-PGF2α formation is a non-enzymatic, free radical-catalyzed peroxidation process of arachidonic acid. In this investigation, urinary 8-iso-PGF2α correlated with plasma homocysteine. These findings suggest that CβS -deficiency stimulates enhanced arachidonic acid peroxidation linking hyperhomocysteinemia with platelet activation, risk for thrombus formation and vascular damage. Folate levels, however, were not measured.

8.3.3 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is an important component of the endogenous antioxidant defense opposing the deleterious vascular effects of free radicals. Blood vessels express 3 isoforms of SOD: cytosolic or copper-zinc SOD (CuZn-SOD), manganese Mn-SOD located in mitochondria, and extracellular SOD (EC-SOD) (Faraci and Didion 2004). More than 90% of interstitial SOD is EC-SOD, a copper- and zinc-containing glycoprotein. Furthermore, circulating EC-SOD is in equilibrium with the EC-SOD on the surface of the endothelium and bound to proteoglycan (Stralin et al. 1996). It has been suggested that EC-SOD is the major determinant of nitric oxide (NO) bioavailability in blood vessels (Fukai et al. 2002). NO reacts with superoxide at a rate 3 times faster than dismutation of superoxide by SOD (Darley-Usmar et al. 1995). Due to the high reactivity (superoxide reacts with NO more

efficiently than with any other known molecule), the local concentration of SOD is an indirect key determinant of NO -bioactivity (i.e. biological half-life). Thus, a major function of SOD is to protect NO and NO-mediated signaling. Steady-state levels of O_2^- are dependent on both its rate of production as well as activity of the various superoxide dismutases (SODs). Another consequence of SOD activity is formation of H_2O_2. The importance of this ROS within vascular cells is becoming increasingly apparent. H_2O_2 is relatively stable and diffusible (including through cell membranes), compared to many other ROS. H_2O_2 can activate select transcription factors and may also function as an endothelium-derived hyperpolarizing factor (EDHF) in some blood vessels, but has also been suggested to be an ED relaxing factor (RF) without functioning as an EDHF (Miura et al. 2003). In combination with some transition metals like iron or copper, H_2O_2 is metabolized to the most reactive ROS, i.e. hydroxyl radical ($^{\cdot}OH$) through the Fenton reaction. This radical reacts with each adjacent biological structure, leading to cellular damage. H_2O_2 mediated effects and local concentrations are regulated by activity of the various glutathione peroxidases (GPx) or catalase. In homocystinuric patients, there is a highly significant, positive relationship between EC-SOD and total Hcy (Wilcken et al. 2000). In 2 newly diagnosed CβS-deficient patients, Hcy-lowering treatment reduced the associated elevated EC-SOD in each by 50%. This relationship could represent a protective antioxidant response to Hcy-induced oxidative damage in the vasculature. There is evidence for Hcy-related ED in homocystinuric patients that is thought to be due to reduced NO production (Celermajer et al. 1993). O_2^- radicals react avidly with NO to form peroxynitrite ($ONOO^-$), a potent oxidant (see below). Another indication of an unbalanced relationship between NO and ROS comes from the observation that ED in homocystinuric patients is ameliorated with ascorbic acid therapy (Pullin et al. 2002). Besides possible direct free radical scavenging, it was suggested that ascorbic acid may also exert its effects by acting directly upon eNOS, enhancing NO production by stabilizing (Heller et al. 2001) and increasing intracellular BH4 concentrations (Huang et al. 2000).

8.3.4 Treatment Effects of Homocystinuria

Treatment to lower the extremely elevated Hcy levels in homocystinuric patients by using pyridoxine, folic acid, cobalamin and, if necessary, trimethylglycine (betaine) dramatically reduces cardiovascular risk in both pyridoxine-responsive (and the more severe pyridoxine-nonresponsive) patients, although Hcy levels remain well above the normal range. (Wilcken and Wilcken 1997; Yap et al. 2001).

Although ineffective on Hcy levels, vitamin supplementation for 2 weeks produced transiently reduced urinary excretion of urinary 8-iso-PGF2α and 11-dehydro-TXB2 in seven homocystinuric patients (Davi et al. 2001).

There are only few studies on these very rare genetic disorders, but the available data suggest that Hcy -treatment with the vitamins mentioned above counteracts Hcy-induced oxidative stress and they may further provide protection through effects unrelated to Hcy -diminution.

8.3.5 Mild Hyperhomocysteinemia

Several functional pathologies associated with mildly elevated Hcy plasma concentrations (>15 μmol/L) have been linked with oxidative stress. Hcy is readily oxidized when added to plasma, principally as a consequence of auto-oxidation leading to the formation of homocystine, homocysteine-mixed disulfides, and homocysteine thiolactone (Loscalzo 1996). Hcy has also been shown to oxidize low density lipoproteins (LDL) by reactions requiring redox-active transition metal ions (Heinecke et al. 1993). In a study using a rat model, acute methionine load-induced HHcy demonstrated elevation of plasma conjugated dienes, lipid hydroperoxides, and thiobarbituric acid-reactive substances (TBARS) (Durand et al. 1997).

8.3.6 Homocysteine and Lipid Peroxidation

In vivo, Hcy has been found to be associated with increased lipid peroxidation (LPO) as measured by plasma F2-isoprostane concentrations (Voutilainen et al. 1999) and decreased antioxidant activity of SOD on endothelial cell surfaces with impaired endothelial function (Yamamoto et al. 2000). HHcy may exert its effects through increased tumor necrosis factor α (TNFα) expression, which enhances O_2^- induced oxidative stress by upregulating a nox1-based NAD(P)H oxidase and inducible NOS (Ungvari et al. 2003) that is associated with increased protein 3-nitrotyrosine content in smooth muscle cells. The effect of decreased NO-bioavailability under conditions of HHcy can be reversed by administration of superoxide scavengers (Ungvari et al. 2002) and ascorbic acid (Chambers et al. 1999). Incubation with Hcy activated IκB kinases (IKKα and IKKβ), leading to phosphorylation and subsequent degradation of IκBα (Kathy et al. 2004). The consequences were NF-κB nuclear translocation, enhanced NF-κB/DNA binding activity, and increased transcriptional activity. There was marked elevation of O_2^- in Hcy-treated cells.

8.3.7 Glutathione Peroxidase

An elevated level of Hcy was found to decrease the activity of cellular GPx in vivo, an intracellular selenocysteine-containing protein and a key antioxidant enzyme that uses glutathione to reduce H_2O_2 and lipid peroxides to their respective alcohols (Huang et al. 2001). GPx may also act as a peroxynitrite reductase (Sies et al. 1997). In an interesting experiment using the CβS-mouse model, overexpression of GPx could compensate for the adverse effects of Hcy on endothelial function, suggesting that the adverse vascular effects of Hcy are at least partly mediated by oxidative inactivation of NO (Weiss et al. 2001). Purified GPx from human plasma is inhibited by physiological free Hcy (1–5 μmol/L) (Chen et al. 2000). Similarly, in cultured endothelial cells, micromolar concentrations of Hcy are sufficient to decrease the

activity of GPx (Weiss et al. 2002; Upchurch et al. 1997). Hcy down-regulates GPx activity by interfering with protein expression (translation) without affecting transcription levels (Handy et al. 2005).

The Hcy-dependent transsulfuration pathway catalyzed by the heme and pyridoxine-dependent CβS leads to cystathionine and subsequently to cysteine and glutathione biosyntheses in addition to providing a catabolic route leading to sulfate. Intracellular glutathione is important for maintenance of the physiological cell redox state and, together with heat shock proteins, plays a key role in mitochondria against adverse effects elicited by Hcy and H_2O_2 (Austin et al. 1998). It is estimated that approximately half of the intracellular glutathione pool in human liver cells is derived from Hcy via the transsulfuration pathway, whose redox sensitivity can be rationalized as an autocorrective response that leads to an increased rate of glutathione synthesis in cells challenged by oxidative stress (Mosharov et al. 2000).

Conditions of chronic oxidative stress may thus impair the synthesis of this major redox buffer. HHcy, induced through methionine loading, is associated with a greater activation of the antioxidant defense as expressed by higher intracellular glutathione content, but was not accompanied by a significant increase of malondialdehyde (MDA) (Campolo et al. 2006). High-dose treatment with 5-MTHF (15 mg/d) improved the antioxidant defense in hyperhomocysteinemic subjects through interaction with glutathione metabolism, i.e. an increased reduction of blood total glutathione that includes oxidized glutathione, protein bound glutathione, glutathione mixed disulfides and reduced free glutathione (Caruso et al. 2006). The reduction of glutathione oxidative forms, however, cannot be explained exclusively by Hcy-diminution or amelioration of glutathione turnover, but may also indicate antioxidative effects of 5-MTHF on the redox state per se, related to glutathione metabolism.

Importantly, it was found that HHcy in healthy young men and elderly men with normal serum folate concentration was not associated with ED or oxidative stress as measured by glutathione, TAS, TBARS, and plasma 8-iso-PGF2α (Hirsch et al. 2004). This finding points toward the hypothesis that folate deficiency is the key factor for ROS and ED, rather than (mild) HHcy. Thus it was postulated that the acute modifications in vascular function induced by folic acid are unrelated to changes in Hcy concentrations. HHcy may, however, help to identify individuals with folate deficiency and target groups that may benefit from supplemental intake.

8.4 Endothelial Dysfunction

ROS play a major role in vascular biology. In general, relatively low concentrations of ROS are thought to act as mediators or modulators of cell signaling and contribute to other key functions, e.g. the regulation of activity of transcription factors and gene expression (Droge 2002). In contrast, higher levels of ROS contribute to vascular dysfunction and abnormal cell growth including hypertrophy of vascular muscle. O_2^- levels are increased in blood vessels in many pathophysiological conditions

including hypertension, diabetes, atherosclerosis, hyperhomocysteinemia, heart failure, sepsis, subarachnoid hemorrhage, and Alzheimer's disease, as well as during aging. Multiple lines of evidence have shown that NO signaling plays a major role in vascular biology. Since the initial evidence that superoxide or other ROS inactivate NO or EDRF, many studies have suggested that inactivation of NO by O_2^- contributes to vascular dysfunction under pathophysiological conditions. In addition to inactivating NO and thus preventing NO-mediated signaling, the reaction of NO with O_2^- produces $ONOO^-$, a potent oxidant with cytotoxic potential (Beckman and Koppenol 1996). Emerging evidence suggests that formation of $ONOO^-$ has multiple effects, including (1) selective nitration of tyrosine residues in proteins, such as prostacyclin synthase and Mn-SOD, (2) activation of poly (ADP-ribose) polymerase (PARP) and expression of inducible NO synthase (iNOS), potentially important mediators of vascular dysfunction in disease states, (3) oxidation of BH4, and (4) oxidation of the zinc–thiolate complex in endothelial NOS (eNOS). The latter two effects can produce eNOS "uncoupling", a condition in which the normal flow of electrons within the enzyme is diverted such that eNOS produces O_2^- rather than NO (Faraci and Didion 2004).

ED, assessed as impaired vasodilator response to mechanical or pharmacological stimuli, has been increasingly recognized as a surrogate end point for cardiovascular risk. Several investigations have found an association between ED and myocardial ischemia (Zeiher et al. 1995; Hasdai et al. 1997). Other reports have demonstrated in long-term follow-up studies, that ED is associated with an increased progression of atherosclerotic disease (Schachinger et al. 2000) and a higher incidence of cardiovascular events (Suwaidi et al. 2000). ED refers mainly to reduced bioavailibility of NO and accumulating evidence suggests that ROS are fundamentally involved in HHcy and folate deficiency associated with ED (Stanger et al. 2002). NO production is determined by cofactors such as BH4, which is oxidized and depleted in conditions of oxidant stress, e.g. by $ONOO^-$. THF deficiency contributes to the uncoupling of NO synthase into an O_2^- radical-producing enzyme.

Importantly, several investigators have demonstrated beneficial effects of folates on endothelial function independent of changes in plasma Hcy levels, suggesting that folates may exert direct antioxidative effects and contribute to restoration of impaired NO metabolism. (Usui et al. 1999; Doshi et al. 2002; Stanger and Weger 2003). More specifically, 5-MTHF above all appears to be associated with endothelial function, independent of Hcy levels (Spijkerman et al. 2005). Using the MTHFR gene polymorphism 677C>T, it was recently demonstrated that vascular 5-MTHF, rather than plasma or vascular Hcy, is the key regulator of eNOS coupling and NO bioavailability in human vessels, further suggesting that plasma Hcy is an indirect marker of 5-MTHF rather than a primary regulator of endothelial function (Antoniades et al. 2009).

In a clinical cross-over study, male patients with angiographically documented coronary artery disease (CAD) received 5 mg of folic acid orally for 12 weeks in an on-off fashion and resistance vessel reactivity was repeatedly assessed by venous occlusion plethysmography. Folic acid supplementation significantly increased plasma folate levels 3.5 fold and improved resistance vessel reactivity (peak

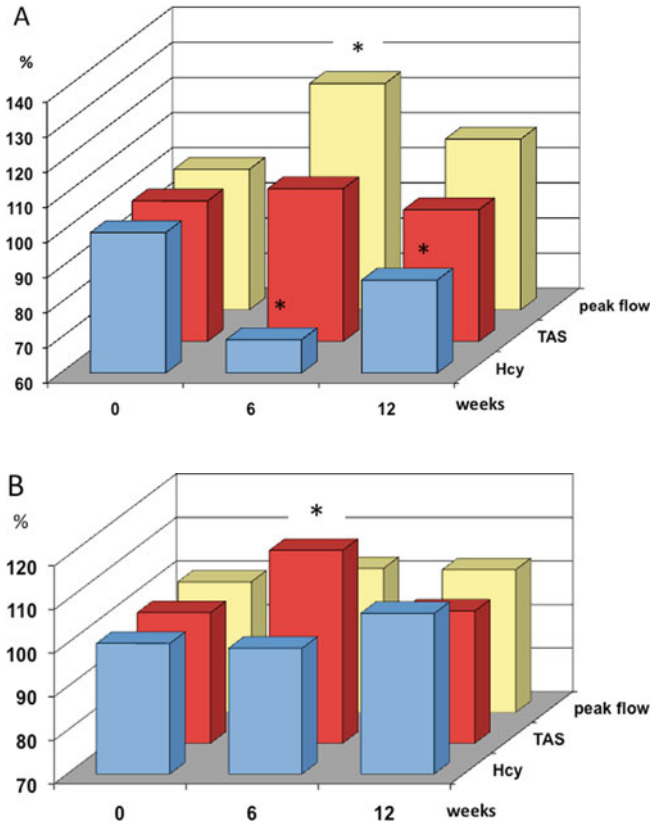

Fig. 8.3 (**a**) Effect of 5 mg/day oral folic acid on TAS, Hcy and resistance vessel reactivity (peak flow) in male patients with CAD after 6 weeks (therapy) and 12 weeks (off-therapy). Changes relative to baseline values (10%). *Indicated significant changes ($p < 0.05$). (**b**) Effect of folate intake on antioxidative capacity as determined by total antioxidant status (TAS) and in resistance vessel reactivity (peak flow) in subjects without significant changes in Hcy levels ($\Delta<2$ μmol/L). Changes relative to baseline values (10%). *Indicated significant changes ($p < 0.05$)

flow), suggesting vasoprotective effects of FA in patients with established ED and atherosclerotic vessel disease (Stanger et al. 2002) (Fig. 8.3a). In subjects without significant changes in Hcy levels ($\Delta<2$ μmol/L), folate administration was associated only with increased antioxidative capacity as determined by total antioxidant status (TAS), suggesting an antioxidative potential for folate (Fig. 8.3b). In other words, unless folate is consumed in the remethylation pathway for Hcy-diminution, folic acid may add antioxidant capacity to plasma.

5-MTHF was suggested to exert direct effects on the enzymatic activity of NOS, which is supported by the identification of a pteridine-binding domain with similarities to the folate binding site of DHF reductase (Hyndman et al. 2002).

Treatment of endothelial cells in vitro with folic acid showed a marked increase of dihydrofolate reductase (DHFR) expression and activity (Gao et al. 2009). Furthermore, folic acid improved the redox status of angiotensin (Ang) II-treated cells by increasing BH4 and NO bioavailability while decreasing O_2^- production. It failed, however, to restore NO levels in DHFR siRNA-transfected or methotrexate pre-treated cells, implicating a specific and intermediate role of DHFR. This has been also demonstrated in mice with oral administration with FA (15 mg/kg/day, 16 days), where endothelial upregulation of DHFR expression and activity occurred in correspondence to improved NO and BH4 bioavailability, and this was highly effective in reducing Ang II infusion-stimulated aortic O_2^- production.

Mice infused with Ang II had markedly increased O_2^- production and reduced NO and BH4 bioavailability. These responses were, however, all significantly reversed by pre-treatment with folic acid. In addition, DHFR expression and activity were increased by FA. This was accompanied by an endothelial downregulation of DHFR. Oral administration of FA significantly improved NO production, while reducing O_2^- levels. Taken together, these data demonstrate that folic acid exerts it beneficial effects in vivo via regulation of DHFR expression and activity. It is important to note that in the endothelium-denuded aortas, DHFR expression was upregulated by Ang II, indicating differential regulation of DHFR in different cell types. This seems consistent as it is known that Ang II stimulates vascular smooth muscle hypertrophy, and that DHFR in general is important for DNA synthesis and cell growth.

In summary, folic acid supplementation was shown to improve endothelial NO bioavailability via upregulation of DHFR expression and activity, and to protect endothelial cells from Ang II-provoked oxidant stress both in vitro and in vivo (Gao et al. 2009).

Growing evidence suggests that potential mechanisms of vascular protection are largely independent of the Hcy-diminution and include a direct superoxide-scavenging capacity of 5-MTHF and effects on eNOS and NO generation. The latter are likely to involve BH4. These effects have been reported for the natural L[6S] and the D[6R] isomer of 5-MTHF, arguing for the possibility that synthetic forms are also biologically active (Stroes et al. 2000; Baggott et al. 2001). Furthermore, beneficial effects of folate administration have also been reported in non-hyperhomocysteinemic subjects, such as the correction of ED in patients with familial hypercholesterolemia (Verhaar et al. 1998) or the prevention of its impairment by postprandial lipaemia (Wilmink et al. 2000).

8.4.1 Interactions

5-MTHF, when given intra-arterially, not only improved flow-mediated dilatation without altering plasma Hcy concentrations but also abolished Hcy-induced increase in intracellular O_2^- generation. Folic acid and BH4 also abolished Hcy-induced intracellular O_2^- production in culture (Doshi et al. 2002). This suggests that folic acid, 5-MTHF and BH4 act in concert to restore endothelial function by

suppressing O_2^- production and enhancing NO generation or increasing its half-life. This is supported by the observation that 5-MTHF had no direct effect on in vitro NO production by eNOS but induced a dose-dependent reduction in eNOS- and xanthine oxidase–induced O_2^- generation and reversed impaired endothelium-dependent vasodilation in patients with familial hypercholesterolemia (Verhaar et al. 1998).

It is evident from the preceding discussion that folate/folic acid, H4B, insulin, and O_2^- interact with each other and significantly influence the generation, stability and action of NO. Folate/folic acid, H4B, and insulin suppress O_2^- production and thus prolong the half-life of NO. The importance of ascorbic acid lies in the fact that it enhances eNOS activity by increasing intracellular levels and by chemical stabilization of BH4 (Heller et al. 2001). Ascorbic acid also enhances the release of NO and thus suppresses the formation of increased amounts of total S-nitroso thiols and S-nitroso albumin (Verhaar et al. 1998).

8.5 Folates as Antioxidants

8.5.1 Folate Effects In Vitro

8.5.1.1 Radical Scavenging

The pulse radiolysis technique showed that folic acid can effectively scavenge such free radicals as $CCL_3O_2^{\bullet}$ (a model peroxyl radical), $N_3^{\bullet}$, $SO_4^{\bullet -}$ and $Br_2^{\bullet -}$ (one electron oxidants), physiologically relevant $^{\bullet}OH$ and O_2^- radicals as well (Joshi et al. 2001).

Folic acid and its reduced forms were further used to study their antioxidant capacities in the TEAC, DPPH and FRAP assays (Gliszczynska-Swiglo 2007). In short, the TEAC (trolox equivalent antioxidant capacity) value is based on the ability of the antioxidant to scavenge the blue-green coloured $ABTS^{\bullet}$ radical cation relative to the $ABTS^{\bullet}$ scavenging ability of the water-soluble vitamin E analogue, Trolox™ (Rice-Evans and Miller 1994). The antioxidant (radical-scavenging) activity can also be measured using 2,2-diphenyl-1-picrylhydrazyl ($DDPH^{\bullet}$) as a free radical. And finally, the FRAP assay is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ) (Benzie and Strain 1996).

The lowest TEAC and FRAP values and the highest IC50 value in the DPPH assay were obtained for folic acid, identifying it as the least potent antioxidant among the folates tested (Gliszczynska-Swiglo 2007). Reduction of folic acid to DHF significantly increased $ABTS^{\bullet}$ radical cation and $DDPH^{\bullet}$-scavenging activity as well as the ability to reduce Fe^{3+} in the FRAP assay. Further reduction of DHF to THF decreased the TEAC and the FRAP values, indicating a decrease in free-radical scavenging activities of THF as compared to DHF (Gliszczynska-Swiglo 2007). In contrast, the $DDPH^{\bullet}$-scavenging activity of THF compared to that of DHF was almost two-fold higher (IC50 values 59,1 μM and 107 μM, respectively). The

$DDPH^{\cdot}$-scavenging activities of 5-MTHF and THF appear comparable and support data obtained by Rzek et al., using the TEAC test, a lipid peroxidation (LPO) assay (Haenen and Bast 1983) and also measuring the peroxynitrite scavenging capacities (Rzek et al. 2003). Their results indicated that 5-MTHF is the most effective $ONOO^-$ scavenger and THF is the most effective inhibitor of LPO among the folates tested (Rzek et al. 2003).

Lucigenin-enhanced chemiluminescence demonstrated that 5-MTHF could reduce O_2^- generation by 2 O_2^--generating systems: xanthine oxidase/hypoxanthine and eNOS (Verhaar et al. 1998). Folate abolished the Hcy-induced increases in endothelial O_2^- (Doshi et al. 2002), and the O_2^--scavenging capacity of 5-MTHF was confirmed by electron paramagnetic resonance, using 5-(diethoxyphosphoryl)-5-methyl-L-pyrroline N-oxide as a spin trap for O_2^- (Stroes et al. 2000). This method also showed that the scavenging potency of 5-MTHF is about 20-fold lower than that of ascorbic acid. The radical scavenging activity of folic acid and its reduced forms, measured in the TEAC assay, was furthermore found to be strongly pH-dependent. Folic acid is a potent radical scavenger at acidic and basic pH in contrast to neutral pH, whereas reduced forms exhibit their radical scavenger activity predominantly at acidic pH rather than at neutral and basic pH values (Gliszcznska-Swiglo and Muzolf 2007). Thus, folate clearly does exert antioxidant effects.

8.5.1.2 Folates and Lipid Peroxidation

Folic acid has poor antioxidant activity in $ONOO^-$ scavenging and LPO inhibition. However, as folic acid is reduced, its antioxidant capacity increases. When folic acid is reduced to DHF, $ONOO^-$ scavenging becomes more pronounced; further reduction to THF and conversion to 5-MTHF enhance the scavenging activity even more. The fully reduced forms, i.e. THF and 5-MTHF, are the most active compounds in inhibiting LPO. (5-MTHF is relatively less active than THF in protecting against LPO, whereas 5-MTHF is the better $ONOO^-$ scavenger than THF).

The activities of folic acid and derivatives against H_2O_2 generation in linoleic acid (poly unsaturated fatty acid) peroxidation may have an early- "prooxidant" and a strong late- "antioxidant" phase (Higashi-Okai et al. 2007). 5-MTHF, but not folic acid, significantly increased the resistance of VLDL and LDL to oxidation in vitro (McEneny et al. 2007).

In summary, the results suggest that folic acid has a poor protective effect against free radicals and lipid peroxidation. Thus, due to its poor activity against LPO, it is likely that the minor antioxidant capacity of folic acid is physiologically irrelevant, though its physiological metabolites are much more active. Indeed, the fully reduced folates, namely THF and 5-MTHF, are the most active forms in scavenging the $DDPH^{\cdot}$ and $ONOO^-$. THF is the most effective inhibitor of LPO, whereas DHF is most effective in scavenging $ABTS^{\cdot}$ radical cation and is the best reductant as expressed by the FRAP value.

8.5.2 The Active Sites of Folates

In determining the active part of the molecule responsible for the antioxidant activity of folic acid, it was suggested that the 4-OH group on the pterin ring has an important role (Joshi et al. 2001). It has been found that the side chain, i.e. N-(p-aminobenzoyl)-L-glutaic acid, is more important, since the products formed by nitration of folic acid were identified as 10-nitrofolate and 12-nitrofolate (Nakamura et al. 2002).

To determine the responsible pharmocophore of the reduced forms of folic acid, the antioxidant activities of 4-hydroxy-2,5,6-triaminopyrimidine (and after removing the 5 amino group or the 5 and 6 amino groups) and structurally related compounds (5-6-7-8-tetrahydro-L-biopterin [BH4], pterin, leucopterin) were measured by $ONOO^-$ scavenging and the inhibition of LPO (expressed as IC50) (Rzek et al. 2003). The minor importance of the side chain for the antioxidant activity is suggested by (a) the absence of antioxidant activity of N-(p-aminobenzoyl)-L-glutaic acid, (b) the large difference in activity of the various folates, which all contain the same side chain, and (c) the antioxidant activity of BH4, which lacks the side chain and whose activity is comparable to that of THF. Therefore, and by comparing structurally related compounds, it was found that the antioxidant pharmacophore of THF and 5-MTHF, i.e. 4-hydroxy-2,5,6-triaminopyrimidine, resides in the pterin moiety (Rzek et al. 2003).

It is important to stress that in vitro assays for determination of the antioxidant activity of compounds under investigation do not reflect in vivo conditions because they do not take into account the presence of other compounds, which may interact with other bioactive molecules and therefore influence the antioxidant capacity of a given compound. Moreover, the relevance of potent antioxidants in biological systems may be strongly related to their bioavailability and metabolism.

The actual antioxidant effect of folates in vivo might depend on both reduction by the enzyme MTHFR and consumption, which is related to cobalamin and Hcy levels, tissue distribution and concentration (see Section 8.2.3), interaction with related compounds and susceptibility of the individual redox balance.

8.5.3 Antioxidant Effects of Folic Acid, Trimethylglycine (Betaine), 5-MTHF and Methionine In Vitro and Ex Vivo

Our group recently investigated the antioxidative potential of four different substances that are involved in the Hcy pathway, i.e. folic acid, trimethylglycine (betaine), 5-MTHF and methionine.

We took three different approaches, using established, commercially available biomarkers, to study whether these substances might (a) increase the lag-time of DPHPA, a fluorescent marker, that reflects the water-soluble antioxidant capacity in biological samples (Protein-Ox®), (b) increase peroxidase-activity (EPA®), an endogenous antioxidant, and (c) have a quenching property to reduce total peroxides (TOC®). The tests are described briefly:

(1) Water-soluble antioxidants: The lag time of ex vivo degradation of the fluorophore 1,6-diphenylhexatriene propionic acid (DPHPA) by ROS in serum was determined with a fluorescent diagnostic assay (Protein-Ox®, Tatzber KG, Klosterneuburg, Austria) according to (Mayer et al. 2001), with modifications to the oxidation part. Briefly, 100 μL sera were incubated with 2 nMol DPHPA and kept under argon at 37°C for 1 h. Oxidation was started via a peroxide (0.004%)/peroxidase (10 U/ml) reaction and the time-dependent decrease in fluorescence intensity at 430 nm (excitation at 360 nm) was monitored on a FluoStar Fluorometer (BMG Lab-Technologies; D-77656 Offenburg, Germany). Results were expressed as "lag time" in minutes.

(2) Serum total peroxidase-activity: Peroxidase-activity in serum was determined by the reaction of endogenous peroxidases with hydrogen peroxide, using TMB as the chromogenic substrate (EPA) supplied by Labor Diagnostic Nord (LDN, Nordhorn, Germany) as previously described (Tatzber et al. 2002). Serum peroxidase-activity was expressed as milliunits per millilitre. Serum reference values are in the range of 5 ± 3 mU/mL.

(3) Serum total peroxides: Serum total peroxide concentrations were determined by a rapid enzymatic in vitro diagnostic assay (TOC®) supplied by Labor Diagnostic Nord (LDN, Nordhorn, Germany) as previously described (Tatzber et al. 2002). The test system was based on a peroxide/peroxidase reaction using 3,5,3′,5′-tetramethylbenzidine (TMB) as substrate. Peroxide levels were specified as "μM H_2O_2 equivalents", because of the different contributions of various peroxides to the reaction. Reference values were reported to be less than 200 μM H_2O_2 equivalents (Lindschinger et al. 2004).

Blood was drawn from volunteers, centrifuged and exposed to a range of reference values as well as supra-optimal concentrations of the respective substances. A lyophilisate of each substance was reconstituted in distilled water to achieve the following concentrations: folic acid (7 ng, 18 ng and 18 μg), betaine (35 μmol/L, 146 μmol/L and 200 μmol/L), 5-MTHF (10 ng/mL, 40 ng/mL and 6 μg/mL) and methionine (47 μmol/L and 100 μmol/L).

For in vitro experiments, we applied the reconstituted stock-solutions and a dilution of 1:1 (v/v) with distilled water (Fig. 8.4b and d). For ex vivo experiments, we diluted stock-solutions 1:1 (v/v) with serum in the microtitre-wells for each biomarker (Fig. 8.4a, c, and e). With regard to peroxidase-activity we used the third standard of the EPA-assay (i.e. 10 mu/mL) as a reference.

None of these substances was able to affect significantly the lag-time of water-soluble antioxidants in serum (Fig. 8.4a). With respect to peroxidase-activity, we observed the maximum decrease (30%) at the peak concentration for methionine (100 μmol/L) and for betaine at a concentration of 146 μmol/L (Fig. 8.4b). Furthermore, betaine was able to decrease peroxidase-activity (20%) in a range from 35 to 146 μmol/L, as was also observed for folic acid at the lowest concentration (3.5 ng/ml). Nevertheless, peak and bottom levels for betaine (200 μmol and 17 μmol) did not affect the enzyme-activity in vitro. Just one case of increased enzyme activity was observed at the utmost concentration of 5-MTHF (6 μg/mL).

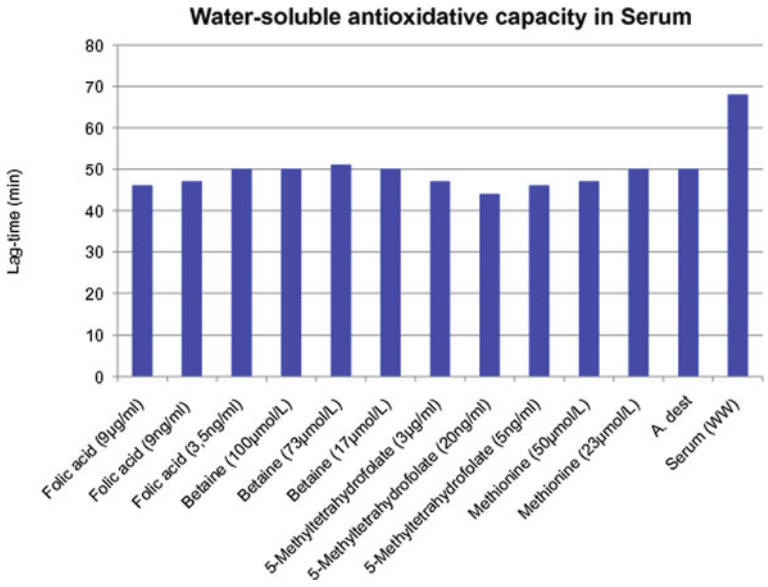

Fig. 8.4 (**a**) Water-soluble antioxidative capacity in serum. Lag-time (min)

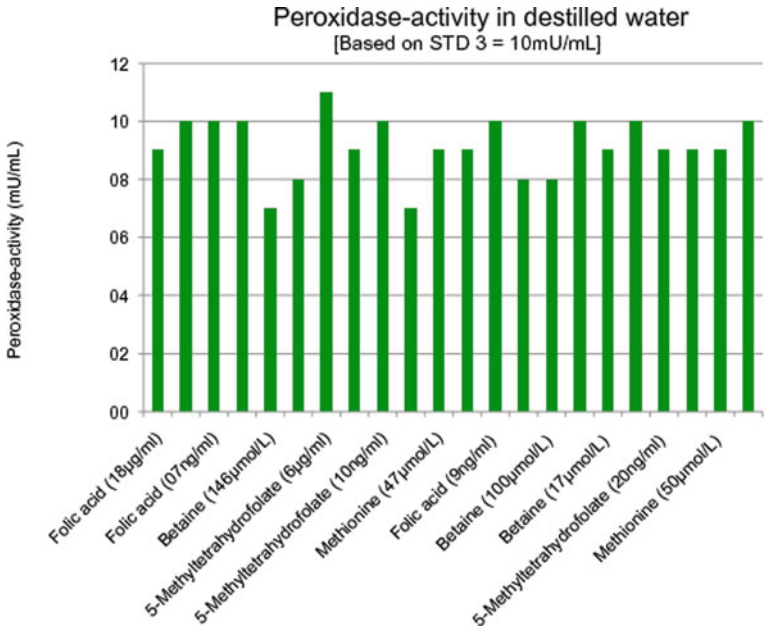

Fig. 8.4 (**b**) Peroxidase-activity in destilled water (mU/mL)

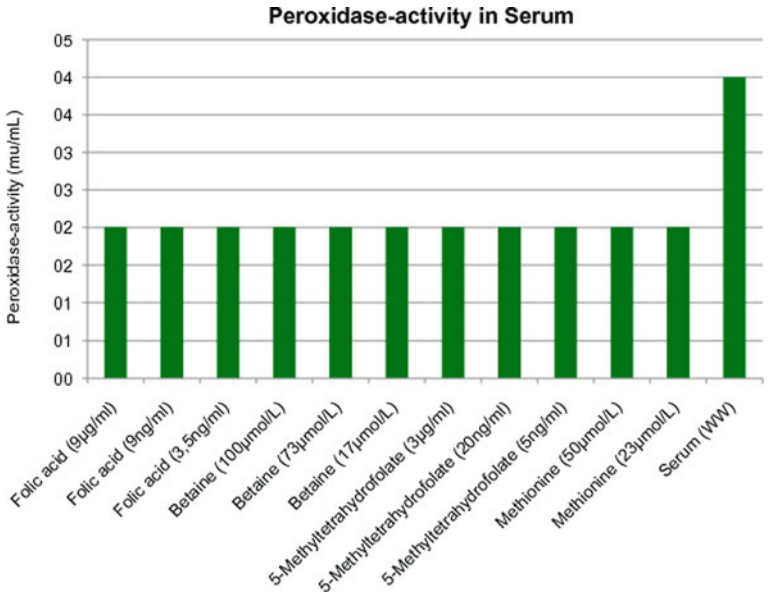

Fig. 8.4 (**c**) Peroxidase-activity in serum (mU/mL)

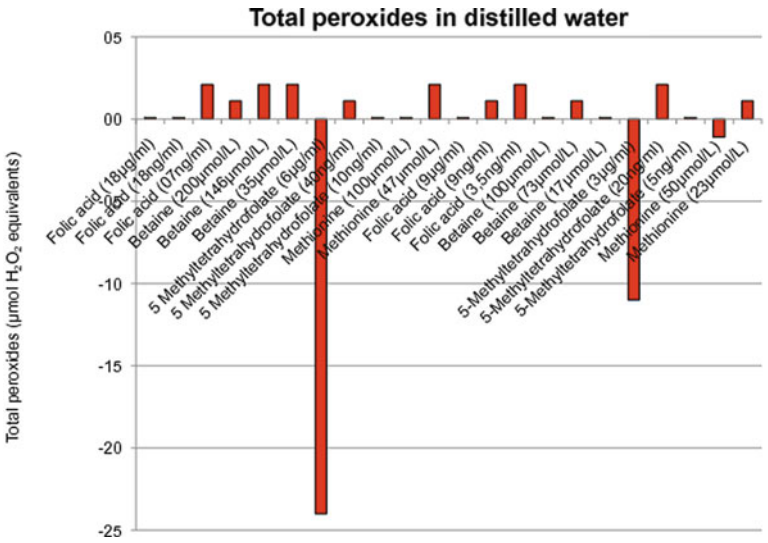

Fig. 8.4 (**d**) Total peroxides in destilled water (µmol H_2O_2 equivalents)

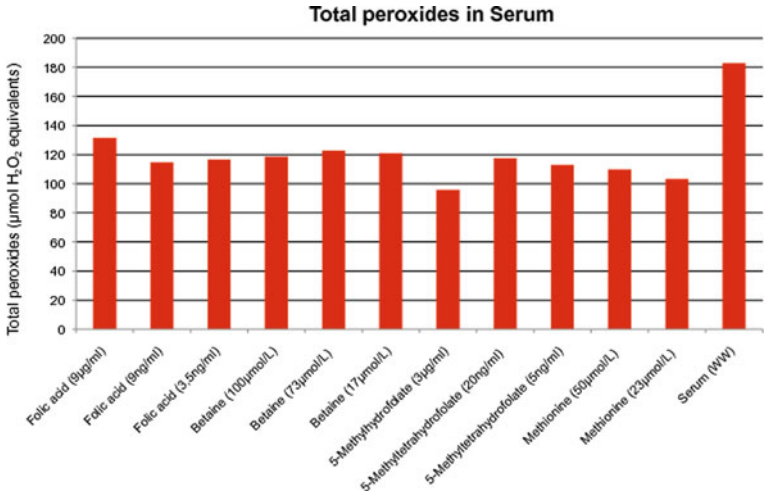

Fig. 8.4 (**e**) Total peroxides in destilled serum (µmol H_2O_2 equivalents)

Nonetheless, peroxidase-activity in serum (ex vivo) was not at all affected by these substances (Fig. 8.4c). The same applied to total peroxides with one exception: 5-MTHF in a micro-molar concentration was able to quench peroxides in a dose-dependent manner (from 3 to 6 µmol/L) as demonstrated in Fig. 8.4d. This effect was confirmed in serum inasmuch as the 3 µmol/L concentration of 5-MTHF was able to quench total peroxides to the same extent as was observed in the in vitro experiment (Fig. 8.4e).

These data suggest that folic acid, betaine, methionine and 5-MTHF have no significant antioxidative effect in vitro and ex vivo, either for the lag-time of water-soluble antioxidants or for the activity of the endogenous antioxidant-enzyme peroxidase.

We did, however, observe a significant quenching effect of 5-MTHF in a micro-molar concentration both in vitro and ex vivo, which supports its capacity to scavenge $ONOO^-$.

8.5.4 Folate Effects In Vivo

After male Wistar rats had been fed with a folate-deficient diet for 4 weeks, a significant elevation of oxidative stress was observed inside the liver mitochondria (mt) with a 77% decrease in mt folate level, a 28% reduction in GPx activity, a 1.2-fold increase in mt protein carbonyls and an accumulated 4834 bp large-scale deletion in mt DNA (Chang et al. 2007). The elicited oxidative injuries in liver mt were associated with a 30% reduction of cytochrome c oxidase activity that coincided with membrane potential dissipation (depolarization) and superoxide overproduction. Interestingly, preincubation with prooxidant copper-sulfate (48 h)

in folate-deficient hepatocytes with supplemental folic acid reversed the mt oxidative defects described and diminished O_2^- overproduction. Increased supplemental levels of folic acid strongly correlated with decreased lipid peroxidation and protein oxidative injuries in prooxidant pretreated mitochondria of hepatocytes.

Lipid peroxidation was significantly inhibited by folic acid in Wistar rat liver microsomes (Joshi et al. 2001). Thus, the reduced physiological forms THF and 5-MTHF may act as effective free radical scavengers and LPO inhibitors in vivo. Their free radical scavenging activities expressed as the TEAC and FRAP values are comparable to those of vitamins C and E. The activities of reduced folates in the DPPH assay are merely 2–6 fold lower than those of vitamin C and E.

Oral intake of folic acid (5–10 mg/d/6–8 weeks) increased total antioxidant capacity (TAC) in patients with established CAD (Stanger et al. 2002), in hypercholesterolemic adults under lovastatin treatment (Shidfar et al. 2009), and in hemodialysis patients (Delfino et al. 2007). Activity of folic acid against radical-mediated oxidative damage in human whole blood has been reported previously (Stocker et al. 2003).

In volunteers with manifest atherosclerotic disease and plasma Hcy >20 μmol/L, 2 months of daily oral treatment with 10 mg folic acid were found to significantly increase activities of GPx and SOD, and decrease MDA (Mayer et al. 2002). Wilmink et al. reported that folate treatment prevented the increase in MDA in urine after an acute fat load (Wilmink et al. 2000). Oral treatment with 1 mg folic acid/day in 12 female subjects significantly increased the resistance of very low density lipoprotein (VLDL) and of low density lipoprotein (LDL) against oxidation in (5-MTHF>FA) and in vivo (McEneny et al. 2007).

F2-isoprostanes, a family of bioactive prostaglandin F2-like compounds derived from arachidonic acid through a non-enzymatic process of LPO, are catalyzed by oxygen free radicals on cell membranes and LDL particles. Increased levels of these compounds have been reported in association with myocardial reperfusion injury and with cardiovascular risk conditions (Patrono and Fitzgerald 1997), including severe HHcy because of homozygous CβS-deficiency (Davi et al. 2001).

5-MTHF (15 mg/d/4 weeks) was given to patients with early-onset thrombotic episodes and the 677TT MTHFR genotype. At baseline, patients in the lowest quartile of the plasma folate distribution (<3.9 μg/L) had the highest urinary 8-iso-PGF2α excretion (Coppola et al. 2005). Urinary 8-iso-PGF2α and 11-dehydro-TXB2 excretions were closely correlated. After 5-MTHF supplementation, the urinary 8-iso-PGF2α levels were significantly lowered, their decrease being proportional to baseline values and maximal in patients with lowest pre-supplementation folate levels. On average, 8-iso-PGF2α was 17% lower after 5-MTHF supplementation and no longer significantly different from healthy individuals.

8.5.5 5-MTHF, NO, BH4 and Peroxynitrite

Inflammation of the vascular endothelium is often associated with an "uncoupling" of eNOS, in which eNOS becomes a source of O_2^- concurrent with a reduction in its

capacity to generate NO (Stanger and Weger 2003). This phenomenon is associated with oxidation of BH4, an obligate cofactor for coupled activity of eNOS. NOS function is determined by the availability of BH4 versus the abundance of BH2, which is inactive for eNOS cofactor function.

$ONOO^-$ is a powerful oxidizing and nitrating agent. Due to its pKA of 6.8, the anion predominates (80% at pH 7.4) under physiological conditions. The conjugated acid homolyzes at a rate of $0.9\tilde{s}1$ (37°C, pH 7.4), giving free hydroxyl and nitrogen dioxide ($^{\bullet}NO_2$) radicals in 30% yield, which can nitrate aromatic residues such as tyrosine and tryptophan. The preferential biotargets of $ONOO^-$ are metal centers, carbon dioxide, and sulfur and selenium compounds (Ferrer-Sueta and Radi 2009). $ONOO^-$ appears to be the chief mediator of this oxidation and recent evidence indicates that $ONOO^-$ can suppress synthesis of BH4 by promoting the ubiquitination and proteasomal degradation of GTP cyclohydrolase, rate-limiting for BH4- synthesis (Xu et al. 2007). Administration of folic acid or 5-MTHF can "recouple" the activity of eNOS and restore generation of NO, leading to restoration of endothelial function and NO-dependent vasodilation (Stanger et al. 2001, 2003; Stanger 2002; Verhaar et al. 2002; Das 2003) with potential implications for ischemia-reperfusion injury.

In an animal study, folic acid, administered per os before ligation of a coronary artery, caused a dramatic reduction in myocardial infarct area (Moens et al. 2004). Importantly, this effect appeared to be mediated by an NOS-dependent mechanism, as it could be entirely inhibited by administration of a NOS inhibitor.

A recent ex vivo study investigating human arteries and veins presented evidence that exposure to 5-MTHF rapidly boosts the level of BH4 and the BH4/total biopterin ratio in endothelial cells, while reversing the uncoupled behavior of eNOS (Antoniades et al. 2006). The improved NO-mediated endothelium-dependent vasomotor response could not be explained by direct O_2^- scavenging by 5-MTHF or by changes in plasma Hcy. Rather, 5-MTHF was shown to be a strong $ONOO^-$ scavenger with increased vascular BH4 availability. This effect may be due to the protective effect of 5-MTHF on $ONOO^-$-induced oxidation of BH4. The findings further suggest that 5-MTHF has primarily specific rather than general antioxidant effects in human atherosclerosis. 5-MTHF could enhance eNOS activity and qualitatively restore eNOS coupling in human vessels. Furthermore, 5-MTHF increased the dimer:monomer ratio in human vessels, suggesting an improvement in eNOS protein dimerization (Antoniades et al. 2006).

Although 5-MTHF may be a rather weak O_2^- scavenger, it might increase intracellular BH4 by preventing intracellular oxidation by $ONOO^-$. It may also interact with the pterin-binding site of eNOS in a fashion analogous to BH4 (Hyndman et al. 2002) or directly stimulate BH4-synthesis from BH2. Oxidation of BH4 in the presence of $ONOO^-$ occurs in two steps, the first step yielding a BH3 radical that can be reconverted to BH4 by interaction with ascorbate (Kuzkaya et al. 2003). Further oxidation yields BH2 (7,8-dihydrobiopterin), which promotes eNOS uncoupling due to competition with BH4 for eNOS binding.

Conversely, DHFR (EC 1.5.1.3.) can generate BH4 from BH2. DHFR is a key enzyme in folate metabolism converting folic acid to DHF, and DHF to THF. More recently, the key role for DHFR in eNOS coupling by maintaining the BH4:BH2 ratio, particularly in conditions of low total biopterin availability, was demonstrated (Crabtree et al. 2009).

Folic acid undergoes modification by $ONOO^-$ (Nakamura et al. 2002). The nitrated folate species were identified as 10-nitro-folate and 12-nitro-folate. Furthermore, the radicals derived from $ONOO^-$, nitrogen dioxide and carbonate radical, were demonstrated to inactivate CβS (Celano et al. 2009). CβS catalyzes the condensation of Hcy with serine to form cystathionine and water in the first step of the transsulfuration pathway that yields cysteine. CβS can also catalyze the formation of hydrogen sulfide, a novel gasotransmitter with signalling and cytoprotective effects. Inhibition of the transsulfuration pathway typically leads to HHcy, and to homocystinuria in its extreme form (CβS-deficiency, see above).

Nevertheless, it was recently questioned whether reduced pterins including folates interact directly with $ONOO^-$ (McCarthy et al. 2009). Rather, like uric acid, they may scavenge the nitrogen dioxide and carbonate radicals that evolve from $ONOO^-$ when the latter reacts with carbon dioxide in biological systems. If this is the case, reduced folates may not influence the oxidizing impact of $ONOO^-$ on transition metals or on cysteine and methionine. The reversible oxidation of acidic cysteine groups in proteins is a major route by which $ONOO^-$ (in its protonated form peroxynitrous acid) functions physiologically to modulate signal transmission pathways and enzyme activities. Nontheless, the nitrogen dioxide and carbonate radicals likely are responsible for a high proportion of the cellular damage inflicted by $ONOO^-$, including mutagenesis, nitration, and PARP activation, so the scope of protection afforded by reduced folates may be broad.

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Chapter 9
Folate-Linked Drugs for the Treatment of Cancer and Inflammatory Diseases

Jun Yang, Erina Vlashi, and Philip Low

Abstract Folic acid, also known as vitamin B9 (Fig. 9.1), is an essential co-enzyme in one-carbon metabolism pathways, including the biosynthesis of nucleotides (i.e. purines, thymidine) and several amino acids. In general, two functionally different systems mediate the cellular uptake of folate: (1) the reduced folate carrier (RFC, Kd $\sim$ 10–6 M), an anion transporter that delivers folates across the plasma membrane in a bidirectional fashion, and (2) the folate receptor (FR, Kd $\sim$ 10–10 M), which internalizes folate through active receptor-mediated endocytosis. The RFC, a membrane-spanning anion transporter, is present in virtually all tissues and is responsible for the majority of folate transport in and out of cells. In contrast, FR expression is largely restricted to malignant cells, activated macrophages, and the proximal tubule cells of the kidneys. Because a variety of important diseases are caused by the former two cell types, interest in exploiting FR for drug targeting applications has rapidly increased. And achievement of this targeting objective, primarily through conjugation of drugs to folic acid is believed to enable (1) enhanced net drug uptake by pathologic cells, and more importantly (2) reduction in drug deposition into non-pathologic cells, thereby mitigating collateral toxicity to normal tissues.

Keywords Activated macrophages · Cancer imaging · Folate receptor targeting · Uptake of folic acid · Vitamin-mediated drug delivery

In this chapter, we will first provide an overview of the biology of the folate receptor, followed by a summary of recent advances and developments in FR-targeted cancer therapy. Finally, FR expression in immune cells and its relevance to the treatment of inflammatory and autoimmune diseases will be discussed.

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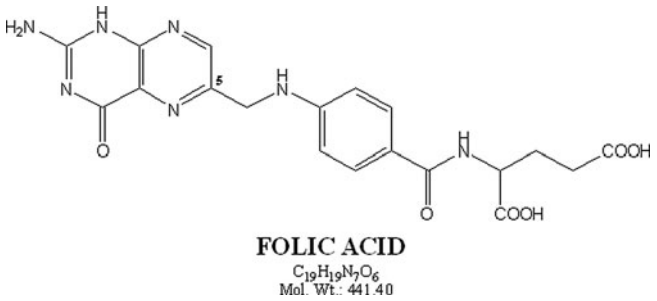

Fig. 9.1 The structure of folic acid

9.1 Biology of the Folate Receptor

The merits for using the folate receptor (FR) as a tumor target are three fold: (i) expression of FR is highly upregulated on cancer cells, (ii) folic acid is a high-affinity, high-specificity ligand for FR and can be chemically modified to enable conjugation with therapeutic and diagnostic agents without impairing its binding to FR, and (iii) FR recycles rapidly between the cell interior and the plasma membrane, allowing delivery of large quantities of folate-linked drugs into FR-expressing cells.

Human folate receptor has at least three well-characterized isoforms (Mr ~ 38 kDa): the α- and β-isoforms are GPI-anchored, whereas the γ isoform is a secreted soluble protein (Shen et al. 1994). The membrane-associated FR-α and -β forms have N-terminal signal sequences of about 20 residues, and characteristic C-terminal hydrophobic segments that serve as the processing signal for GPI-anchor attachment at Ser234 and Asn230, respectively (Yan et al. 1998). Additional acylation may also account for the hydrophobicity of the C-terminal region (Luhrs et al. 1987). All isoforms of FR have 16 conserved cysteine residues, which are believed to form 8 disulfide bridges based on the crystal structure of a closely related protein, namely riboflavin binding protein (Maziarz et al. 1999; Zheng et al. 1988). There are three potential sites for N-linked glycosylation in FR-α (two in FR-β), all of which appear to contribute to cell surface presentation of a functional FR protein. Glycosylation, however appears to be unnecessary for folate binding after FR is already properly folded (Luhrs 1991; Roberts et al. 1998; Shen et al. 1997b). Although capable of mediating uptake of folate (and its conjugates), both α- and β- forms of the FR appear to have somewhat different binding patterns. For example, FR-α binds 6S folinic acid (also known as isovorin, or the 6S diastereoisomer of N5-methyltetrahydrofolate) with an affinity comparable to folic acid, while FR-β displays more than 50-fold lower affinity for the 6S isomer of folinic acid (Wang et al. 1992). Based on studies of a series of chimeric FR variants, such isoform-specific binding selectivity is thought to derive from a sequence divergence involving residues Leu49 in FR-β and Ala49, Val104 and Glu-166 in FR-α (Maziarz et al. 1999; Shen et al. 1997a).

9.2 Expression of the Folate Receptor

Expression of each human FR gene occurs with distinct and narrow tissue specificity, although it has been long believed that elevated FR levels are associated with cells/tissues in need of excess folate (i.e. rapidly dividing cancer cells). According to a comprehensive immunohistochemical analysis of normal tissues by Weitman et al. (1992a) using monoclonal antibodies to FR-α (MOv18 and MOv19), FR-α expression is restricted to certain epithelial cells, including epithelia of the choroid plexus, proximal tubules of the kidney, fallopian tubes, uterus, epididymis, the acinar cells of the breast, submandibular salivary and bronchial glands, type I and II pneumocytes in the lung, and trophoblasts in placenta. Further studies, employing other methods, generally confirm these results (Buist et al. 1995; Garin-Chesa et al. 1993; Stein et al. 1991; Veggian et al. 1989; Wu et al. 1999), and more careful analyses of the anatomic distribution of FR has indicated that the receptor in these tissues is almost exclusively restricted to the apical (lumenally facing) membranes of polarized epithelial cells (Weitman et al. 1992b). Because adherens and tight junctions prevent diffusion of folate conjugates across these epithelia, parenterally administered folate conjugates do not generally gain access to these FR (except in the cases of the kidney and malignant cells). It has, thus been observed that most FRs on normal tissues are inaccessible to folate-targeted agents administered in the blood stream, and therefore, most normal epithelia should remain unaffected by folate therapy.

As noted above, one exception to the rule that normal epithelia are not accessible to folate-linked drugs occurs in normal kidneys, where FR-α is expressed on the apical surface of the proximal tubules. In this case, low molecular weight folate-linked agents, which are filtered into the urine by the glomerulus, are subsequently captured by FR-α on the apical surface of the proximal tubules. These folate conjugates are then transported back to the blood as the FR travels from the apical to the basolateral side of the proximal tubule, a process known as re-absorption transcytosis (Sandoval et al. 2004). The question naturally arises as to whether folate-targeted drugs will be released into the kidney and thus cause renal toxicity. Even though both preclinical and early clinical studies of various folate-targeted therapeutic agents suggest that FR targeting significantly reduces side effects of otherwise toxic compounds without conferring appreciable damage to the kidney (Leamon et al. 2005; Reddy et al. 2006), additional studies are still warranted in order to confirm such conclusions.

Folate receptor expression is also significantly up-regulated in numerous malignancies of epithelial origin, especially ovarian and endometrial cancers. A large number of studies have confirmed that FR-α is consistently over-expressed in ovarian and uterine adenocarcinomas, non-small cell lung cancers, testicular choriocarcinomas, ependymal brain tumors and nonfunctional pituitary adenomas, expressed frequently in malignant pleural mesothelioma and less frequently in breast, colon and renal carcinomas (Buist et al. 1995; Campbell et al. 1991; Evans et al. 2001; Garin-Chesa et al. 1993; Parker et al. 2005; Ross et al. 1994; Toffoli et al. 1997; Veggian et al. 1989; Weitman et al. 1992b; Wu et al. 1999). The ability of FR-α, expressed in malignant tissues, to bind and transport folate into cells has also been

demonstrated (Ross et al. 1994; Weitman et al. 1992b), although other speculated functions remain a matter of debate. It also appears from these studies that there is a quantitative increase in FR-α expression associated with the more poorly differentiated tumors (Orr et al. 1995; Wu et al. 1999). In a more recent study, Low and Hartmann (unpublished observations) demonstrated a clear inverse correlation between FR-α levels in primary breast tumors and 4-year survival rates, raising the possibility that FR is somehow involved in tumor progression and may serve as a prognostic marker for aggressive breast cancers. It should also be noted that a considerable variability in the level of FR over-expression can exist among tumor masses within the same patient and among the same tumor types in different patients (Wu et al. 1999).

9.3 Recycling of the Folate Receptor

An important benefit of folate receptor-targeted drug delivery derives from the fact that FR recycles between the cell surface and intracellular organelles constitutively (Kamen and Smith 2004; Sabharanjak and Mayor 2004). Thus, ligand-bound FR will unload folate conjugates upon endocytosis and the resulting vacant receptors will subsequently return to the cell surface, allowing multiple rounds of folate uptake and the consequent amplification of drug delivery (Fig. 9.2). In cancer cells this rate of FR recycling from the cell surface to internal endosomes and back to the cell surface again has been measured at 8–12 h (Paulos et al. 2004b). In contrast, the rate of FR recycling in activated macrophages has been found to require only ∼15 min (unpublished observations). Although a considerable number of studies have confirmed the passage of FR through various acidic endosomal compartments, the mechanistic details pertaining to FR trafficking seem to vary significantly depending on the experimental system (e.g. cell lines) employed, and thus the precise mechanisms underlying FR-mediated endocytosis remain a matter of debate.

Studying endocytosis of FR-α in a cultured cell line that endogenously expresses FR, namely KB cells, Rijnboutt and coworkers (Rijnboutt et al. 1996) showed that FR was internalized via non-coated membrane invaginations into fluid-filled endosomes. Endocytic studies in Chinese Hamster Ovary (CHO) cells permanently transfected with FR have demonstrated that FR is segregated from other endocytic membrane molecules (e.g. transferrin) at the onset of endocytosis. In fact, immediately after internalization (within a few minutes) GPI-anchored FR are detected in GPI-anchored protein enriched endocytic compartments (GEEC) that are void of markers of clathrin-mediated endocytosis (i.e. transferrin receptor (TfR) and C6-NBD-sphingomyelin) (Sabharanjak et al. 2002; Turek et al. 1993). To summarize multiple studies, it is now concluded that FR-α is segregated from most transmembrane-anchored receptors at the cell surface and internalized via a clathrin/dynamin-independent, Cdc42-regulated endocytic pathway (Higgs and Pollard 2001; Takenawa and Miki 2001).

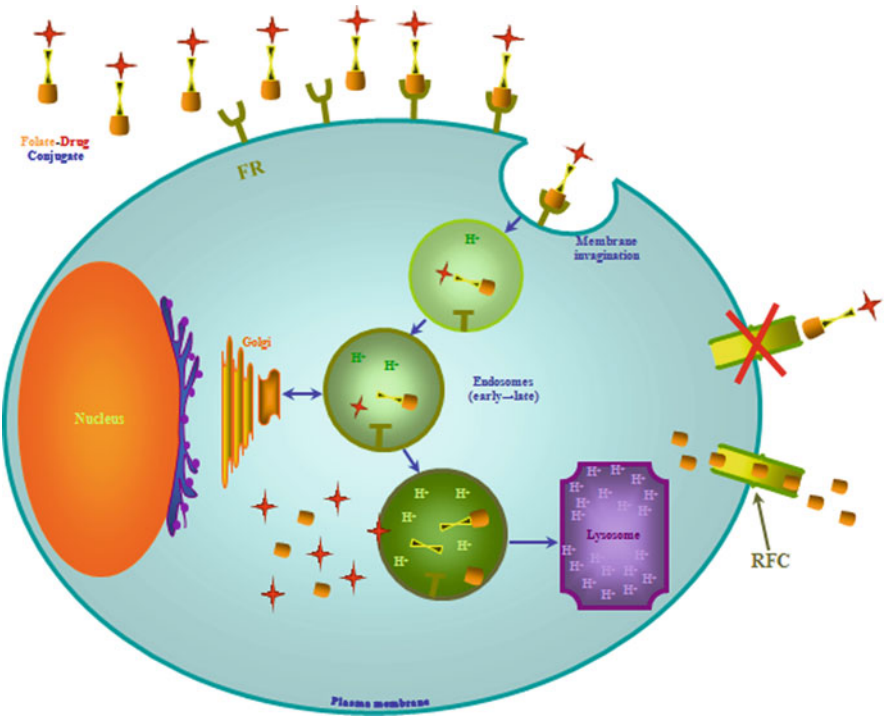

Fig. 9.2 Folate-mediated delivery of therapeutic agents to folate receptor-positive cancer cells. Folates can enter cells via either the reduced folate carrier (RFC), which is present on all cells, or the folate receptor (FR), which is over-expressed on cancer cells. Folate conjugates bind to and enter cells only via the folate receptor. As FR undergoes endocytosis the receptor associated folate–drug conjugates will traffic into the cancer cells. Drugs that require access to intracellular targets can be then delivered in substantial quantities to cytosolic locations via such endocytic pathway

After delivery to the GEECs, FR-α and other GPI-anchored proteins then migrate to a pericentriolar TfR-positive recycling center (REC), from which both eventually return to the plasma membrane (Sabharanjak et al. 2002; Turek et al. 1993). However, comparison of trafficking kinetics of TfR and FR from the REC to the cell surface indicates that GPI-anchored proteins (including FR) are recycled at 3-fold slower rates than their membrane-spanning counterparts (Chatterjee et al. 2001; Mayor et al. 1998), suggesting that the exit of FR from the REC may involve carriers distinct from those for TfR. The question then naturally arises as whether this variability in intracellular trafficking is a consequence of GPI-mediated association with lipid rafts on the cell surface. Consistent with this hypothesis, depletion of lipid raft components (i.e. cholesterol or sphingolipid) has been found to increase the FR recycling rate to a level comparable to TfR by removing the prolonged retention of GPI-anchored proteins in the REC.

Further, Mayor and coworkers (Mayor et al. 1998) have demonstrated that FR (but not a transmembrane isoform) exist in submicron-sized domains (<70 nm)

that are dependent upon cellular cholesterol levels. This highly compact organization of FR on the plasma membrane was also confirmed by Friedrichson and Kurzchalia (Friedrichson and Kurzchalia, 1998) who demonstrated that FR can be chemically crosslinked by an ~ 11 Å bifunctional crosslinker. In contrast, studies by Kenworthy and colleagues (Kenworthy and Edidin, 1998; Kenworthy et al. 2000) have failed to find evidence for a clustered distribution of FR. It was also shown that the intracellular sorting of GPI-anchored FR is dependent on sphingolipid and cholesterol levels, and the involvement of "raft-like" entities during endocytic trafficking of FR has been implied, although the structural and functional correlates of these rafts remain elusive (Parton and Richards 2003; van Meer and Sprong 2004).

9.4 Folate Receptor-Mediated Targeting of Cytotoxic Agents to FR+ tumors

A wide variety of folate-linked molecules and complexes have been designed to enable selective delivery of therapeutic or diagnostic agents to FR-positive (FR+) cancer cells in vivo, including small molecule chemotherapeutics, radiopharmaceutical agents, immunotherapeutic agents (i.e. antibodies), proteins and protein toxins, liposomes with entrapped drugs, plasmids, aptamers, antisense oligonucleotides and nanoparticles (see Advanced Drug Delivery Reviews, volume 56, issue 8, for a comprehensive collection of reviews on recent advances in folate-targeted drug delivery). Readers interested in folate-targeted cancer imaging agents are also directed to an excellent review by Green et al. (Ke et al. 2004).

The first cytotoxic agents conjugated to folic acid were two protein synthesis inhibitors, namely momordin and Pseudomonas exotoxin (PE), prepared in the early 1990s (Leamon and Low 1992; Leamon et al. 1993). When tested in vitro, folate-momordin (IC50 ~1 nM) was shown to selectively kill FR+ cancer cells. Furthermore, selective modification of functional residues in the folate-PE conjugate, which resulted in facilitated escape of the toxin from the endosomal compartment following endocytosis, enhanced its cytotoxicity 10-fold (Leamon et al. 1993). Thus, it was demonstrated that if the protein toxin were provided with a release/escape mechanism from the internal vesicular compartment, it would be extremely beneficial to its effective cytotoxicity. This result highlighted a very important conclusion, which has since been confirmed with other folate-drug conjugates: release of the drug, in its functional form, from a folate-drug construct constitutes a critical step in accelerating and enhancing therapeutic efficacy. Due to their extraordinary toxicity (only one or two molecules of a protein toxin are needed to kill a cell), folate-targeted protein toxins have been largely successful in vitro, and moderately successful in treating "liquid" malignancies in vivo (Pan et al. 2002). However, most folate-targeted therapies based on protein toxins and other macromolecular complexes have failed to produce satisfying results when tested in vivo on mice bearing solid tumors. It is now appreciated that large proteins and other macromolecules may penetrate solid tumors too poorly to accumulate in sufficient quantities for a significant therapeutic effect (Dreher et al. 2006; Evans et al. 2001).

Appreciating the fact that a large fraction of the FR+ cancers are in fact solid tumors, conventional low molecular weight chemotherapeutic agents have become an attractive alternative for their treatment and diagnostic imaging (Henne et al. 2006; Ladino et al. 1997; Leamon et al. 2005). Although generally less potent than protein toxins, small chemotherapeutic agents have the benefit that they penetrate solid tumors easily and are rapidly cleared from the body if they are not captured by a cell surface FR. For example, treatment of solid, FR+ tumor-bearing mice with folate-mitomycin C was found to significantly extend their lifespan in the absence of any evidence of toxicity to normal tissues (Leamon et al. 2005). Meanwhile, the administration of the same doses of nontargeted mitomycin-C showed no antitumor effect, and led to profound impairment of renal and hepatic functions. Together with other similar studies, these results constitute evidence that folate receptor-mediated drug delivery selectively targets therapeutic agents to FR+ cancer tissues, thus reducing general toxicity to normal cells.

In an attempt to increase the amount of small chemotherapeutic drugs delivered to an FR+ tumor, folate-targeted liposomes have been employed. Folate can be anchored to the surface of a liposome via the incorporation of a folate-phospholipid conjugate into the liposome bilayer. Folate targeted liposomes have, in fact, been used to deliver a wide variety of compounds to tumor cells, including small molecular weight drugs (Anderson et al. 2001; Ni et al. 2002; Pan and Lee 2005; Pan et al. 2002; Stevens et al. 2004); genes and antisense oligonucleotides (Hofland et al. 2002; Reddy et al. 2002), neutron capture agents (Stephenson et al. 2003; Sudimack et al. 2002), and radionuclides (Henriksen et al. 2004). Often times, folate is conjugated to the distal end of a longer PEGylated phospholipid (Mr $\sim$ 3,350), such that the folate molecule protrudes further from the polyethylene glycol (PEG) coat of the "stealth" liposome (Mr $\sim$ 2,000), thus, allowing for proper presentation of folate to the FR on the target cell. It has also been documented that maximal uptake is achieved at a rather low concentration of the folate-PEG-lipid conjugate (i.e. 0.03% of total lipid) (Reddy et al. 1999a). As mentioned above, it is critical that the folate-cargo is released and escapes from the endosomal compartment after endocytosis occurs. To this end, pH sensitive lipids and peptides have been employed in some cases, to improve endosomal release of the contents of folate-targeted liposomes (Ghaghada et al. 2005; Reddy and Low; 2000; Turk et al. 2002).

9.5 Folate-Receptor Mediated Immunotherapy

Tumor-specific expression and extracellular presentation of the folate receptor renders FR an especially attractive candidate for targeted immunotherapy. Immunotherapeutic strategies involving FR were first explored in studies where two murine monoclonal antibodies were raised against a membrane preparation of ovarian cancer tissue and were found to recognize FR-α (Coney et al. 1991). Subsequent use of these antibodies included: (1) treating with un-conjugated anti-FR antibodies in order to opsonize the FR+ cancer cells in vivo (Buist et al. 1995; van Zanten-Przybysz et al. 2002); (2) concentrating harmful radiation to the site of cancer by

administration of anti-FR antibodies conjugated to ionizing radionuclides (131I, 211At, 111I) (Andersson et al. 2001; van Zanten-Przybysz et al. 2000); (3) forcing T cell recognition of FR-expressing tumors via the use of anti-FR/anti-T cell receptor bispecific antibodies (Mezzanzanica et al. 1991); and (4) administration of anti-FR immunocytokine constructs (IL-2/MOv19 scFv) to activate immune cells at the tumor cell surface (Melani et al. 1998). These strategies however have their shortcomings due to the same reason highlighted in the previous section: macromolecules (i.e. antibodies) seem to penetrate solid tumors poorly, thus compromising their therapeutic potential.

To avoid these limitations associated with size, a new strategy involving the targeting of highly immunogenic folate-hapten conjugates to tumor cell surface FR has been recently designed and tested in both animals and humans (Lu and Low 2003). In this multi-step process aimed at increasing the immunogenicity of FR+ tumors, the patient/animal is first vaccinated against a specific hapten (such as fluorescein), and then administered the folate-hapten conjugate which binds to and labels all FR-positive tumor cells with the foreign hapten. The tumor-bound haptens are then seen to be rapidly recognized by the anti-hapten antibodies generated by the vaccination, leading to removal of the antibody-coated tumor cells by Fc receptor-bearing immune cells (Fig. 9.3).

In immune competent mice previously vaccinated against fluorescein, administration of a folate-fluorescein conjugate has been shown to result in complete

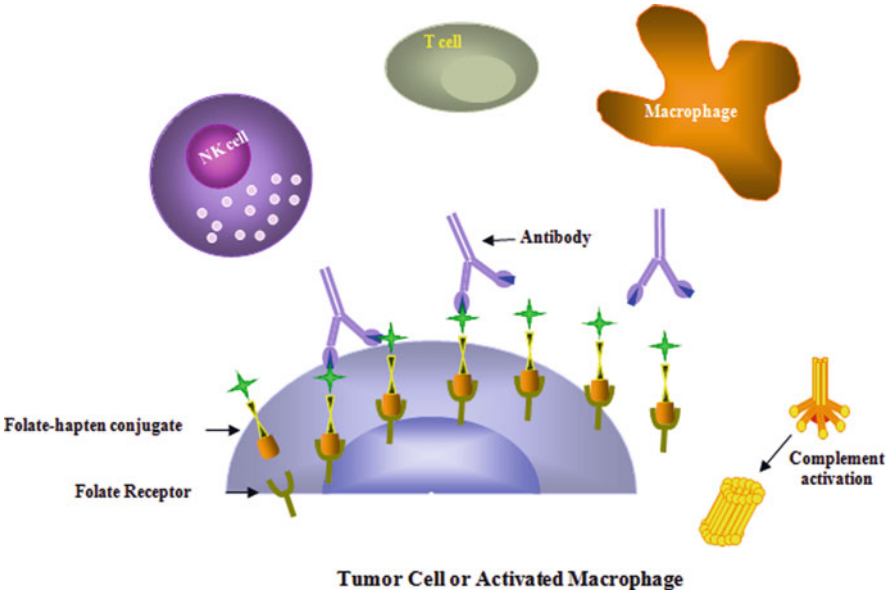

Fig. 9.3 Folate–hapten targeted immunotherapy: retargeting of circulating anti-hapten antibodies to FR-positive tumor cells or activated macrophages via a folate–hapten conjugate. The immune effector cells responsible for the removal of antibody-coated cells are also shown

eradication of established tumors, especially when the immune system was mildly stimulated with cytokines (e.g. IL-2 and IFN-α) (Lu and Low 2002). Perhaps, the most important result to emerge from these studies has been the fact that mice completely cured of their initial tumor burdens have been found to reject subsequent challenges with the same tumor cells, even in the absence of any additional folate-FITC treatment, thus strongly suggesting that a tumor-specific cellular immunity is developed during the initial hapten-mediated folate-targeted therapy, and that this cellular immunity enables subsequent recognition of the same tumor cells (i.e. recurring tumor) without the aid of additional hapten labeling. Recent mechanistic studies have, in fact, confirmed this hypothesis (Lu et al. 2005). They have also shown that depletion of macrophages or CD4+ or CD8+ T cells leads to a substantial compromise of the therapeutic effect, implying involvement of both humoral and cellular immune components (Lu et al. 2005).

9.6 Additional Considerations for the Design of Folate Receptor-Targeted Therapies

Some unusual characteristics of the folate receptor need to be considered when designing a folate conjugate for treatment of cancer. First, most folate conjugates bind FR with sub-nanomolar affinity, often resulting in retention of a significant fraction of the folate-linked agent on FR as it recycles back to the cell surface. Since such a mechanism allows for continuous presentation of the folate-hapten on the surface, it is believed to be beneficial to the immunotherapy. However, in the case of FR-targeted chemotherapy, release of drug inside the cell is crucial for the therapeutic effect. If the folate conjugate returns to the plasma membrane still bound to FR, less drug will not only be delivered to the cell, but fewer FR will be available for the next round of delivery. In order to address this problem, it has been proposed to replace folate with an analogous ligand such as 5-methyltetrahydrofolate, which dissociates more efficiently upon endosomal acidification during the FR recycling (Fig. 9.2). Alternatively, the drug can be attached to folate via a cleavable linker (Fig. 9.4), thus facilitating its release from folate following endocytosis (Leamon and Low 1994; Leamon et al. 1993, 2005).

A variety of releasable linkers, including disulfide bonds, acid sensitive linkers, and enzymatically cleavable bonds, have either been tested or proposed to bridge

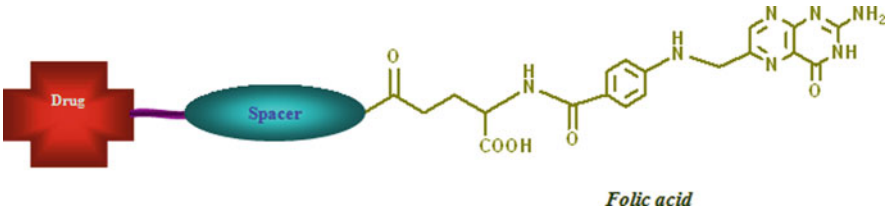

Fig. 9.4 Model fluorescent folate-targeted conjugate for in vitro and in vivo imaging

folate to therapeutic moieties (Hamann et al. 2002; Ladino et al. 1997; Leamon and Reddy 2004; Reddy et al. 2006). Such release mechanisms are especially required in the case when the delivered drug is only active in its unmodified form. Secondly, although many human cancer cells express up to 107 FR/cell, it has also been documented that, in cancer cells, FR recycling is somewhat inefficient. In fact, only ~1 to 3 × 105 molecules/h are delivered inside many cancer cells via FR-mediated endocytosis (Paulos et al. 2004b). Because weakly active chemotherapeutic agents are ineffective at these concentrations, it is usually necessary to select a highly potent chemotherapeutic drug (i.e. IC50 ~ 10 nM) to assure measurable therapeutic efficacy. Finally, it is important to point out that once folate is conjugated to the desired drug, the physical/chemical properties of the drug can dictate the pharmacological behavior and distribution of the conjugate, potentially interfering with FR-mediated drug targeting. Thus, depending on the therapeutic moiety, unanticipated problems with serum protein binding, nonspecific uptake by the liver, and undesirable pharmacokinetics can be influenced by the nature of the payload molecule.

9.7 Folate Receptor Expression in Leukocytes

As mentioned previously, expression of human FR-α is highly restricted to certain normal epithelia and tumors of epithelial origin. FR-β, on the other hand, is expressed in the placenta and in cells of hematopoietic origin or associated malignancies, such as leukemias. For example, FR-β is detected in the myelomonocytic lineage and during neutrophil maturation, where it can be co-expressed with CD14, CD33, CD13, and CD11b, but it is not found on cell expressing lymphocyte markers, CD3 and CD19 (Pan et al. 2002). Curiously, the FR-β expressed on most of these myelocytic cells appears to be functionally inactive, since cells expressing FR-β rarely bind radiolabeled folate or its conjugates. It is now clear that while the receptor can be readily detected by antibodies, the receptor is inactive except when the myelocytic cell becomes activated (Reddy et al. 1999b; Ross et al. 1999). In contrast, functional FR-β is consistently expressed on chronic myelogenous leukemia (CML) cells (Ross et al. 1999). In acute myelogenous leukemia (AML), approximately 70% of the leukemias are FR-β positive (Pan et al. 2002), while acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and hairy cell leukemia cells do not express FR-β (Ross et al. 1999).

A careful examination of FR-expressing cells of the myeloid lineage has revealed that a functional FR-β is often co-expressed with cell surface markers characteristic of a highly activated state (Xia et al. 2009). Consistent with the fact that macrophage/monocyte activation usually occurs only at sites of inflammation, functional FR-β has also been observed on activated (but not quiescent) synovial macrophages in rheumatoid arthritis (RA) in humans (Chen et al. 2005; Nakashima-Matsushita et al. 1999; Reddy et al. 1999b), as well as in a rat arthritic model of the disease (Turk et al. 2002). These studies reveal that FR-β mRNA and FR-β protein are much more abundant in activated monocytes and RA synovial macrophages than

in their naïve counterparts from healthy individuals, and that this receptor can bind folate-fluorophore conjugates with high affinity (Turk et al. 2002). Taken together, these data imply that expression of a functional FR-β is associated with activation of macrophages and monocytes during acute and chronic inflammation, although the nature of the activation process and the molecular mechanism underlying acquisition of binding affinity still remain unclear.

9.8 Folate Receptor-Mediated Therapy for Inflammatory Diseases

As summarized above, activated, but not resting macrophages express a functionally active folate receptor, only in this case the receptor isoform is FR-β. Furthermore, these activated cells seem to contribute prominently to many autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis, multiple sclerosis, Crohn's disease, psoriasis, ulcerative colitis, pulmonary fibrosis, sarcoidosis, scleroderma, and graft versus host disease, etc. The inflammation-associated expression of FR-β has also been demonstrated by imaging studies on both arthritic animals and human patients using EC20, a folate-targeted 99mTc-based radioactive tracer. In these studies, EC20 is seen to concentrate in the arthritic extremities of diseased rats, but with a much reduced intensity in healthy animals. Further, the livers and spleens of arthritic animals also show elevated accumulation of EC20 compared to their normal counterparts (Paulos et al. 2004a). The same results have been obtained in independent experiments on collagen-induced arthritic (CIA) mice. Furthermore, during clinical trials with two different folate-targeted radiopharmaceuticals (folate-DTPA-111In and 99mTc-labeled-folate, EC20) aimed at imaging malignant tissues in cancer patients, arthritic knees, but not healthy knees, of patients also suffering from arthritis can readily be seen in the nuclear images (Fig. 9.5). More recently, analogous results have also been observed in humans with clinically diagnosed rheumatoid arthritis (unpublished observations), suggesting that folate can be exploited for targeting drugs to pathogenic macrophages in a variety of human inflammatory diseases.

As discussed above, the most significant advantage to emerge from FR-mediated cancer therapies has been the surprisingly low level of toxicity to normal tissues. Thus, folate targeting efficiently abrogates uptake of otherwise cytotoxic agents by non-pathologic cells. By the same token, the design of therapeutic strategies to target only the FR+ activated macrophages should result in a reduction of inflammation while minimizing systemic toxicity to nonpathologic cells.

To test this hypothesis, Low et al. have evaluated the aforementioned FR-mediated cancer immunotherapy (Fig. 9.3) on antigen-induced arthritic (AIA) rats. In this application, a folate-hapten conjugate (i.e. folate-FITC) was administered to diseased animals, previously immunized with a KLH-FITC, resulting in significant suppression of both local and systemic inflammation symptoms (e.g. greatly reduced bone and cartilage degradation, reduced paw swelling, smaller spleen size, etc.) in two different advanced-stage models of rheumatoid arthritis. Presumably,

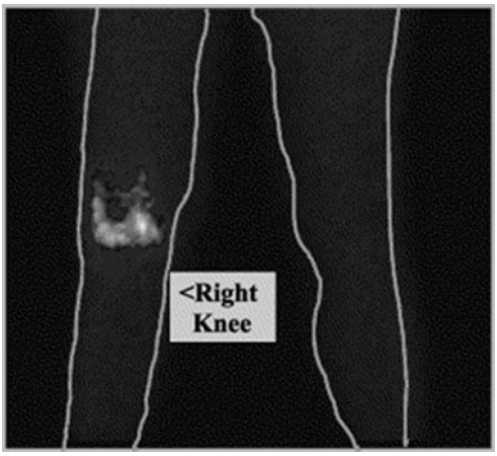

Fig. 9.5 Scintigraphic image of a cancer patient diagnosed with arthritis in the right knee (profile of legs is outlined in *gray*). Four hours following intravenous administration of 2 mg 111In-DTPA-folate, the patient was imaged. This fortuitous observation demonstrated for the first time that folate targeting could be used to deliver drugs to the arthritic extremities of humans with arthritis. Reproduced with permission from Endocyte

specific decoration of FR+ activated macrophages with folate-hapten conjugates promotes selective recognition of the cells by anti-hapten antibodies in immunized animals, leading to elimination of the macrophages via antibody-dependent cell cytotoxicity (ADCC) (Fig. 9.3). As seen in Fig. 9.6 (i) paw swelling, (ii) bone erosion, (iii) systemic inflammation, (iv) splenomegaly, (v) elevation of arthritis score, and (vi) body weight loss associated with untreated arthritis in two different arthritic models (in rats and in mice) can all be essentially prevented/reversed by treatment with folate-hapten conjugates. More importantly, when compared with other standard arthritis treatments in the clinic, such as methotrexate, celecoxib, etanercept and anakinra, folate-hapten therapy yielded better response rates with lower toxicity (Paulos et al. 2006). It should also be pointed out that similar results have been obtained in arthritic dogs, with no observable systemic toxicity even when the folate –hapten treatment lasted for more than 2 years.

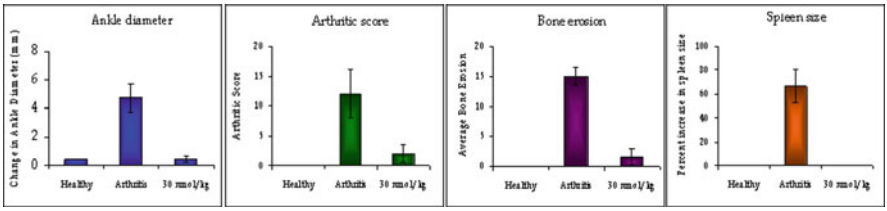

Fig. 9.6 Symptoms associated with rheumatoid arthritis can be prevented/reversed with folate-targeted immunotherapy. The optimum dose for the folate-hapten is as low as 30 nmoles/kg

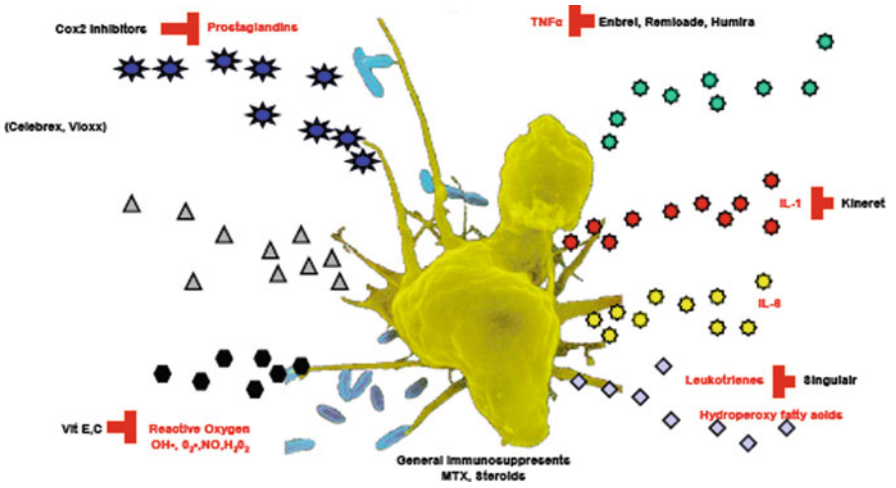

Fig. 9.7 Common anti-inflammatory therapies specifically inhibit a single secretion product of the activated macrophage. In contrast, the folate-targeted therapy enables selective elimination of the activated macrophage responsible for these destructive inflammatory products

9.9 Conclusion

In conclusion, it is now well-accepted that the activated macrophage is the principle contributor to the induction and aggravation of rheumatoid arthritis, as well as many other inflammatory pathologies (listed above). Therefore, this cell constitutes a primary target for therapeutic intervention in numerous inflammatory and autoimmune diseases. The major advantage of the folate-targeting approach lies in the selectivity of the therapy for activated macrophages, a major cause of inflammatory diseases. All other common anti-inflammatory therapies specifically inhibit a single secretion product of the activated macrophage (Fig. 9.7), allowing the macrophage to continue secretion of its other inflammatory mediators. In contrast, the folate-targeted therapy enables selective elimination of a cell population responsible for destructive inflammatory products without damaging other healthy cells required for normal organismal function.

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Chapter 10
Folate in Skin Cancer Prevention

J.D. Williams, Elaine L. Jacobson, H. Kim, M. Kim, and M.K. Jacobson

Abstract Skin, the largest, most exposed organ of the body, provides a protective interface between humans and the environment. One of its primary roles is protection against exposure to sunlight, a major source of skin damage where the UV radiation (UVR) component functions as a complete carcinogen. Melanin pigmentation and the evolution of dark skin is an adaptive protective mechanism against high levels of UVR exposure. Recently, the hypothesis that skin pigmentation balances folate preservation and Vitamin D production has emerged. Both micronutrients are essential for reproductive success. Photodegradation of bioactive folates suggests a mechanism for the increased tendency of populations of low melanin pigmentation residing in areas of high UV exposure to develop skin cancers. Folate is proposed as a cancer prevention target for its role in providing precursors for DNA repair and replication, as well as its ability to promote genomic integrity through the generation of methyl groups needed for control of gene expression. The cancer prevention potential of folate has been demonstrated by large-scale epidemiological and nutritional studies indicating that decreased folate status increases the risk of developing certain cancers. While folate deficiency has been extensively documented by analysis of human plasma, folate status within skin has not been widely investigated. Nevertheless, inefficient delivery of micronutrients to skin and photolysis of folate argue that documented folate deficiencies will be present if not exacerbated in skin. Our studies indicate a critical role for folate in skin and the potential to protect sun exposed skin by effective topical delivery as a strategy for cancer prevention.

Keywords Cancer prevention · DNA repair · Folate · Folic acid · Skin · Topical delivery strategy · UV light

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10.1 Human Skin and UV Radiation

The skin provides a direct interface for the human body with the environment and plays many roles in protection against physical, chemical, and microbial insults. The average human skin area exceeds m^2 and is generally less than 2 mm thick (Goldsmith 1991). Skin protects against most solar UV radiation (UVR), regulates body temperature through surface blood flow control and sweating, allows detection of the ambient and physical environments through feel and touch, and participates in social communication through the display of information such as age, health, and ancestry (Jablonski 2004). Human skin is a complex laminar structure comprised of many different cell types organized into the outer epidermal layer and an inner dermal layer. The epidermis provides the barrier properties of the skin and is composed of keratinocytes (structure), melanocytes (pigmentation), Langerhans cells (immune system), and Merkel cells (sense of touch). Keratinocytes are the primary cell type in the epidermis and originate in the stratum basale layer from the continual division of stem cells. They migrate outward through the epidermis while undergoing differentiation until they reach the stratum corneum where they form a layer of dead, flattened, highly keratinized cells called squamous cells. The epidermis is avascular and contributes to the elasticity and toughness of the skin. At the stratum corneum, keratinocytes are continuously shed and replaced (Goldsmith 1991) generating significant metabolic demands for energy and nutrient consumption. The dermis is a dense fibroelastic connective tissue composed of collagen fibers, elastic fibers, and an interfibrillar gel which comprises most of the skin thickness. Fibroblasts are the primary cell type in the dermis and are collagen-rich. Collagen is a major skin component and accounts for the tensile strength of the skin. Interwoven with the collagen is a network of elastic fibers. The dermis appears to be of equal thickness in people with dark or light pigmentation (Taylor 2002).

10.2 UVR, DNA Damage, and Skin Cancer

Skin damage, particularly that derived from sunlight, constitutes a major public health problem. Non-melanoma skin cancers (NMSC) are the most frequent malignancies in the United States with more than 1,000,000 cases diagnosed annually (Karagas et al. 1999). Melanoma skin cancer is the most rapidly increasing type of cancer. Additionally, actinic keratosis (AK), skin lesions that can progress to NMSC are far more prevalent than skin cancers. DNA damage and cellular responses to DNA damage play a central role in skin damage (Ames 2001). Sunlight is the major source of skin damage as it leads to DNA damage directly via formation of pyrimidine dimers and other photoproducts (Ullrich 2002) and indirectly via generation of reactive oxygen species (ROS) and reactive carbonyl species (RCS) by photooxidation and photosensitization reactions (Wondrak et al. 2002a, b) (Wondrak et al. 2003). UVR is a complete carcinogen. UVB (280–320 nm) which penetrates into the epidermis, is responsible for most of the direct DNA damage and is the most

effective at initiation of squamous cell carcinoma (SCC). UVA (320–400 nm) which penetrates into the dermis, induces ROS and causes SCC (Pentland et al. 1999; Agar et al. 2004).

Chronic DNA damage results in progressive losses of genomic integrity and end stage skin damage in the form of skin cancer involving altered growth properties of keratinocytes such as unresponsiveness to terminal differentiation signals leading to epidermal hyperplasia and progressively to actinic keratosis (Jeffes and Tang 2000; Lober et al. 2000). Cell populations present in actinic keratosis lesions progress to transformed cell populations that represent epidermal carcinoma in situ (Guenthner et al., 1999; Hurwitz and Monger 1995; Kobayashi et al., 2000). Subsequent cellular changes occur including induction of matrix proteases that facilitate disruption of the integrity of the epidermal barrier leading to invasion of the dermis, the point at which the damage process is diagnosed as SCC. A second major consequence of DNA damage in skin is the suppression of immune responses that would normally detect and remove damaged cells. The consequences of sunlight-induced DNA damage are depicted in Fig. 10.1. While mechanisms of immune suppression extend beyond DNA damage, DNA damage is a major factor. The consequences of genotoxic stress include altered migration, antigen presentation by Langerhans cells, and

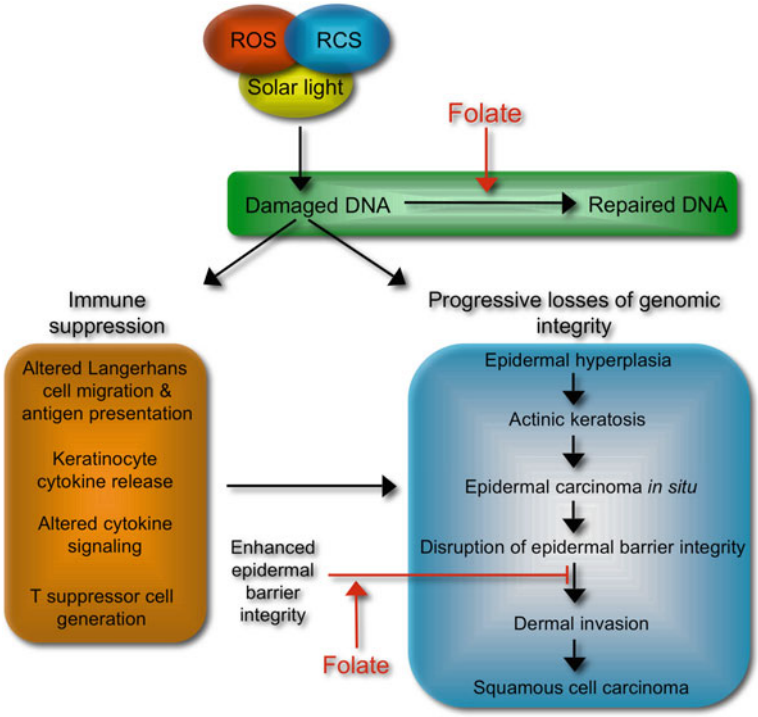


Fig. 10.1 Sunlight-induced DNA damage pathways and opportunities for micronutrient modulation

stimulation of cytokine release by keratinocytes that likely alters cytokine signaling required for normal immune surveillance including generation of T suppressor cells. Given the complexity of damage pathways and the down stream effects, opportunities to modulate the consequences of genotoxic stress include preventing DNA damage, enhancing DNA repair, and strengthening the integrity of the epidermal barrier to prevent migration of transformed cells from the epidermis (depicted in Fig. 10.1). A compelling body of evidence now indicates that several key micronutrients are candidates for skin damage prevention. This chapter will focus on the role of folate in skin health.

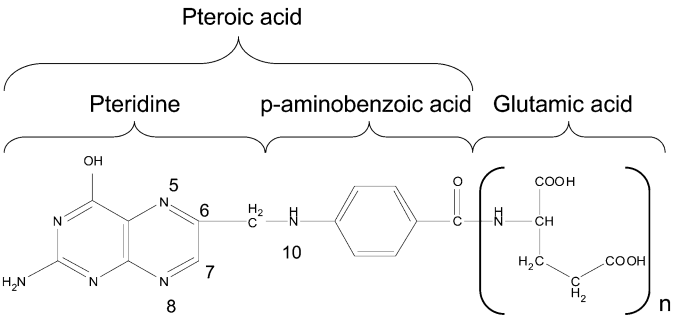
10.3 Folate Metabolism

In 1931, it was demonstrated by Lucy Wills that yeast extract was effective in treating tropical macrocytic anemia observed during late pregnancy (Wills 1978). This observation led to the isolation and structural determination of the B vitamin folate, named after the Latin word – folium (leaf). The terms folate and B9 vitamins refer to a large family of chemically similar compounds, the most widely known of which is folic acid. Folic acid or pteroylmonoglutamate (PteGlu), is a synthetic folate analog utilized preferentially due to its enhanced chemical stability and rapid conversion to the bioactive forms upon uptake. Natural folates vary in the one-carbon substituent at the N10 and N5 positions as well as in the number of glutamic acid residues conjugated via gamma glutamyl bonds to form the polyglutamate tail. Natural folates also vary in the oxidation state of the pteridine ring, with the reduced, unsubstituted dihydrofolate (DHF, $H_2PteGlu_n$) and tetrahydrofolate (THF, $H_4PteGlu_n$) forms being particularly prone to biological inactivation due to cleavage of the C-9 and N-10 bond (Blakley 1969). The structure of folic acid and its reduced, native forms are illustrated in Fig. 10.2.

Humans do not synthesize folate, and thus are dependant upon a variety of dietary sources. The overall folate nutritional status of the population is believed to have been positively influenced since the implementation of the United States federal government's requirement to fortify cereal-grain products in 1998 (Dietrich et al. 2005); however, this effect may be waning due to current low carbohydrate diet fads. Natural folates are a mixture of reduced folate polyglutamates which vary in the number of glutamic acid residues. The main folate found in foods is 5methyltetrahydrofolate (5-methyl-$H_4PteGlu_n$, 5MTHF) and the monoglutamyl form is the primary form of folate which enters the circulating plasma (Thien et al. 1977; Herbert et al. 1962).

The transport and metabolism of the synthetic folic acid and it's active folate metabolites have been extensively characterized. Figure 10.3 depicts an overview of folate metabolism which along with the role of folate in disease processes is expertly reviewed in (Lucock 2000).

In the cell, folates serve to carry and transfer one-carbon units of various oxidation levels during biosynthetic reactions that occur within the cell. Of particular



Native folate	Substituent at	
	N-5	N-10
THF	-H at 5,6,7, & 8	-H
DHF	-H at 7 & 8	-H
5FormylTHF	-HCO	-H
5MethylTHF	$-CH_3$	-H
10FormylTHF	-H	-HCO
5,10MethenylTHF	$=CH^+-$	
5,10MethyleneTHF	$-CH_2^-$	

Fig. 10.2 The chemical structure of folic acid and its various derivatives

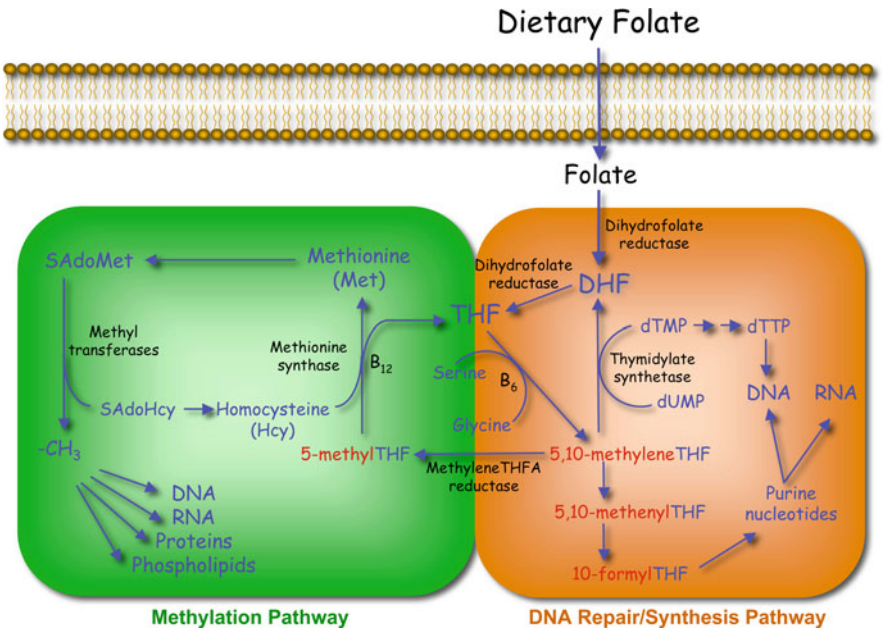


Fig. 10.3 Overview of folate metabolism

importance is the role that folates play in purine and pyrimidine nucleotide biosynthesis to supply precursors for DNA repair and synthesis. Also of importance is the role that 5MTHF plays in the homocysteine (Hcy) remethylation cycle and de novo methionine biosynthesis. De novo synthesized methionine can then

be activated by ATP and the enzyme methionine adenosyltransferase to yield the methyl donor Sadenosylmethionine (SAM). SAM serves as the methyl donor for the majority of methyltransferases that modify DNA, RNA, histones and other proteins, contributing to replicational, transcriptional and translational fidelity, mismatch repair, chromatin remodeling, epigenetic modifications and imprinting, which are all topics of great interest and importance in cancer research and aging.

10.4 UVR at the Earth's Surface and Photodegradation of Folates

UVR levels reaching the Earth's surface are affected by numerous factors such as latitude, altitude, season, moisture content, cloud cover, and the depth of the ozone column (Madronich et al. 1998). The shortest UV wavelengths (UV-C, 100–280 nm) are essentially completely blocked or absorbed by atmospheric oxygen (O_2) and ozone (O_3). Wavelengths in the UV-B range (280–320 nm) are partially absorbed by O_3 and thus reach the Earth's surface while UV-A wavelengths (320–400 nm) are only weakly absorbed by O_3 and are most easily transmitted through the atmosphere. At latitudes further away from the Equator, the angle of solar elevation decreases and the thickness of the atmosphere and ozone layer through which sunlight must pass before reaching the Earth's surface increases which results in attenuation of UVR levels. It is now well established that folic acid is photosensitive. Folic acid has absorption peaks at 280 and 350 nm with shoulders at 300 and 370 nm. The absorption peak at 350 nm indicates that folic acid absorbs UVA radiation which is very prevalent at the Earth's surface. Folic acid solutions exposed to biologically relevant levels of UVA radiation demonstrated that UVR exposure results in the cleavage of folic acid to form p-aminiobenzoyl-L-glutamic acid and 6-forml pterin (Off et al. 2005). Bioactive folate derivatives are also UV labile. The most common bioactive folate, 5MTHF, has an absorption maximum at 290 nm. Exposure of 5MTHF to UVB at 312 nm results in oxidation to 5-methyldihydrofolate (5MDHF), which upon continued irradiation is further broken down to a pterin residue and paminobenzoylglutamate (Lucock et al. 2003). While the breakdown of 5MTHF results in irreversible loss of vitamin activity by cleavage of the C9-N10 bond, even oxidation to 5MDHF may decrease vitamin activity. UVB does not penetrate deeply into the skin as depicted in Fig. 10.4, and therefore, does not reach the blood stream. Thus, direct photodegradation of folates may not be the mechanism of biological significance. However, the oxidation of 5MTHF to 5MDHF is stimulated by exposure to light in the presence of photosensitizers (Steindal et al. 2006). This implicates ROS in folate degradation. ROS are produced in human tissues by UVA radiation (Fig. 10.4) and possibly radiation of even longer wavelengths which would penetrate the skin and blood vessels to a much greater extent than UVB. ROS production by longer wavelength radiation is enhanced in the presence of photosensitizers, and numerous endogenous photosensitizers are present in both the skin and blood.

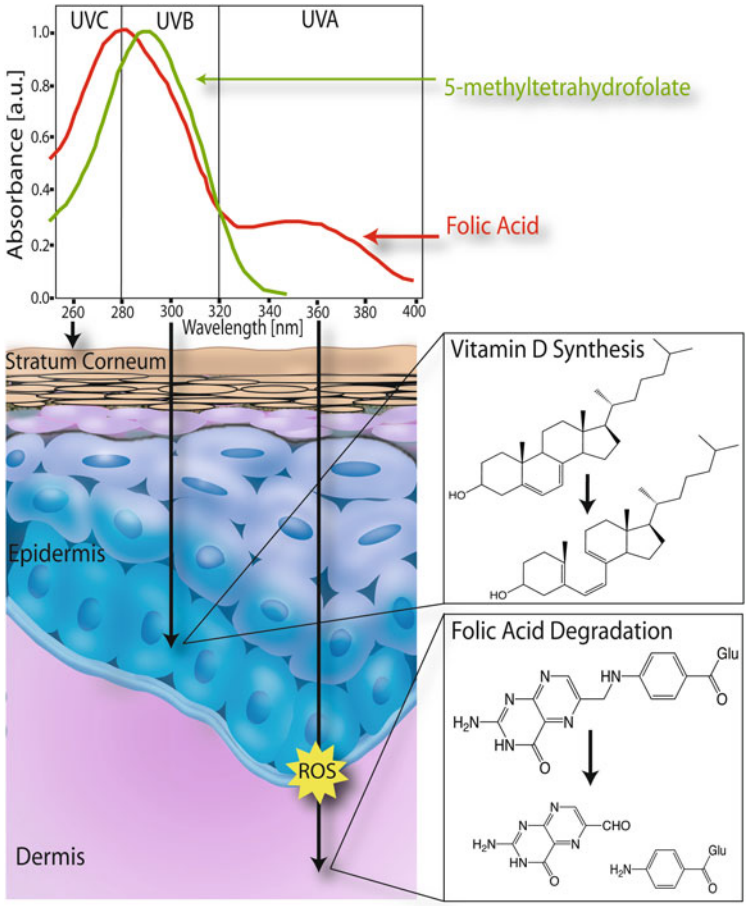


Fig. 10.4 Effects of UV radiation on lightly pigmented skin

10.5 Variations in Human Skin Pigmentation

It has been long recognized that skin colors among indigenous populations show a direct relationship to the intensity of the local UV spectrum. Recently, Jablonski and Chaplin have dramatically advanced the study of the proposed relationships by correlation and regression analyses of quantitative skin color measurements obtained by skin reflectance spectrophotometry with newly available remotely sensed data on levels of UVR, total solar radiation, temperature, humidity, precipitation, and other environmental variables at the Earth's surface (Jablonski 2004). The strongest association to skin color was found to be latitude, which corresponds to an effect of UVR intensity. A strong correlation was found between latitude and annual

average minimal erythemal dose of UVR (UVMED), and thus between annual average UVMED and skin reflectance using UVMED data from the Earth's surface (Jablonski and Chaplin 2000). A subsequent study of the influence of minimum, maximum, and seasonal levels of UVR showed that skin reflectance was correlated with autumn levels of UVMED, and that skin reflectance could be almost fully modeled as a linear effect of this variable alone (Jablonski 2004). Dark pigmentation was found to be primarily a function of UVMED (Jablonski and Chaplin 2000), with regression analysis demonstrating that autumn UVMED levels have the strongest effect. Interestingly, their data indicated that skin color is more strongly correlated with UVA, which is consistently higher throughout the year at all latitudes, than with UVB (Jablonski 2004).

10.6 The Evolution of Skin Pigmentation and the Micronutrient Hypothesis

The evolution of human skin color has invited many possible explanations. Enhanced resistance to sunburn is clearly a major hypothesis. Devastating consequences for albinos in topical regions of intense UV light exposure including early fatalities from both SCC and acute injury from solar exposure leading to denudation and resultant infections directly support this reasoning (Cohn 1998). The benefits of lightly pigmented skin have been related to the synthesis of vitamin D. It is estimated that most humans utilize sunlight exposure to obtain nearly 100% of their vitamin D which is optimally stimulated by exposure to UVB photons with wavelengths of 295,300 nm (Holick 2003; Holick et al. 1981). The current hypothesis states that light skin pigmentation is necessary in regions of low UVR in order to permit vitamin D biosynthesis (Loomis 1967) and is an adaptation to resist cold injury (Post et al. 1975). Clearly, melanin pigmentation is an adaptation to some attribute of the physical environment. The genetic characteristic of skin pigmentation is believed to be governed by at least three to five loci which would require continued positive selection for its maintenance. This points to a sustained evolutionary pressure which acts to favor retention of pigmentation characteristics (Cohn 1998). Adaptive explanations for a given phenotypic trait require demonstration that the trait increases the real or probable reproductive success of the organism. Adaptive explanations such as resistance to skin cancer or protection from cold injuries attributed to different levels of melanin pigmentation in human skin have suffered from an inability to demonstrate probable or real differences in survivorship and reproduction of different skin color phenotypes (Jablonski 2004). The original hypothesis that dark skin pigmentation arose to protect against skin cancer induced by UV exposure does not account for selective reproductive pressure as fatal repercussions of skin cancers tend to develop after reproductive age. In this reasoning, the adaptive response of skin pigmentation toward the adequate protection or production of micronutrients which play key roles in the success of the reproductive process has gained much credibility. The evolution of dark skin to protect against folate photo-degradation

serves a direct reproductive influence as folate is now known to be essential for fetal development and fertility. The attenuation of skin pigment levels to allow for adequate vitamin D production also serves a direct reproductive influence in fetal bone development and maternal bone health. This model in which skin pigmentation balances availability of essential micronutrients is of much interest as shifting world populations result in many people residing in areas of UV exposure that are significantly different from those to which their skin tone has been adapted.

10.7 Melanin and UV Penetrance

Melanocytes in the epidermis produce the skin's primary pigment, melanin. In mammals there are two types of melanin pigments, eumelanin which is black-brown and pheomelanin which is yellow-reddish (Thody et al. 1991). Melanins are produced in highly organized elliptic membrane bound cytoplasmic organelles of melanocytes called melanosomes. Melanocytes project their dendrites into the neighboring keratinocytes where they then transfer mature melanosomes. Once transferred to the keratinocytes, melanosomes aggregate and are surrounded by a membrane in a melanosome complex (Szabo et al. 1969). The variation in skin color among various races is determined mainly by the number, melanin content, and distribution of melanosomes within the keratinocytes (Jimbow et al. 1976). Natural selection has produced a gradient of skin pigmentations in response to two opposing factors. The first factor results in a cline of photoprotection that grades from darkly pigmented skin at the Equator to lightly pigmented skin near the Poles. The second factor results in a cline of vitamin D photosynthesis that grades from lightly pigmented near the Poles to darkly pigmented at the Equator. In the middle of the two clines we find peoples with enhanced abilities to develop facultative pigmentation according to seasonal UVR levels (Jablonski 2004). The concentration, depth, and distribution of skin melanin is strongly affected by UVR exposure. The tanning response observed in peoples able to develop facultative pigmentation results from distribution of melanosomes throughout the epidermis from the basal layer. Melanin has a broad absorption spectrum stretching from the UV to the near infrared regions. Recently it has been noted that not only the quantity of melanin present but also the depth and distribution of the melanosomes impact the amount of UVR penetrating to the living cells of the epidermis (Nielsen et al. 2006). Within keratinocytes, melanin granules accumulate above the nuclei and act as a sunscreen absorbing harmful UVR before it can reach the nucleus and damage the DNA.

Melanin serves to protect at least in part from mutations that might be caused by UVR exposure (Ohnishi and Mori 1998). In addition to DNA protection, it has also been shown that darkly pigmented skin generates a larger reflectance of wavelengths below 300 nm than lightly pigmented skin due to scattering of light by the spheroid shaped melanosomes (Nielsen et al. 2004). Scattering and absorption of light in this range shows that melanin and melanosomes play an important role as natural sunscreens in moderating UVR related health effects.

10.8 Folate Deficiency

Naturally occurring folates are water soluble, labile compounds that rapidly lose activity in foods over periods of days or weeks, consequently it is estimated that half or even three-quarters of initial folate activity may be lost between harvest and consumption (John Scott 2000). In view of the dietary behavior of many individuals, inadequate folate intake is likely and supplementation of folates is strongly recommended. This usually involves consumption of synthetic folic acid, which is more chemically stable and bioavailable than the natural folates.

10.9 Folate Deficiencies in Skin

Folate deficiency has been extensively documented in human plasma. However, folate status within skin has not been widely investigated. Delivery to the skin via the blood circulation of nutrients taken orally is inherently inefficient since this delivery is distal to other organs, particularly the liver, which removes many agents by first pass metabolism. In addition, the epidermal layer of the skin is non-vascular. The inefficiency of delivery of nutrients to skin argues that documented folate deficiencies will extend to skin. Folate is sensitive to photolysis by exposure to UVR. Exposure to sunlight and UVR in particular is expected to lower folate levels in at least the superficial layers of the skin. Photolysis of folate stores in the skin may be extensive enough to result in systemic depletion of folate. Indeed it has been reported that fair skinned patients undergoing photochemotherapy for dermatological conditions have low serum folate concentrations, suggesting that folate depletion may occur in vivo (Branda and Eaton 1978). However, in another experiment where volunteers were exposed to UVA radiation in a solarium, no connection between UVA exposure and plasma folate status was found (Gambichler et al. 2001). This study has since been criticized for use of non-specific bioassays for folate quantification, which serves to exemplify the point that much research is left to be done (Lucock et al. 2003).

10.10 Folate Deficiencies and Human Disease

Folate deficiencies and genetic folate metabolism alterations have been linked to a wide range of conditions including megaloblastic anemia (Lucock 2000), mood alterations (Godfrey et al. 1990), Alzheimer's disease (Clarke et al. 1998), and numerous athero/thrombogenic phenomena (Brattstrom and Wilcken 2000). Folate deficiencies have also been shown to play a role in Downs syndrome (Hobbs et al. 2000), neural tube defects (MRC vitamin study research group 1991), pregnancy complications (Rajkovic et al. 1997), and male infertility (Wong et al. 2002), which positions folate as a limiting factor in the evolution of the human species. Finally

folate deficiencies have been linked to an array of cancers including those of the colon (Slattery et al. 1999), breast (Zhang et al. 1999), pancreas (Stolzenberg-Solomon et al. 2001), stomach (Fang et al. 1997), cervix (Butterworth 1993), bronchus (Kamei et al. 1993), and blood (Skibola et al. 1999).

10.11 Cancer Prevention by Enhancing Genomic Integrity and Repair

The major putative relationship between cancer and folate status relates to the role of folate in providing precursors for DNA repair and synthesis. Folate may also promote genomic integrity through its role in the generation of methyl groups needed for regulation of gene expression via CpG methylation patterns. The cancer protection potential of folates has been demonstrated by large-scale epidemiological and nutritional studies indicating that decreased folate status increases the risk of developing certain cancers. Consistent with a role in DNA repair, chromosome breaks and centrosome abnormalities have been observed in patients deficient in folate (Heath 1966; Chen et al. 1989). In vitro, DNA strand breakage and uracil misincorporation increased in a time and concentration dependent manner after human lymphocytes were cultured with decreasing amounts of folate (Duthie and Hawdon 1998). Moreover, folate deficiency impaired DNA excision repair in specific tissues excised from animal models (Choi et al. 1998). These data indicate that folic acid deficiency affects the stability of cellular DNA at the chromosomal and molecular levels (Choi and Mason 2000). Folate supplementation, particularity localized to areas of elevated demand, is thus proposed as a strategy to enhance genomic integrity and prevent the development of cancer.

10.12 Cancer Prevention by Enhancing the Epidermal Barrier

A terminally differentiated epidermal barrier with high integrity is crucial to cancer prevention. Several points need to be considered with regard to micronutrients in epidermal barrier development. First, there is a growing body of evidence indicating that a significant percentage of the American population is deficient in a number of micronutrients and the constant turnover of the epidermis makes this tissue particularly vulnerable to micronutrient depletion. Micronutrient deficiencies observed in plasma are observed also in skin (Peng et al. 1993). Second, the constant renewal of the epidermal compartment places an important nutrient requirement on the organism. Thus, the nutritional status of micronutrients such as folic acid whose bioactive forms play important roles in the generation of components necessary for optimal cell growth and protein expression regulation are important to the integrity of the epidermal barrier. Third, the non-vascular nature of epidermal compartment makes micronutrient delivery to this compartment inherently inefficient. The above considerations lead to the hypothesis that optimal micronutrient status will strengthen

integrity of the epidermal barrier which in turn can lead to a decrease in skin cancer. Studies have shown that cell populations with altered growth properties within actinic keratosis lesions can be recognized by immune surveillance and removed. Alternatively, cell populations within such lesions can progress to cell populations (carcinoma in situ) that secrete proteases and other factors that allow escape from the epidermis. Thus, the degree of integrity of the epidermal barrier can be a deciding factor between the ultimate fates of removal or escape of abnormal cell populations from the epidermal compartment.

10.13 Generation of Folate-Restricted Skin Cell Culture Model

HaCaT keratinocytes have been used to create a cell culture model of folate deficiency. The HaCaT cell line derives from normal human abdominal skin and exhibits a differentiation profile comparable with normal human keratinocytes despite an altered and unlimited growth potential. HaCaT cells exhibit UV-B type-specific mutations on the p53 tumor suppressor gene (Stampfer et al. 1993), representative of an early stage precancerous skin cell. To analyze the role of folate in skin cells during exposure to UV photodamage or photooxidative stress, we have developed a cell culture method in which folate concentrations in the culture medium are controlled by restricting folate and dialyzing the fetal bovine serum. This significantly reduces growth rates of HaCaT keratinocytes, as shown in Fig. 10.5. Because these cells cannot synthesize folates de novo, folate levels are rapidly depleted during cell division.

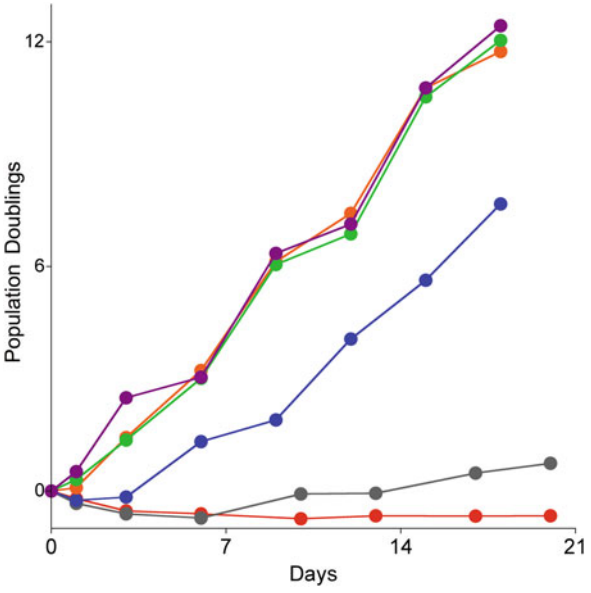


Fig. 10.5 HaCaT keratinocytes were depleted of folate for 14 days. Cell growth was measured after folate repletion at varying concentrations. Non-Depleted Control (9100 nM Folate), Depleted (9100 nM Folate), Depleted (910 nM Folate), Depleted (91 nM Folate), Depleted (9.1 nM Folate), Depleted (0 nM Folate)

We have found that through folate restriction the population doubling times of cells in culture are increased in a direct relationship to folate depletion over a critical range of concentrations (Fig. 10.5). Normal growth rates were reestablished upon folate supplementation. Interestingly, under conditions of complete folate deprivation, HaCaT cells survive without dividing for an extended period of time stalled in the S phase of the cell cycle.

10.14 Topical Micronutrient Delivery for Skin Damage Prevention

Described here is evidence that folate is a candidate for skin damage prevention involving several different mechanisms. However, a major challenge for the development of micronutrient prevention strategies for skin damage is delivery of micronutrients to skin. Folic acid is a good candidate for topical micronutrient delivery based on the roles for the bioactive forms of this nutrient in maintenance of genomic integrity via enhancement of DNA synthesis, DNA repair, and maintenance of epigenetic regulation. Our studies have demonstrated that a topical micronutrient delivery strategy to limit skin damage is feasible. Since folate is hydrophilic, developing a lipophilic form would enhance delivery through the stratum corneum, which is very lipophilic. Pro-folate compounds designed for targeted delivery were synthesized. Once delivered to the epidermis, the abundant esterases present there rapidly cleave the pro-folates to the parent compound. We designed the delivery properties to provide a slow, continuous supply of folate to skin cells to allow increased uptake by the cells. This strategy takes into consideration two distinct barriers that influence the delivery of a micronutrient to skin, lipophilicity of the stratum corneum and skin metabolic activity. The formulation strategy controls the rate of partitioning of the pro-folate into the stratum corneum by optimizing lipophilicity. Figure 10.6 shows a multiple compartment model that served as the framework for the development of this delivery strategy.

The pro-folate must effectively partition from the topical formulation into the stratum corneum. The highly lipophilic nature of the stratum corneum dictates that the profolate be sufficiently lipophilic to effectively partition into the stratum corneum from the delivery vehicle, e.g. a skin cream of lotion (arrow #1 in Fig. 10.6). The pro-folatet must partition from the stratum corneum into the epidermis at an optimal rate to achieve effective delivery to the cellular components of skin. (#2 in Fig. 10.6). Studies of drug structure-penetration relationships have provided useful information concerning this criterion (Tsai et al. 1992; Weber et al. 1994). A correlation between skin permeability and the physicochemical properties of the drug, such as octanol/water partition coefficient (Poct/w) have proven to be of great value in predicting drug transport across skin. A linear correlation between skin permeability of many compounds and their log (Poct/w) has been established (Anderson et al. 1998; Roberts et al. 1978). These findings allow a correlation between prodrug lipophilicity and drug delivery.

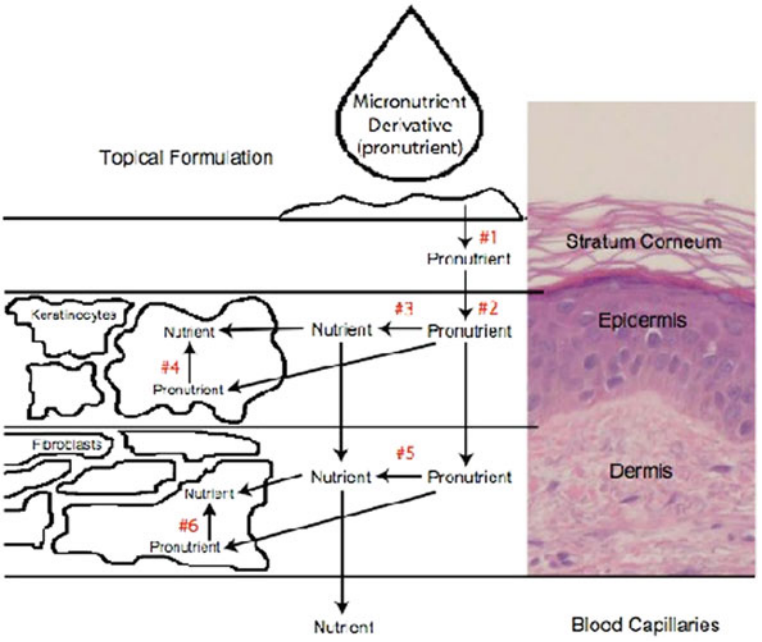


Fig. 10.6 Tropical micronutriment delivery: a multiple compartment model

10.15 Summary

The discussion presented above suggests that a great deal is yet to be learned regarding folate metabolism in skin. Using the in vitro model described along with experiments in human reconstructed skin models and animal models, addressing this dearth of information should be possible. Furthermore, the strategy to optimize folate status by directed pro-folate topical delivery mechanisms in sun exposed skin may provide an opportunity to prevent skin cancer development and progression.

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Chapter 11
Thiamin(e): The Spark of Life

Derrick Lonsdale

Abstract One of the earliest vitamins to be discovered and synthesized, thiamin was originally spelled with an "e". The terminal "e" was dropped when it was found that it was not an amine. It is still spelled with and without the "e" depending on the text. This chapter provides a brief historical review of the association of thiamin with the ancient scourge of beriberi. It emphasizes that beriberi is the model for high calorie malnutrition because of its occurrence in predominantly white rice consuming cultures. Some of the symptomatology of this ancient scourge is described, emphasizing the difference from that seen in starvation. High calorie malnutrition, due to excessive ingestion of simple carbohydrates, is widely encountered in the U.S.A. today. Thiamin deficiency is commonly associated with this, largely because of its cofactor status in the metabolism of glucose. The biochemistry of the three phosphorylated esters of thiamin and the transporters are discussed and the pathophysiology of thiamin deficiency reviewed. The role of thiamin, and particularly its synthetic derivatives as therapeutic agents, is not fully appreciated in Western civilization and a clinical section describes some of the unusual cases described in the scientific literature and some experienced by the author. The possible role of high calorie malnutrition and related thiamin deficiency in juvenile crime is hypothesized.

11.1 History

It is now well known that thiamin deficiency is the major cause of beriberi, a disease that had affected humans for centuries. The name "Kakke" was the term used for the disease in Japan and this word can be found in documents as early as 808 (Inouye and Katsura 1965). Until the 17th century the majority of the population in Japan took unpolished rice as the staple food. Polished rice was associated with relative affluence, since it looked better on the table when served. Epidemics of beriberi have been known to occur in association with increased affluence simply because it was expensive to take the rice to the mill. When white rice was served to friends, it became a signature of the newly acquired affluence. As the ingestion of

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well-milled white rice became nation wide, so the incidence of the disease increased. This is because the B group vitamins are in the discarded husks. The first national statistics on mortality appeared in 1899 and showed a death rate of 20 per 100,000. This dropped to 0.5 in 1959 after its nutritional association was discovered. Of considerable interest to us today, for reasons that will appear later in the chapter, the peak incidence of the disease occurred in August and September every year. Factory workers would take their lunch between factory buildings. If the sun came round so that it shone into the corridor, some workers would get the first symptoms of the disease. It was evident that the sun's rays would stress them sufficiently to initiate these symptoms. It was therefore hardly surprising that the etiology in the early 1900s, before its nutritional association became common knowledge, was considered to be from infection.

The discovery of the relationship of thiamin with malnutrition came in the late decades of the 19th century, but it was many years before scientific knowledge caught up. A Japanese naval surgeon by the name of Takaki studied in England from 1875 to 1880. He noted that beriberi was less common in the British Royal Navy than in navy personnel in Japan where the diet on ships was very different. In 1882 a Japanese naval vessel sailed on a 272 day voyage. On its return, 61% of the crew had succumbed to beriberi. Two years later, another ship completed the same voyage, but was provided with an ample supply of dried milk and meat, giving a carbon to nitrogen ration of 16:1. Only 14 crew members had developed beriberi. Takaki concluded that a lack of nitrogenous food was the cause of the disease, a notable contribution before vitamins were known.

In 1890, Eijkman found that polished rice, given to pigeons, caused polyneuritis and the histopathology was similar to that seen in humans in beriberi. Funk and Cooper isolated an "antiberiberi factor" from rice polishing in 1910 and this was crystallized in 1926 and called Vitamine (Jansen and Donath 1926). It was not until 1936 that thiamin was synthesized (Williams 1938) leading to an explosion of basic science and clinical experimentation. The work of Sir Rudolph Peters exposed the vitally important association of thiamin with what was later to become the science of oxidative metabolism (Peters 1936).

11.2 Clinical Facts About Beriberi and Thiamin Deficiency

Although a great many symptoms of beriberi have been described, none of them is considered to be pathognomonic (Inouye and Katsura 1965). Generally it is classified into the "wet" or edematous type and "dry", both being chronic in their course. The peracute and extremely lethal form is known in Japan as "Shoshin". Infantile beriberi is associated with sudden death and the clinical picture is startlingly similar to that of Sudden Infant Death Syndrome that is familiar to us today (Lonsdale 2001). Wernicke encephalopathy, usually associated with alcoholism, is at least one form of the disease that affects brain function in adults. A complete review of the clinical aspects of beriberi was published and interested readers are encouraged to

look in this publication or a similar one since the complete information is readily available (Lonsdale 1975). It is not necessary to provide the details again here, but there are some aspects that need to be emphasized because it focuses on some of our modern day clinical problems that are being sadly overlooked.

Many readers might be of the opinion that the classical forms of nutritional deficiency diseases have faded into the background of interesting history. This has caused their diverse symptoms to be neglected by most modern physicians since vitamin enrichment of many foods automatically erases them from their thinking about differential diagnosis. It must be emphasized that beriberi is a disease where the caloric intake is high, particularly in the form of simple carbohydrate foods such as starch. Indeed, it can be superficially compared to a car engine that is being fuelled by a rich mixture of gasoline with insufficient air and/or inefficient spark plugs. It is the lack of vitamin content, particularly thiamin, that results in inefficient oxidative metabolism. Since the brain and heart have a high requirement for oxygen, it is easy to understand why the brain, nervous system and heart are the organs that are affected primarily.

Early investigators found that the arterial oxygen concentration in beriberi patients was relatively low while venous oxygen concentration was relatively high (Inouye and Katsura 1965). This means, of course, that tissue oxygen exchange by cells is defective in the disease. Edema, a common clinical problem today, is one of the important, but not exclusive, signs of beriberi and is always present in the early stages of the disease. A classical sign is tenderness of the calf muscles when gripped with the hand. Anasarca is, however, rare even though there may be profound edema. It should enter into differential diagnosis when cardiovascular and neurological symptoms, especially if there is edema, are present together. Exertion or mental stress results often in cardiac palpitations and disproportionate dyspnea. The typical cardiac enlargement that is easily found by chest roentgenography can easily be mistaken for a viral myocardiopathy and might even lead to consideration of heart transplant if the correct diagnosis is missed. Cardiac beriberi was reported in England in 1971, (McIntyre and Stanley 1971). The authors emphasized the need for it to be considered in the differential diagnosis of heart failure, even when the cardiac output is low. Pang et al. considered that the Shoshin form of beriberi was probably underdiagnosed in the West (Pang et al. 1986).

Perhaps the most important thing to emphasize is the effect of beriberi on the autonomic nervous system (Inouye and Katsura 1965). This is altered early in the disease and produces changes in vasomotor function. The diastolic blood pressure falls and may even reach zero. The systolic pressure may rise or remain normal. In classic beriberi the passage of blood through the femoral artery could be heard, even by an observer standing near the patient. Beriberi victims could be divided into certain categories by their chemistry. Platt, an early investigator, reported that many victims had normoglycemia and were easily treated with thiamin (Platt 1967). More severe cases had hyperglycemia and responded to thiamin with greater difficulty, while others had hypoglycemia and some did not respond at all to the vitamin. Presumably this would reflect the severity and chronicity of the biochemical changes incurred and this should be noted since hyperglycemia

would automatically be considered evidence of diabetes in the modern world. Some patients had achlorhydria and others had hyperchlorhydria. Curiously, under treatment with thiamin, those with achlorhydria became hyperchlorhydric before normal acid content was established. The exact opposite effect was seen in the hyperchlorhydric patients. Although the mechanism is unknown, it has suggested that such phenomena might be related to instability and imbalance of autonomic nervous system signaling. At any rate, it certainly introduces the fact that beriberi symptomatology is extremely complex and not a matter of simply giving a few milligrams of thiamin. In fact, it took huge doses of the vitamin for months to abolish the symptoms. We have found many of these facts to be relevant today, bearing them in mind when confronted with symptoms and signs that can be confusing in a differential diagnosis, as will be discussed later in the clinical section.

In assessing the role of thiamin deficiency in the modern world, it was thought that a description of the basic distribution of the autonomic nervous system was a necessary addition to a book devoted to thiamin metabolism (Lonsdale 1987). It would seem that an example of clinical blindness to the importance of vitamin supplementation and therapy is that an extremely detailed and erudite book entitled "Diseases of the Autonomic Nervous System" was edited and published by Sir Roger Bannister (Bannister 1984). Not one word on malnutrition appeared in that book, in our opinion a very important oversight.

After the discovery of thiamin in its application to treatment of a scourge that had existed for thousands of years and still occurs where rice is a staple food, it was natural that a vast amount of research was initiated. In 1962 a symposium was published to commemorate the synthesis of the vitamin. Zbinden reported 696 published papers reporting attempted thiamin therapy in more than 230 different diseases with varying degrees of success (Zbinden 1962). It is probably pertinent to suggest why such information, important to the health of millions, was more or less abandoned until relatively recently when vitamins have reemerged for their true nutrient value. Because so many different diagnosed conditions sometimes responded to the administration of thiamin as a single entity, it offended the emerging model for disease. Each disease is researched to find a cure that is specific for a given condition, sometimes referred to as the "silver bullet". Thiamin provided symptomatic responses in many different disease entities, indicating that it was addressing the underlying biochemical lesion. Perhaps, with the relatively new explosion in nutritional therapy, it is possible to start thinking that our disease model is catastrophically wrong. Since healing is a function of the body itself, all it requires is energy in order to carry out this role and it seems that a new model for disease is required (Lonsdal 2006).

Vitamins fell into relative disrepute and even today they are often spurned by physicians who regard their pharmaceutical use as absurd. This is because even current teaching often provides the concept that vitamins, as cofactors, only work up to their minute physiological concentration. Pharmaceutical doses are conceived as being discarded and excreted without considering the necessary repair to damage that occurs in the enzyme/cofactor bonding as a result of prolonged deficiency.

11.3 Biochemistry

Thiamin consists of a pyrimidine ring (2,5- dimethyl-6-aminopyrimidine) and a thiazolium ring (4-methyl-5-hydroxy ethyl thiazole) joined by a methylene bridge. The naturally occurring vitamin is found in lean pork and other meats, wheat germ, liver and other organ meats, poultry, eggs, fish, beans and peas, nuts and whole grains. Dairy products, fruit and vegetables are not good sources. The RDA is 0.5 mg per 1,000 kcal. Although this may be adequate for a healthy individual consuming a healthy diet, it may only be a marginal consumption. Considerable losses occur during cooking or other heat-processing of food. Polyphenolic compounds in coffee and tea can inactivate thiamin so that heavy use of these beverages could compromise thiamin nutrition.

11.4 Thiamin Monophosphate

Whether thiamin monophophate (TMP) has a specific function in any cells is still unknown. It is formed from free thiamin to synthesize the monophosphate which is then phosphorylated again by a kinase to form thiamin diphosphate, known as the pyrophosphate (TPP). It may be purely analogous to the stepwise phosphorylation of adenosine, since a third phosphorylation of TPP forms thiamin triphosphate (TTP).

11.5 Thiamin Pyrophosphate (TPP)

Also known as cocarboxylase, this is the form of thiamin that is best known. Its biochemical roles in energy metabolism are multiple. It is a cofactor for enzymatic reactions that cleave alpha-keto acids and it has long been known that it activates decarboxylation of pyruvate in the pyruvate dehydrogenase complex. The complex is a group of enzymes and cofactors that form acetyl CoA. This condenses with oxaloacetate to form citrate, the first component of the citric acid cycle. Since pyruvate is derived from glucose via the Embden-Meyerhof pathway, it should be emphasized that the energy drive from oxidation of glucose is dependent upon TPP. It has the same role in the decarboxylating component of alpha-ketoglutarate dehydrogenase, a link in the sequential metabolism of pyruvate through the citric acid cycle. A third enzyme complex, similar in structure to pyruvate dehydrogenase is that which decarboxylates the three branched chain amino acids, leucine, isoleucine and valine From a clinical standpoint, enzymatic failure and thiamin dependency has been described in both of these enzyme complexes and will be discussed in the clinical section.

A fourth enzyme that requires TPP is transketolase that is present twice in the pentose shunt. The functions of this pathway are to provide pentose phosphate for nucleotide synthesis and to supply reduced NADP for various synthetic pathways. Since the pentose shunt is found in erythrocytes, an easily obtained tissue, measurement of the activity of this enzyme has become used as an important laboratory test

for thiamin deficiency. It is to be emphasized that it gives information only on TPP deficiency. It does not indicate deficiency of TTP. The reaction is highly dependent upon the presence of magnesium, an important point in the study of patients in the clinical setting.

11.6 Thiamin Triphosphate (TTP)

Although this component is still poorly understood, there is no doubt that it plays a significant part in thiamin metabolism. In 1938 Minz first suggested a relationship between thiamin and nervous excitation when he observed that thiamin was released into the bathing medium when the pneumoagastric nerve from an ox was stimulated (Minz 1938). In 1979 Cooper and Pincus reviewed the evidence that there was a possibility that thiamin has a function in the nervous system distinct from its activity as a cofactor to enzymes (Cooper and Pincus 1979). Like Minz, they found that nerve stimulation in experimental animal systems resulted in decline of the level of TPP and TTP from the stimulated nerve. The released metabolites were in the form of TMP and free thiamin, making it difficult to interpret the function of the vitamin in nerve conduction. They found that thiamin appeared to be uniformly distributed in nervous tissue and was highly localized in membrane structures. After intracerebroventricular injection of radioactive thiamin into rats, the distribution of the esters was found to be: thiamin 8–12%, TMP 12–14%, TPP 72–74% and TTP 2–3% (Iwata et al. 1985).

Bettendorff and associates reported their research on TTP, using the electric organ of the eel Electrophorus electricus, rat brain vesicles and neuroblastoma cells (Bettendorff et al. 1987, 1993, 1994). They found that 87% of the total thiamin content of the electric organ in the eel was in the form of TTP, suggesting the great importance of this ester in nerve physiology since this organ is an adaptation of a synapse to create a condenser. They indicated that the real substrate of TTP phosphatase is probably a 1:1 complex of Mg^{2+} and TTP. Incubation of rat brain homogenates with thiamin and TPP led to synthesis of TTP that appeared to be an activator of chloride channels having a large unit conductance. In mammalian tissues TTP concentrations are regulated by a specific thiamin triphosphatase (Makarchikov et al. 2003). It must be stated, however, that the role of TTP is still incompletely understood.

11.7 Thiamin Transporter

The SLC gene family of solute carriers is a family of three transporter proteins with significant structural similarity, transporting substrates with different structure and ionic charge. SLC19A1 mediates the transport of reduced folate and its analogs and SLC19A2 mediates the transport of thiamin. SLC19A3 is also capable of transporting thiamin (Ganapathy et al. 2004; Nabokina and Said 2004).

11.8 Pathophysiology of Thiamin Deficiency

The clinical results seen in proven thiamin deficiency are now well known. Its etiological association with beriberi and Wernicke Korsakoff encephalopathy is accepted by all. Largely because of our remaining ignorance of the role of TMP and TTP, however, the pathophysiology is still incompletely understood. There is no doubt that thiamin, in all its forms, has a vital role in many different aspects of energy metabolism since it catalyzes the normal use of oxygen. This is very well illustrated by the fact, discussed in relation to the symptoms of beriberi, that arterial oxygen concentration is relatively low while that of venous oxygen is relatively high. It is also pretty clear that TPP is possibly the rate limiting factor in entry of pyruvate to the citric acid cycle. Thus, besides being compared with a spark plug in an engine. it can also be compared to a throttle or accelerator.

A conference was sponsored by the New York Academy of Sciences in 1982 to discuss the pathophysiology of thiamin deficiency and existing knowledge of the vitamin at that time (Sable and Gubler 1982). In pyrithiamin-induced thiamin deficiency in animals the net levels of phosphocreatine in the lateral vestibular nucleus and other nuclei of brain were increased selectively, suggesting to the author that this represented under utilization of this source of ATP synthesis (McCandless 1982). Gibson and associates reported evidence of deterioration in the cholinergic system with thiamin deficiency (Gibson et al. 1982) and Meador and associates found evidence for a central cholinergic effect from administration of large doses of thiamin (Meador et al. 1993a). Lonsdale (Lonsdale 1982) found that intraperitoneal injection of thiamin tetrahydrofurfuryl disulfide, a biologically active derivative of thiamin, prolonged the period of sound stimulated seizures in DBA/J2 mice weanlings. Audiogenic seizures in rodents are known to be cholinergic in origin and this type of seizure undergoes spontaneous remission after only a few days of sensitivity in this strain of mouse. The increase in severity of the seizures and the prolongation beyond the normal period of audiogenic seizure initiation supported the role of thiamin in its stimulation of cholinergic CNS activity.

Two publications have indicated that thiamin supplementation has a mild clinical effect in Alzheimer's disease (AD) where the abnormality of the cholinergic system is part of the pathophysiology (Blass et al. 1988; Meador et al. 1993b). Mastrogiacoma and associates studied thiamin, its phosphate esters and its metabolizing enzymes in autopsied specimens of brain in AD and controls (Mastrogiacoma et al. 1996). In the AD group the mean levels of free thiamin and TMP were normal. Concentrations of TPP were reduced by 18–21% although the TPP metabolizing enzymes were normal. The authors hypothesized ATP deficiency since that is required for increased phosphorylation in thiamin esters. Thiamin deficiency in rats caused encephalopathy and DNA synthesis decreased significantly in cortex, brain stem, cerebellum and subcortical structures. This was reversible by the administration of thiamin (Henderson and Schenker 1975).

Creatinuria has been noted in beriberi as well as in experimentally induced thiamin deficiency. An observation in rats showed that creatinuria occurred as a result of protein calorie deficiency caused by experimental starvation. Where

anorexia was caused by thiamin deprivation the creatinuria occurred much sooner and was statistically greater in concentration (Lonsdale 1987). This was mindful of the greater degree of creatinuria observed in calorically starved T3 injected rats than in calorically sufficient animals injected with T3, suggesting that membrane physiology was involved in these two differently stimulated mechanisms. As will be indicated in the clinical section of this chapter, TTP remains as the outstanding mystery of thiamin metabolism, particularly in brain.

11.9 Laboratory Methodology

As in the case of other vitamins, blood and urine measurements of thiamin are unreliable. By far the best test for thiamin deficiency is measurement of transketolase (Boni et al. 1980; Jeyasingham et al. 1987). As already indicated, this enzyme occurs twice in the pentose shunt, a biochemical pathway that exists in erythrocytes. In the first part of the test, the product of the enzymatic reaction, sedoheptulose-7-phosphate is measured per unit of blood per minute by spectrophotometry A baseline activity is then calculated. The reaction is then repeated after the addition of TPP. The test is then reported in two sections, the first being the baseline activity. This is referred to as transketolase activity (TKA). The normal range of this in our laboratory is 42–82 mU/L/min, originally derived from apparently healthy asymptomatic subjects. In the second part of the test, if it is found that TKA increases as a result of the addition of cofactor, it is reported as a percentage increase over that detected at baseline. This is referred to as the thiamin pyrophosphate effect (TPPE). In our laboratory, this is considered to be acceptable up to 18% acceleration. In theory, there should be no acceleration and the TPPE should be zero if the enzyme is saturated with its cofactor. It is possible that the "normal subjects" had not reached a stage of deficiency that caused symptoms. It should be regarded as a continuum, progressing from ideal thiamin cofactor status, through marginal deficiency to increasingly severe deficiency generating symptoms in the affected subject. This test should be readily available in clinical laboratories, but is often neglected because vitamin deficiencies are generally considered to be rare or even non-existent. It has a number of important relationships. Sometimes the TKA is low while the TPPE is normal and this would suggest that there is some abnormality in the enzyme (Blass and Gibson 1977). In most cases of thiamin deficiency in our experience the TPPE is increased, sometimes remarkably so. In a more severe deficiency state the TKA is low and the TPPE is high. Correlating the patient's symptoms with a fall in the TPPE is an excellent way of proving the clinical effect of thiamin supplementation. This can be complicated since the enzyme complex is also dependent on magnesium and other non caloric nutrients (Eisinger et al. 1994). Magnesium depletion aggravates the clinical effects of thiamin deficiency (Dyckner et al. 1985). Magnesium and calcium deficiency affects the distribution of thiamin in rat brain (Kimura and Itokawa 1977). We have recently found several patients whose TPPE could not be corrected until they had received an adequate supplementation with magnesium (Lonsdale

2006c). There is still much to learn about the inter-relationships between vitamins and minerals in the overall management of oxidative metabolism. An increase in TKA was found in B12 deficient patients but not in those where the anemia was due to folate deficiency (Markannen and Kalliomaki 1966; Wells et al. 1968).

In 1969 Cooper and associates (Cooper et al. 1969) published their finding of TTP deficiency in Leigh's disease, also known as subacute necrotizing encephalomyelopathy. The pathophysiology of this disease is similar, but not identical, to that of Wernicke's disease. Diagnosis depended upon their finding of a substance in urine that reportedly inhibited the formation of TTP (Cooper et al. 1970). The substance was never identified and this important research was eventually discontinued for lack of funding. Urine from several patients suspected of thiamin deficiency symptoms were sent to Cooper's laboratory, were reported positive for this test and responded to thiamin supplementation. None of these patients could be considered to be examples of Leigh's disease (Lonsdale Unpublished observations). It was the only laboratory study ever reported to indicate TTP deficiency and there is presently no known clinically available method of depicting this vitally important link in our knowledge of thiamin metabolism in brain.

11.10 Clinical Application of Thiamin in Modern Nutrition

It is important to emphasize once again that vitamin supplementation fell into disrepute after the studies reported in the middle decades of the last century. Very little clinical research has been done since then and major medical journals have consistently rejected what clinical research has been reported. This book indicates that may be changing, so it is apt to refer back to some of the excellent studies that were published 50 or more years ago. One of the most important ones in the study of pure thiamin deficiency (as distinct from the nutritional aspects of beriberi) was performed in 1943 (Williams et al. 1943) and I shall refer to this in some detail since it has great relevance to modern nutritional deficiencies. Severe thiamin deprivation resulted in depressed mental states, generalized weakness, giddiness, backache, soreness of muscles, insomnia, anorexia, nausea, vomiting, weight loss, poor muscular tone, low blood pressure and bradycardia with the subjects at rest. On exertion, heart palpitation and precordial distress (pseudoangina) occurred. Tachycardia and sinus arrhythmia were observed. The investigators reported electrocardiographic changes and impairment of gastro intestinal motility.

Moderate, prolonged restriction of thiamin, but not of calories, resulted in emotional instability, reflected by irritability, moodiness, quarrelsome behavior, lack of cooperation, vague fears and agitation, mental depression, variable restriction of activity and numerous somatic complaints. The effects on one subject, a 48 year old woman, were described in detail after 120 days of this deprivation. Blood pressure was between 90 and 98 and the diastolic between 50 and 60 mm Hg. Heart rate 50–60 bpm and there was marked sinus arrhythmia. Pallor and giddiness were

observed when standing from the sitting position and rising from the squatting position could be accomplished only with assistance. The patellar tendon reflexes were hypoactive but could be increased through reinforcement and the Achilles tendon reflex was absent. The comments of the authors included the statement that symptoms suggestive of dysfunction of the central and peripheral nervous pathways preceded by months the gross signs of neurologic dysfunction.

This is an old study and it would not be possible to perform a dangerous experiment of this nature now. But we should look seriously at the symptomatology since a discerning practitioner will find clinical effects of this nature today. A simple diet history often will produce clues that make the diagnosis surprisingly clear. Because of the widespread nature of marginal malnutrition in the U.S. where there is a heavy emphasis on sweet foods containing the simple carbohydrates, particularly sucrose and fructose, findings of this nature are often relevant. Limited data are available on the relation between thiamin requirements and the intake of simple carbohydrate in human physiology, but a study was reported that investigated the influence of stepwise increases of carbohydrate intake on the status of thiamin in healthy volunteers under isocaloric conditions (Elmadfa et al. 2001). An increase of dietary carbohydrate intake caused a decrease of plasma and urine levels of thiamin without affecting enzyme activities. A publication in 1962 (Bhuvaneswaran and Sreenivaran 1962) discussed problems of thiamin deficiency states and their treatment. Quoted in this paper is a reference to Yudkin who "showed that, despite the absence of thiamin, rats can survive for many months if carbohydrate is excluded from the diet. Polyneuritis and death followed the addition to the diet of as little as 5% carbohydrate".

The major point to make is that a patient with complaints of the nature described by Williams and associates is so often regarded today as having psychosomatic disease and is either referred to a psychiatrist or treated empirically with a drug. Some simple clinical observations and laboratory studies remove the stigma that is usually associated with this diagnosis, at least by the patient. As already mentioned above, diet history is frequently revealing to the point of wonder at the excesses involving sugar, soft drinks and alcohol, to name a few of the myriad temptations surrounding the public.

There are several publications that support the contention that high calorie malnutrition of this nature is responsible for a huge amount of so-called psychosomatic disease (Lonsdale 1975, 1990; Lonsdale and Shamberger 1980). It is interesting to note that I received a call from the editor of the Journal for the 1990 paper. She was nervous about its publication and suggested that the word "possible" should be introduced into the title. A subsequent letter to the editor castigated the Journal for publishing it at all. This is just a reflection of the incredible resistance that has accompanied evidence of vitamin deficiency disease for years in contemporary society. Lonsdale and Shamberger (1980) described 20 adolescents with proven evidence of metabolic disturbance associated with the symptoms that are so common in this age group today (Lonsdale and Shamberger 1980). Transketolase abnormalities occurred in all of them and were corrected by the use of supplementary thiamin. Many of them had abnormalities in serum folate and vitamin B12 that were just as

important as the transketolase in interpreting the biochemical effects of malnutrition in these individuals. The editor would only publish this article if it was reviewed by two acknowledged experts in the field who criticized it heavily in two editorials that appeared in the issue.

A revealing note should be added to the question of the concentration of thiamin relative to the intake of glucose. A woman was receiving intravenous hyperalimentation in a hospital setting. The injected fluid contained 24 mg of thiamin a day, together with a total intake of 5% dextrose water, yielding a calculated 1,036 calories from this source alone. She died and the autopsy revealed Wernicke encephalopathy (Lonsdale 1978). This letter to the editor included the statement that there were no less than six cases of Wernicke's encephalopathy found in the same hospital some 2 years previously. All of them had been receiving supplementary vitamin.

Autonomic dysfunction, as already discussed, is an important expression of symptomatology in beriberi and has been associated with modern high calorie malnutrition (Lonsdale 1981). This was noted to be asymmetric in some cases (Lonsdale 1990). One of them was a girl whose main complaint came from her mother. She had violent temper tantrums, but each one was accompanied by a set of unusual symptoms and signs. She would complain of a migraine-like headache. One pupil would dilate, the other one did not. She would become white on one half of her body and pink on the other, mindful of a harlequin. Sweat could be obtained by electrophoresis on one side, but not the other. Sleep spindles were observed on one side of the brain by electroencephalogram, but not on the other. When stressed by an intravenous injection of epinephrine the pulse pressures were widely different in the two arms when it was measured at the same time by two people (Lonsdale 1987). By abolishing her appalling diet and providing her with vitamin supplementation, that included thiamin, she was retested a year later and the asymmetric functional changes had disappeared.

Any pediatrician knows only too well that there are huge numbers of children seen in the U.S. today with symptoms that are considered to be psychological in nature. Relatively few of them are seen as examples of limbic system biochemical abnormalities that have ravaged them from their awful diet (Lonsdale 2001). The limbic system is really the primitive brain and is responsible for our emotional reactions. It can certainly be regarded as a computer that presides over our ability to adapt to the environment. Inefficient metabolism in this part of the brain would be expected to cause untoward emotional reactions and autonomic/endocrine signals that might justifiably be defined as "maladaptive". Though efforts are often made by parents now to provide a nutritious diet, the devastation usually comes from the ad lib ingestion of "junk" (empty calories) that the children get between meals. Thiamin deficiency is relative to the increased calorie content from simple carbohydrates and the reason for referring to this disastrous menace as "high calorie malnutrition". The peer pressure is enormous and the temptations provided by a greedy food industry are all too obvious in the shape of advertising and promotion. Many citizens in the U.S. are deeply concerned with some of the mysteries of behavior that beset the generation of children and adolescents. Vandalism is irrational and is extraordinarily common. Destructive juvenile behavior is written off

without considering the underlying mechanisms of anger. Much has been published in regard to the connection between ingestion of "junk" food and juvenile crime (Schauss 1981; Lonsdale 1992a, 1994) It is an unacceptable societal factor at present because it offends the concept that crime is always a cold blooded voluntary act.

A case report (Lonsdale 1992b) dealt in detail with a crime in a young African American who had a crime-free background. The Public Defender felt that the incident was brought about by biochemical changes in the brain of this young man. His diet was appalling, involving a huge amount of carbohydrate and alcohol. Biochemical studies revealed profound changes that included an increase in urinary catecholamines. In thiamin deficient rats, catecholamine contents in the cerebral cortex, in the atria and ventricles of the heart and in the spleen were significantly increased compared with those of control animals. There was a significant reduction of noradrenaline metabolism in these tissues. Monoamine oxidase activities were depressed in tissues in which the catecholamine content had been found raised during thiamin deficiency. A close relationship exists between possible changes in enzyme mechanisms for destroying catecholamines and thiamin deficiency (Iwata et al. 1969a, b).

The possibility of thiamin deficiency secondary to diuretic therapy has received only scant attention. A study showing furosemide induced thiamin deficiency in rats (Yui et al. 1978) prompted a clinical study (Seligman et al. 1991) to ascertain whether this commonly used diuretic would induce thiamin deficiency in 23 furosemide treated patients with congestive heart failure. The authors reported a high TPPE indicating deficient thiamin status in 21 of the patients as compared with only two control patients. Thiamin excretion in urine was higher than in controls. Clinical improvement and normalization of TPPE occurred with thiamin supplementation. The metabolic and hemodynamic features of the failing heart can be further aggravated by thiamin deficiency and the authors noted that it was thus possible that in patients with advanced congestive heart failure, particularly those receiving long-term furosemide therapy, thiamin deficiency is one determinant of their poor clinical status

11.11 Thiamin Dependency

When a cofactor such as thiamin is required in huge doses in order to produce enzymatic function it is termed dependency. It is clinically deceptive because, even if the symptoms are regarded as related to vitamin cofactor action, a physiologic dose of the "missing" vitamin might be prescribed on the basic understanding that it would be curative. When no clinical response is observed, the therapist might conclude that the concept was wrong and the vitamin discontinued.

A 6 year old child with intermittent episodes of cerebellar ataxia proved to be the first case of thiamin dependent defective pyruvic dehydrogenase activity (Lonsdale et al. 1969; Blass 1972). Although thiamin responsiveness could not be demonstrated in vitro, the child's episodes were prevented by the ingestion of 600 mg of thiamin a day. If he should succumb to a simple infection the daily dose of thiamin

would have to be doubled. At least one intermittent metabolic disorder has been reported where a "stress factor" such as infection could initiate the disease (Dancis et al. 1967) and thiamin plays a role in the decarboxylation of branched chain amino acids (Elsas and Danner 1982).

Some important facts need to be mentioned in regard to this thiamin dependent child who actually imitated a classic example of childhood beriberi. Episodes of ataxia were almost always initiated by a simple infection. Other "stress factors" included head injury and inoculation. Even an acute ambient temperature change could trigger symptoms. On one occasion he entered an air conditioned store from a 90° ambient temperature and succumbed to asthma, something that he had never experienced before. On yet another occasion the air conditioning in the car was turned on and he succumbed again. The inevitable conclusion was that some form of "stress" would initiate a response where metabolic efficiency was lacking. This is a concept that might apply to many different conditions where metabolic efficiency is marginal, as for example, with excess empty calories in the diet.

One of these ataxic episodes was studied in detail. Without any treatment, it resolved spontaneously over about a 10 day period, reaching a clinical climax at about the 6th day. Daytime urinary concentrations of alanine and pyruvate were much higher by day than night throughout the episode and were inversely proportional to urinary concentrations of aspartate and glutamate. This led to two conclusions: first that the mechanism was related to circadian rhythm and secondly that this represented an abnormal redox where pyruvate was being reversely transaminated to the corresponding keto acids, siphoning off glutamate and aspartate from brain function. With thiamin treatment his optic atrophy and learning disability gradually improved. Although his younger brother had the same defect, he did not experience ataxia. As an adolescent, however, he had a relatively mild head injury that rendered him unconscious. He was taken to an emergency room where it was virtually impossible to get the physician to understand the nature of the underlying risk and his requirement for an injection of thiamin.

11.12 Thiamin Transporter Disease

The SLC19 gene family of solute carriers have been described (Ganapathy et al. 2004; Nabokina and Said 2004), opening up a new aspect of thiamin metabolism. It had long been noted that megaloblastic anemia could occur that was treatable by the use of supplementary thiamin (Rogers et al. 1969). The discovery of the transporters made the etiology much clearer. Thiamin responsive megaloblastic anemia (TRMA) syndrome is now known to be an autosomal recessive disorder with features that include this form of anemia, mild thrombocytopenia, leucopenia, sensorineural deafness and diabetes mellitus (Ozdemir et al. 2002). Mutations in the SLC19A2 gene encoding a high-affinity thiamin transporter protein THTR-1 are responsible for the clinical features of this syndrome (Lagarde et al. 2004).

Three TRMA patients have been reported with heart rhythm abnormalities and structural cardiac anomalies (Lorber et al. 2003). This suggested to the authors that knowledge of the TRMA syndrome might shed light on more commonly observed cardiac disease where an etiology is unclear. These authors also agreed that the harmless nature of thiamin administration might even suggest that the vitamin could be used empirically for routine supplementation in cardiac failure where etiology is unknown.

A mouse model has been generated lacking functional SLC19A2. The mouse was unexpectedly found to have a male-specific sterility phenotype. Spermatogenic failure was reversed by injections of large doses of thiamin, giving a new insight into the possible association of thiamin responsive human infertility (Oishi et al. 2004).

11.13 Thiaminase

In 1941 Fujita (Fujita 1941) was engaged in determining the vitamin content of Japanese foodstuffs. The thiamin content of some shellfish and crustacea was zero. When thiamin was added, it could not be recovered because it was being destroyed by an enzyme that occurred in the tissues. He named the enzyme aneurinase. Subsequently he was able to identify two enzymes that destroyed thiamin and might be relevant to human disease. Thiaminase I (EC2.5.1.2) is produced by Clostridium thiaminolyticum, an anerobic organism found in human small intestine and by Bacillus thiaminolyticus, an aerobic organism that occurs in the colon. This enzyme splits the pyrimidine ring of thiamin from the thiazolium ring at the methylene bridge and adds a base compound to the pyridinium to create an analogue inhibitor of thiamin in its metabolic role. Bacillus aneurinolyticus is aerobic and is found in human colon. It produces thiaminase II (EC 3.5.99.2) that functions in the same way as thiaminase I but does not add a base compound to the pyrimidine ring.

A chapter on thiaminase (Murata 1965) can be found in a book that was written by the Vitamin B Research Committee of Japan. After the discovery of the two enzymes it was to be expected that there would be studies performed to ascertain whether they were relevant in human disease. A case of “thiaminase disease” was reported. The patient had beriberi and potent thiaminase activity was found in the feces. In another patient with “thiaminase disease” a bacterium was found subsequently to be responsible. Mentioned by Murata in this chapter, an anaerobic organism was found in human feces and named Clostridium thiaminolyticum. It was also reported that a strain of Candida isolated from the oral cavity of a human subject produced thiaminase II.

Thiaminase I was found in the ruminal contents of animals affected by cerebrocortical necrosis (CCN) (Edwin and Jackman 1970). When this enzyme is purified it does not have thiamin splitting activity without the presence of a cofactor. When these investigators dialysed CCN rumen liquor against phosphate buffer, all thiaminase activity was lost. It could be restored by the addition of various cofactors, the most potent being nicotinic acid. The subsequent metabolite,

pyrimidinyl-nicotinic acid, was isolated. They concluded that this might have antithiamin properties since it has elements of similarity to pyrithiamine and amprolium, both known to be thiamin antagonists (McCandless 1982; Brin 1964)). The disease could be produced experimentally in calves by administration of amprolium (Markson et al. 1974). All 60 strains of Cl sporogenes subsequently examined (Shreeve and Edwin 1974) produced thiaminase. This organism has been recognized under various names since it was first described by Metchnikoff in 1908 and is commonly found in soil and in intestinal contents of man and animals. Shreve and Edwin noted in their paper that Clostridium thiaminolyticum, isolated from human feces was later considered to be a member of the Cl sporogenes species.

An epidemic of seasonal ataxia was reported in Western Nigeria (Adamolekum and Ndububa 1994). This disease was later found to be due to activity of thiaminase I extracted from the pupae of an African silkworm that is consumed as a source of protein (Nishimune et al. 2000). The significance of these enzymes in human disease is unclear and little or no research has been performed on the subject since this early work was reported. A letter to the editor (Loew 1974) suggested that CCN might be a model for the experimental study of human disease, in particular Wernicke's encephalopathy and Leigh's subacute necrotizing encephalomyelopathy, both of which are etiologically associated with aspects of thiamin metabolism.

11.14 Thiamin Derivatives

A notable advance in research of thiamin metabolism in Japan was the discovery of allithiamin (Fujiwara 1965). Allicin, the compound that gives garlic its characteristic odor, is produced from alliin by the action of alliinase during the grinding of fresh garlic bulbs and conjugates with thiamin in an alkaline medium to form allithiamin (2′-methyl-4′amino-pyrimidyl-(5′)methylformamino-5-hydroxy-2-pentenyl-(S) allyl disulfide). It had been found that the aqueous solution thus formed lost the characteristic thiochrome reaction given by thiamin but exerted full thiamin activity when administered to animals. The addition of cysteine restored the thiachrome reaction, thus demonstrating that the molecule was reduced to thiamin. These disulfide derivatives were tested extensively for their chemical and biological properties by the Vitamin B Research Committee of Japan. Their then unknown characteristics were found to be quite different from thiamin. Propyl-allithiamin (TPD) was said to have the most satisfactory actions. This compound, however, produced a profound garlic odor from both animals and human subjects. This later led to synthesis of the tetrahydrofurfuryl disulfide (TTFD) (Fig. 11.1) which did not produce the garlic odor. It is now marketed in Japan as a prescription item named Alinamin F (odorless). The biololgical properties of TPD and TTFD are otherwise the same.

When allithiamin is administered by mouth, urinary excretion occurs exclusively in the form of free thiamin, not in the form of allithiamin. This is because it is easily reduced to thiamin by cysteine or glutathione.

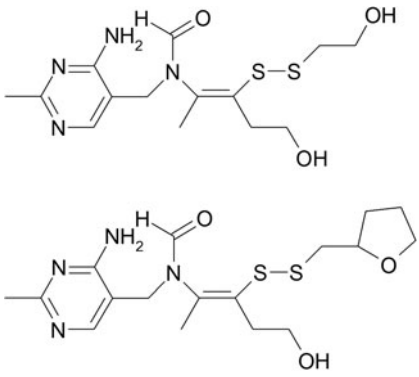

Fig. 11.1 Synthetic allithiamin homologs

Allithiamin + L-cysteine ◊ thiamin + S-allymercapto-L- cysteine

The concentration of excreted urinary thiamin is much greater than occurs with an equivalent dose of a water soluble thiamin salt such as thiamin hydrochloride. This is because its absorption from the intestine is far superior to that of the thiamin salt. After parenteral injection of TPD into healthy male subjects, blood samples showed much greater concentration of thiamin than an equivalent dose of a thiamin salt By the use of 35S-labled TPD, it was shown that the mercaptan fragment remained in the plasma whereas the thiamin segment entered the blood cells. Thus, the open thiazolium ring in allithiamin derivatives closes to form an intact molecule of intracellular thiamin where its vitamin activity is required. When mice were pretreated with TPD in a dose of 1 mg. they found that they were partially protected from potassium cyanide poisoning, given in a dose of 150 μg per 10 g body weight. The death rate in pretreated mice occurred in only 11.7% as compared with 70.6% in those that were not pretreated. It was also found that TPD was effective in prevention of trichloroethylene or lead intoxication in rabbits. It also resulted in striking reduction in liver damage after administration of carbon tetrachloride.

An important experiment was reported in dogs. A segment of jejunum was disconnected from its mesenteric innervation. When TPD was applied topically or given intravenously a marked stimulatory effect on peristalsis was observed. This effect did not occur at all with a thiamin salt. Body weight curves of albino rats were far superior when given TPD than with thiamin hydrochloride. A poorly known therapeutic use of TTFD may well be in its anti-inflammatory effect. Intraperitoneally injected TTFD and TPD showed a strong inhibitory effect in carrageenin induced rat paw edema (Kitzushima 1967). This effect was supported by finding that TTFD reversed the gradual increase in coronary blood flow in the heart-lung preparation of a dog by inhibiting the arachidonic acid cascade activation (Matsui et al. 1985).

All the allithiamin homologues are disulfide derivatives and their easy reduction and consequent absorption of intracellular thiamin is dependent on this. A large number of S-acyl derivatives were synthesized and the Vitamin B Research Committee selected three of them for detailed study (Fig. 11.2).

Figures 11.1 and 11.2 reproduced with permission from Lonsdale D. A review of the biochemistry and clinical benefits of thiamin(e) and its derivatives. eCAM 2006;3(1):49–59.

All of the disulfide (S-S) and S-acyl (S-ac) derivatives were shown to be absorbed from the intestine, at a rate comparable to TPD, far more readily than a thiamin salt. It was found, however, that the S-ac forms were not absorbed into blood cells any better than thiamin hydrochloride. Neither did they have the preventive effect against the various toxins that TPD pretreated animals experienced. They ascribed this major difference to the absence of the S-S bond in S-ac thiamins. These, unlike the disulfide compounds, require enzymatic action in liver or kidney to be reduced to thiamine. An important aspect of the metabolism of these derivatives is what happens to the fragment left outside the cell on hydrolysis. The mercaptan from TTFD has been well studied (Kikuchi et al. 1970; Fujita et al. 1973a, b). Its pharmaceutical effect, if any, is unknown. It was shown to be non toxic and it breaks down through a series of enzymatic actions to sulfates and sulfones when it is excreted in urine. The fragment from the S-ac derivatives has not been studied to my knowledge.

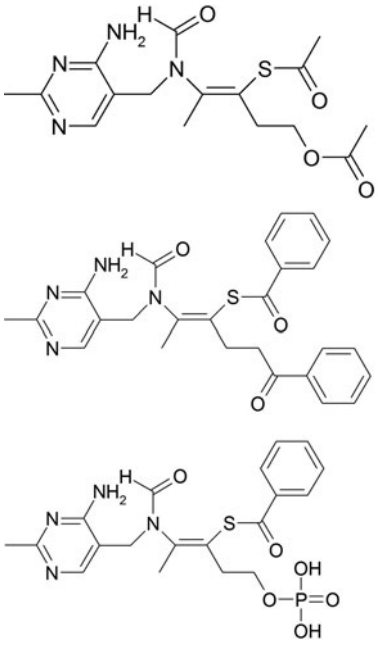

Fig. 11.2 S-acyl-thiamin derivatives

11.15 Clinical Studies

Over the years we have found some unusual aspects of thiamin metabolism in disease. To illustrate the fallacy of relying on a repetitive symptomatology in alerting a physician to the possibility of thiamin deficiency/dependency, studies on two children with recurrent febrile lymphadenopathy were published (Lonsdale 1980). One child had a normal transketolase but was found to have the TTP inhibitor substance in urine (Cooper et al. 1969). His recurrent episodes of high fever and massively enlarged cervical glands had been treated for 2 years with antibiotics on the assumption that they were due to recurrent infection. They ceased with thiamin supplementation and without antibiotic use. To complicate the involved biochemistry, serum folate and B12 were both markedly increased, but fell into the normal range after thiamin was started. The folate and B12 increased and symptoms returned when thiamin was temporarily discontinued. Restoration of thiamin again abolished symptoms: serum folate and B12 concentrations returned again to normal. Approximately a year later his symptoms returned but vanished again when a multivitamin was added as a supplement. The other child had an uncomplicated abnormal transketolase and responded to thiamin supplementation.

Some form of thiamin dependency was depicted in an infant that had the potential clinical hallmarks of a chromosomal abnormality, studied at the age of 6 weeks. Chromosomal testing was normal but the infant had an abnormal transketolase. For this reason, thiamin supplementation was commenced and she began to flourish. Her mother, who has subsequently been found to have symptoms associated with abnormal transketolase, found that her daughter needed more and more thiamin to treat recurrent symptoms as she grew. The dose, given without medical advice by the mother, gradually rose to as high as 7 g a day. Though she was never completely normal in behavior and learning capacity, she was able to graduate from high school where she played an instrument in the marching band. She died at the age of 27 years from what was reported as toxic shock syndrome after an infection.

Another remarkable experience was with a child with recurrent life-threatening croup. His three siblings had had a similar history for the first 4 years of their lives. An abnormal transketolase suggested thiamin treatment and the recurrent croup ceased. One year later, the mother asked whether thiamin supplementation should be continued. It was discontinued on the assumption that he had “outgrown” the problem. Within 3 weeks he required admission to hospital with another attack of croup and thiamin was restored. It could only be assumed that the pathology might have involved the recurrent laryngeal nerve (Lonsdale Unpublished observations)

A family was reported where sleep apnea occurred in three of six siblings between the ages of 18 and 26 months. Twin females first had irregular respiration and episodes of apnea. One twin succumbed to an apneic episode while asleep and the diagnosis of sleep apnea was made in the other twin at a sleep clinic. She also died in an apneic episode 3 months later. A male sibling also died in a similar fashion. The investigators also reported a child with sleep apnea at the age of 7 weeks who died at 31 months. Lesions were confined to the respiratory centers of the lower brain stem. The connection with thiamin was underlined by the discovery of the TTP

inhibitor substance (Cooper et al. 1969) in one of these children and other family members (Adickes et al. 1986). Although this cannot be stated as proven, it raises the very important question of the association of thiamin metabolism in Sudden Infant Death Syndrome (SIDS). As long ago as 1944, it was pointed out that sudden death, with a peak incidence at about 4 months of age, the same time frame as is usual in modern SIDS, occurred as a result of thiamin deficiency in breast fed infants of Chinese mothers in Hong Kong (Fehily 1944). Although the etiology in modern SIDS is still argued, it should be possible to see it in terms of the combination of genetics (or epigenetics), poor nutrition and an incidental stress factor (Lonsdale 2001). Certainly thiamin metabolism has been very much underlined but largely ignored as a simple and effective therapeutic intervention in this tragic and preventable event (Jeffrey et al. 1985; Lonsdale and Mercer 1972; Lonsdale 1977, 1990; Lonsdale et al. 1979, 1982). The association of TTP with SIDS was supported by the finding of its deficiency in the phrenic nerve of autopsied SIDS victims (Barker and Jordan 1982). Magnesium deficiency has been reported (Caddell 1972) in SIDS and the strong association with thiamin metabolism already mentioned would make this a logical relationship that should be emphasized strongly.

Four case reports of encephalopathy were published where thiamine metabolism appeared to be involved in the etiology (Lonsdale and Price 1973). All of them had elevation of lactic and pyruvic acids in blood or urine and three of them had TPP inhibitor substance in urine as described by Cooper and Pincus. None of the three had Leigh's disease. One of the children described in this publication was of more than passing interest since it implies that an adequate knowledge of glucose metabolism is often required in perceiving this kind of biochemical lesion. This girl had severe psychomotor retardation and failure to thrive. This followed from a low Apgar score at birth, failure of the suck reflex, cyanosis and vomiting in the neonatal and infancy era. At the age of 9 months she began self mutilation of the lower lip that produced severe damage requiring removal of the upper incisors and transplant of the parotid duct because of excessive drooling. Although she had increased urinary excretion of uric acid and at least one episode of uric acidemia, the genetic defect in Lesch Nyhan syndrome, where compulsive lip biting is characteristic (Lesch and Nyhan 1964), is sex-linked recessive and the defective enzyme, hypoxanthine-guanine phosphoribosyltransferase responsible for this disease was found to be normal in the child's erythrocytes. The fact that she had an increase in pyruvic acid in blood and urine suggested obstruction of pyruvate into the citric acid cycle, perhaps with an overflow of glucose through the pentose pathway, resulting in increased production of purines and uric acid. She was treated empirically with thiamine for this reason. Five months later, the urinary pyruvic acid concentration had decreased significantly and there was definite clinical improvement evidenced by increased activity, interest in her surroundings and alertness. Granted that this did not constitute a solid proof, but it must be regarded as a challenge to others to explore the biochemical etiology of such a disaster as our knowledge increases. Since the body is a biochemical "machine", it is the "biochemical lesion" rather than the symptomatology that must be plumbed for real progress in the treatment of disease.

Branched chain ketoaciduria (Maple syrup urine disease) has been reported due to thiamine dependency, as already mentioned above. The biochemical abnormalities were reversed by administration of pharmacological doses of thiamine (Scriver et al. 1971; Duran et al. 1978). This disease has been reported to occur intermittently (Dancis et al. 1967). Clinical episodes occurred in association with infections in the same way as the intermittent cerebellar ataxia occurred in the case reported by Lonsdale and associates (Lonsdale et al. 1969). The infection is deemed to be an attack that results in mobilization of the metabolic response and is regarded as a form of "stress". This results in an exposure of the underlying defect that is adequate when the subject is not under attack. The concept of "stress" would apply to other forms of attack and is considered to include head injury and even inoculation as described in the episodes of cerebellar ataxia reported by Lonsdale et al. This concept was introduced earlier in the chapter when the first symptoms of beriberi occurred in Chinese workers when they were suddenly exposed to sunlight. As already mentioned, it also strongly suggests that the disaster of SIDS is brought about by the combination of a "stress" event (e.g. a cold), immature brainstem control of vital function and a deficiency of an equally important nutrient such as thiamine or magnesium (Lonsdale 2001). It has been hypothesized that this concept might be studied further by the use of Boolean algebra that involves the interlocking of circles and published as the Three Circles of Health (Lonsdale 1994). The three circles are entitled Stress, Genetics and Nutrition.

11.16 Clinical Use of Thiamine Tetrahydrofurfuryl Disulfide (TTFD)

I have had an Independent Investigator License for the clinical use of TTFD since 1973. Consequently, I have been able to treat hundreds of patients with it and have reported these activities in a number of reviews and case reports, summarized recently (Lonsdale 2006). Its therapeutic benefits are not well known, at least in the U.S.A. where it is not approved by the FDA. Details of an unusual case of cardiac beriberi were published in a book that is now out of print but available on line (Lonsdale 1987, 2006). The boy in question had not been normal since birth and was mildly mentally retarded. The reason for recurrent episodes of gastro enteritis was never solved, but had always been treated as infections. After one of these episodes the parents of the then 13-year old boy took him up into mountainous terrain for a picnic. While sitting in the sun he developed hemiparesis that recovered spontaneously as he was being driven to the nearest hospital. It is to be noted that this onset of symptoms was similar to the onset of first symptoms of beriberi in Chinese factory workers when exposed to sunshine, as discussed previously. The major discovery on arrival at the hospital was a grossly enlarged heart and he was transferred to our institution where his condition was recognized as beriberi heart disease. He responded dramatically to TTFD for a while but gradually became resistant to its effects and died. Unfortunately, the parents were adamant in denying

autopsy studies and the basic lesion was never defined, for it was clear that this was not due to simple dietary thiamin deficiency. In the light of more modern knowledge it would be possible to postulate an abnormality in thiamin metabolism such as transporter disease.

Some years ago, there were almost annual epidemics of Reye's syndrome, eventually recognized as severe repercussions from the use of aspirin given to children with one of the many different viral diseases. One child that came in to our institution was an 18-month old girl easily recognized as an example of this syndrome. The treatment given at that time failed and she was comatose and surviving with the application of life sustaining machinery. It was agreed by all that she had little hope of survival. Very large doses of TTFD were given both by mouth and intravenously. The first sign of recovery was reappearance of lip vermilion and pink cheeks. As treatment continued she passed through the stage of the peculiar state of partial consciousness known as coma vigilum. In this state she accepted food and followed the faces of attending personnel with her eyes. Consciousness gradually returned and she was able to walk out of the hospital. There was unfortunately not enough factual evidence for publication of this case (Lonsdale Unpublished observations). Since Reye's syndrome produced its lethal effects by attacking brainstem controls, it can be easily suggested that the recovery in this child was due to restoration of oxidative metabolism in this crucial part of the brain on which depends the survival of each one of us.

It was consideration of the role of thiamin in the brainstem and limbic system that led to its experimental use in the treatment of threatened SIDS (Lonsdale 1977). My colleagues and I were able to show that brainstem auditory evoked potentials (BAEP) were abnormal in many children who presented with symptoms of threatened SIDS (Lonsdale et al. 1979). The concept of "threatened" in this syndrome was, for a long time, unaccepted, since the symptomatology is vague and non specific and the syndrome was regarded as being impossible to forecast. Nevertheless, we encountered a number of infants whose history of life threatening apnea described by the parents had been sometimes regarded in emergency rooms as parental over-reaction when the infant was found to be normal on examination. We described the cases of four infants whose history of near-death episodes ceased and clinical recovery occurred after the administration of TTFD (Lonsdale et al. 1982). In one patient the BAEP was found to be extremely abnormal but partially corrected after administration of intravenous TTFD and went on to a more complete recovery with orally administered TTFD. Although her subsequent life was associated with mild mental problems, she is now a married woman. Of particular interest in this discussion, her mother had consumed a great deal of cola drinks during her pregnancy.

The chromosomal etiology in Down's syndrome is well known as trisomy 21. The biochemical effects are less well known but are brought about by the overexpression of genes occurring on chromosome 21. A clinical study in a group of randomly chosen children with Down's syndrome showed modest improvement in the measured IQ of some of them (Lonsdale and Kissling 1987). A current epidemic of infantile autism is being encountered and our own experience has shown that

about 10–20% of these children have an abnormal TPPE, thought to be a significant marker for oxidative stress (Gibson and Zhang 2002). It prompted a pilot study in the treatment of 10 of these children with TTFD (Lonsdale et al. 2002). Because the enteric coated tablets made by Takeda Chemical Industries were not available, the powdered form of TTFD was found to be completely unpalatable and rejected by anyone who tried tasting it, no matter what kind of taste disguise was used. It was therefore administered in the form of rectal suppositories. Using computer-read special symptom forms prepared and tested by the Autism Research Institute in San Diego the symptom scores improved in 8 of the 10 children. This would certainly be a good reason to perform a well designed complete study.

It has now been possible to create a TTFD containing cream that can be applied to the skin, obviating the oral route completely. A by-product of its use is that the patient often will produce a body odor that is constantly likened to the characteristic odor of skunk secretion. The secretion of skunks is known to be due to a mixture of organic mercaptans and the prosthetic group derived from the reduction of TTFD is a mercaptan. By simple trial it has been found that the administration of 10 mg of biotin given each day by mouth will often reduce or remove the odor. Another agent that reduces this odor frequently, discovered by the mother of an autistic child, is the application of fresh lemon juice to the skin. Although scientific study of the mechanism of action has not been performed, it has become clear that this body odor from the patient gradually decreases and may disappear altogether as clinical improvement is observed and the biotin/lemon juice combination is no longer necessary, even though treatment with TTFD may continue. It has been known for some time that thiamin removes lead from experimentally induced lead toxicity in animal studies (Olkowski et al. 1991). Our study of TTFD in autistic children revealed that treatment was associated with increases in urinary lead, arsenic, cadmium and mercury, the SH-reactive heavy metals. This strongly suggests that it is the thiol nature of TTFD that gives it its chelating action of these metals.

An open trial with TTFD was performed on 44 patients with polyneuropathy. Thirty-four patients showed improvement of motor function and some restoration of sensory function. Of 18 patients re-examined electrophysiologically 3 months later, 6 showed remarkable improvement. There were no side effects noted (Djoenaidi and Notermans 1990) The same authors reported three patients with beriberi who presented with different clinical manifestations. The cardiac symptoms responded dramatically to TTFD and there were some improvements in their polyneuropathy (Djoenaidi et al. 1992). In view of the harmless nature of the treatment, these authors also suggested the routine administration of TTFD should be given to all patients in whom heart failure is present without clear evidence of cause. In a 12 week open trial in Alzheimer's disease TTFD at an oral dose of 100 mg/day resulted in a mild beneficial effect on emotional symptoms and intellectual function (Mimori et al. 1996)

A test known as the intravenous olfaction test with TPD is a simple procedure widely used in Japan (Harada et al. 2002). An olfactory stimulus is provided by intravenous injection of TPD. The subject smells n-propyl mercaptan, the prosthetic group derived from hydrolysis. This is discharged from blood into the alveoli and

expired. By electroencephalography the authors found alpha-2 and beta-2 waves to be activated over the frontal and temporal regions during the olfactory stimulation. The EEGs returned to pre-stimulus levels after disappearance of the olfactory sensation.

The S acyl derivative known as benzoyl thiamine monophosphate (BTMP), also known as Benfotiamine has recently received attention and is reported to have clinical benefit in the complications of diabetes (Bitsch et al. 1991, Thornalley 2003; Beltramo et al. 2004; Haupt et al. 2005). Alteration of thiamine pharmakoinetics by end stage renal disease improved with administration of BTMP, suggesting that this derivative might be beneficial in end stage renal disease (Frank et al. 1999). It was reported that an open thiazole ring thiol form of thiamine (unspecified) released nitric oxide from S-nitrosoglutathione (Stepuro et al. 2005). This might explain the reported benefit of thiamine derivatives in the microangiopathy of diabetes and certainly deserves further research and clinical trials.

11.17 Conclusion and Hypothesis

The use of thiamin, and its disulfide derivatives in particular, is much neglected in Western medicine. It was one of the earliest vitamins to be discovered and synthesized and it is surprising that it has not been featured more in clinical reports and reviews involving nutritional therapy. The nutritional diseases have been long associated with poverty and starvation. Starvation, however, represents loss of both calorie yielding food components as well as the non-caloric nutrients, usually a slow attrition to death. If calories are maintained without appropriate vitamin and mineral content (empty calories), the outcome is quite different. Beriberi is the classic form of empty calorie disease since it is strongly associated with white rice in Eastern cultures. Modern Western diets, particularly in children and adolescents are loaded with simple carbohydrates, placing a metabolic strain on the mechanisms of oxidative metabolism. The disease outcome is therefore often seen as marginal beriberi.

My experience of more than 30 years of nutritional therapy in the U.S.A. has clearly shown that malnutrition is extremely widespread. Thiamin figures high on the list of nutrient deficiencies, although it can also be said that is more an excess of simple carbohydrates that overwhelms the oxidative system rather than a strictly defined vitamin deficiency. Because of vitamin fortification of processed foods and the relative affluence of our present culture, we are not ready to consider that obscure symptoms, particularly those that are generally termed functional, are of dietary origin. They are frequently the direct result of years of dietary abuse and the subsequent deterioration in enzyme action is not easily repaired. Physiological doses have no effect since the enzyme/cofactor bonding appears to be damaged or partially atrophied. Thus, physicians often become disenchanted when they suspect vitamin deficiency, treat a patient with low-dose supplementation and see no benefit. The RDA of thiamin is 1–1.5 mg per 1,000 kcal but only in a biochemically healthy individual. Like the RDA of vitamin C is the minimal amount to prevent

scurvy, so is the RDA of thiamin the minimal to prevent beriberi. As was pointed out in discussion of beriberi, occurring in epidemic form, it took months of high dose thiamin to reverse the symptoms and sometimes it was irreversible.

One important aspect of this kind of malnutrition is the effect that it has on the emotional characteristics of behavior. Anger is easily invoked and temper tantrums may be much more violent and persistent in children. The hypothesis is that the limbic system of the brain becomes much more responsive to incoming stimuli when its oxidative metabolism becomes inefficient. Primitive reflex activity in this system might then occur under stress when the supervisory action of the cognitive brain is overwhelmed. Juvenile crime has been linked with high calorie malnutrition (Schauss 1981; Gray 1987), and the role of thiamin deficiency accentuated (Lonsdale 1992a, 1994) in view of the nature of dietary excesses as considered earlier in this chapter.

Legal circles have addressed this association in only a very limited manner because our concept of criminal behavior is that it is purely volitional and performed in cold blood. It is entirely possible, however, that a perceived anger might give rise to irrational behavior that might explain the presently inexplicable problem of school shootings, for example. To my knowledge no newspaper account has ever included any discussion on diet in any one of these insane incidents since it never enters the consciousness of anyone to make the enquiries. Temporary insanity, at least in the State of Ohio, can be used in court if it can be shown that a criminal "knew what he was doing but was unable to stop himself doing it". The human body is a fuel burning "machine" with the incredible ability to heal itself. Assuming a genetically, or epigenetically, determined cellular "blueprint", all it requires is the appropriate fuel and the catalysts that enable it to go through the complex mechanisms of oxidative metabolism, particularly in the brain. As we move further and further from our biologic origins, so our risks incurred by appalling planet earth stewardship increase accordingly. The aim of the food industry is profit and it appeals to our modern urge to avoid kitchen duty as much as possible by processed convenience foods that provide taste pleasure irrespective of their nutritional quality.

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Chapter 12
Riboflavin in Development and Cell Fate

Hilary J. Powers, B.M. Corfe, and E. Nakano

Abstract Riboflavin (7,8-dimethyl-10-ribitylisoalloxazine; vitamin B2) is a water-soluble vitamin, cofactor derivatives of which (FAD, FMN) act as electron acceptors in the oxidative metabolism of carbohydrate, amino acids and fatty acids and which in the reduced state can donate electrons to complex II of the electron transport chain. This means that riboflavin is essential for energy generation in the aerobic cell, through oxidative phosphorylation. The classic effects of riboflavin deficiency on growth and development have generally been explained in terms of these functions. However, research also suggests that riboflavin may have specific functions associated with cell fate determination, which would have implications for growth and development. In particular, riboflavin depletion interferes with the normal progression of the cell cycle, probably through effects on the expression of regulatory genes, exerted at both the transcriptional and proteomic level.

Keywords Riboflavin · Cell fate · Gene expression · Cell cycle

12.1 Riboflavin Structure, Classical Biochemistry and Conventional Wisdom Regarding Effects of Deficiency

Riboflavin (7,8-dimethyl-10-ribitylisoalloxazine; vitamin B2) is a water-soluble vitamin with a well-established role in redox metabolism. It is obtained from the diet as the 'free' riboflavin and in the forms of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which participate in numerous redox reactions in intermediary metabolism. This activity is a function of the ability of the isoalloxazine ring of these flavins to lose or gain electrons (Fig. 12.1). Attention has been drawn to the remarkable versatility of flavoproteins, attributed to the wide variation in redox and electronic states in which the isoalloxazine ring of flavins can exist (Miura 2001). FAD and FMN act as electron acceptors in the oxidative metabolism of carbohydrate, amino acids and fatty acids and in the reduced state

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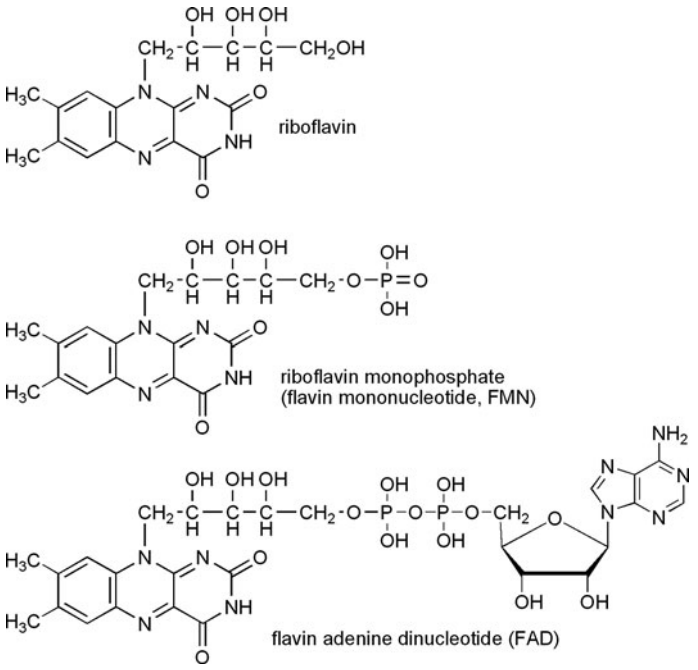


Fig. 12.1 Riboflavin and coenzymes (reprinted from: Human Nutrition, Geissler C and Powers H, eds. P189, 2005, with permission from Elsevier)

can donate electrons to complex II of the electron transport chain. This ensures that dietary riboflavin is essential for energy generation in the aerobic cell, through oxidative phosphorylation, and a lack of riboflavin would therefore be expected to compromise cellular energetics.

12.1.1 What is the Evidence that Riboflavin Deficiency Compromises the Energetics of the Cell?

The importance of riboflavin derivatives to mitochondrial function was demonstrated in the 1970s and 1980s in studies conducted in riboflavin-deficient animals. Mice fed a riboflavin diet from weaning developed morphological abnormalities of hepatic mitochondria, associated with an impaired oxidative metabolism (Hoppel and Tandler 1975). Riboflavin depletion in weanling rats leads to a reduction in the activity of various mitochondrial flavin-dependent enzymes in hepatic tissue, including succinate dehydrogenase and a ketoglutarate dehydrogenase. Riboflavin depletion has the capacity therefore to impact on various different components of the energy-yielding apparatus of the cell and there is evidence of a hierarchic response of the metabolism of an organism to riboflavin depletion. This was first proposed by Prentice and Bates during their work in riboflavin deficient rats

(Prentice and Bates 1981) and has been more fully characterised more recently (Ross and Hansen 1992). The electron transport chain is clearly of high priority to the cell and its activity is preserved at the expense of other flavin-dependent pathways including mitochondrial β-oxidation of fatty acids, although the peroxisomal oxidation of fatty acids seems little affected. Importantly, an early response of animal models to riboflavin depletion is an alteration in the relative activities of the flavin-metabolising enzymes. The fall in flavokinase activity is more pronounced than that of FAD pyrophosphorylase, which ensures that concentrations of FAD are sustained at the expense of FMN (Fass and Rivlin 1969). It is no coincidence that the majority of flavin-dependent enzymes in tissues are FAD-dependent.

Mitochondrial fatty acid oxidation is impaired in riboflavin-deficient rodents through inhibition of FAD – dependent acyl CoA dehydrogenases (Brady and Hoppel 1985; Olpin and Bates 1982). This leads to an accumulation of dicarboxylic acids, which are excreted in the urine (Goodman 1981) and would also explain the altered fatty acid profile in triacylglycerols and phospholipids in riboflavin deficiency (Olpin and Bates 1982). Gene defects leading to flavin-dependent acyl CoA dehydrogenase deficiencies in humans are associated with profound abnormalities of mitochondrial metabolism, characterised by defects in fatty acid ß-oxidation and reduced activity of the electron transport chain (Gianazza et al. 2006). People carrying these gene defects are responsive to high dose riboflavin or of the cofactors FAD or FMN (Antozzi et al. 1994). Genetic acyl CoA dehydrogenase deficiency reported in a human pregnancy, associated with abnormally elevated urinary excretion of riboflavin, led to deaths of the offspring in the neonatal period or early infancy. High riboflavin intake in the third trimester, and in infancy, prevented the syndrome (Harpey et al. 1983).

The mitochondrial oxidation of carboxylic acid intermediates of the tricarboxylic acid cycle and of ketone bodies has also been shown to be impaired in riboflavin-deficient rats (Zaman and Verwilghen 1975). Furthermore, patients with glutaryl CoA dehydrogenase deficiency, an autosomal recessive disorder of mitochondrial amino acid metabolism, have also been shown to be responsive to pharmacological doses of riboflavin (Chalmers et al. 2006).

12.1.2 Implications for Metabolic Rate and Growth

Metabolic rate: As flavoproteins are central to mitochondrial energy-generating pathways, riboflavin status would be expected to be a key determinant of whole body metabolic rate. Oxygen consumption is impaired in hepatic mitochondria of riboflavin-deficient rats (Olpin and Bates 1982) and in mitochondria isolated from brown adipose tissue, which is important for non-shivering thermogenesis (Duerden and Bates 1985). Metabolic rate, measured as basal whole body oxygen consumption, and in response to nor-adrenaline, which is the physiological stimulus to brown adipose tissue thermogenesis, is depressed in riboflavin-deficient suckling rat pups (Patterson and Bates 1989). In contrast, riboflavin depletion in weanling rats elicited an increase in metabolic rate, in association with a marked elevation in

Table 12.1 Effect of riboflavin depletion on weight gain per g food consumed Wistar rats were placed on a riboflavin-deficient diet from weaning and rats receiving a complete diet were weight matched (Williams et al. 1996a)

	Weight gain/g food consumed mean (SEM)	
Days from weaning	Riboflavin-deficient (n=6)	Weight-matched (n=6)
0	–	–
7	0.26* (0.021)	0.33 (0.018)
14	0.17* (0.021)	0.24 (0.015)
21	0.19* (0.013)	0.25 (0.010)

*Significantly different from weight-matched controls ($P < 0.05$)

mitochondrial GDP binding, which suggests an increase in the thermogenic capacity of brown adipose tissue. The functional effects of riboflavin depletion evidently depend upon the stage of development. Babies born prematurely are at increased risk of riboflavin deficiency but riboflavin supplements given to premature infants did not bring about any change in the rate of 1-13C-octanoic acid metabolism (Patterson et al. 1989).

Growth: Riboflavin depletion in young rats leads to growth impairment, which is partly due to inanition but thought also to be causally related to the role of riboflavin in cellular energetics. When weanling rats are fed a riboflavin deficient diet from weaning and animals receiving a control diet are weight matched to riboflavin deficient animals the latter show a reduced weight gain per gram of diet consumed, indicating an effect on the efficiency of energy production from fuel utilisation (Williams et al. 1996a) (Table 12.1).

Effects of riboflavin deficiency on human growth are less easy to determine, as riboflavin deficiency in isolation is unusual in populations in which riboflavin deficiency is endemic. Riboflavin deficiency in infants and children in developing countries (Bates et al. 1982) is associated with poor growth, which is likely to be a manifestation of numerous chronic nutrient deficiencies including riboflavin (Bates et al. 1993). Nevertheless, a supplement of 0.5 mg riboflavin given to Gambian infants for 12 weeks mitigated the deterioration in growth, measured as length-for-age Z score, seen in infants receiving a placebo (unpublished observations, Fig. 12.2).

12.1.3 Are There Other Reasons Why Riboflavin Deficiency Might Impair Growth?

Effects of riboflavin deficiency on growth are classically related to flavin-dependent redox reactions in the pathways of intermediary metabolism but other mechanisms may also be involved. The oxidative folding of secretory proteins in the endoplasmic reticulum requires flavin-dependent oxidases (Tu et al. 2000). Riboflavin-depletion of Jurkat cells and HepG2 cells in culture is associated with impaired folding and

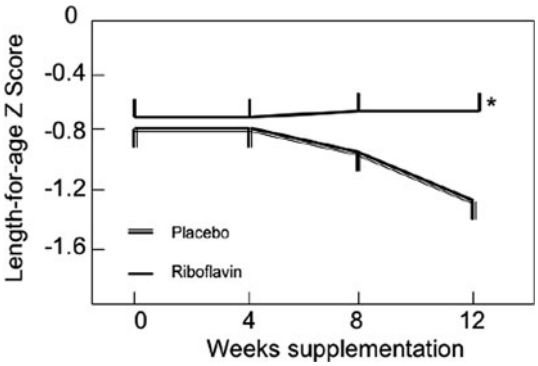


Fig. 12.2 Effect of a daily supplement of 0.5 mg riboflavin on growth in Gambian infants. *Significantly different from placebo ($P < 0.05$)

secretion of proteins and a consequent accumulation of unfolded proteins in the endoplasmic reticulum of these cells. This elicits a stress response (Camporeale and Zempleni 2003; Manthey et al. 2005) known as the Unfolded Protein Response. There are various possible downstream effects of the unfolded protein response, including increased phosphorylation of eukaryotic initiation factor 2α, which leads to an overall suppression of protein synthesis (Pahl 1999).

12.2 Riboflavin in Differentiation and Development

Our understanding of the essentiality of riboflavin for normal fetal development stems from the isolation of a riboflavin carrier protein (RCP) from chicken eggs. RCP is an oestrogen-inducible protein, immunoneutralisation of which leads to fetal resorption in mammalian species (Adiga and Murty 1983). Studies conducted by (Rao et al. 2000) suggested that the fetal resorption observed in immunoneutralised animals was due mainly to abnormal embryo implantation. The requirement for this riboflavin carrier protein during normal fetal development indicated its crucial role in materno-fetal riboflavin transport in avian and mammalian species. Riboflavin carrier proteins have also been measured in amniotic fluid during human pregnancy, (Prasad 1992), as well as in maternal serum (Natraj et al. 1988), and shown to exhibit significant cross-reactivity with chicken riboflavin carrier protein (Visweswariah and Adiga 1987) indicating conservation of the protein during evolution. RCPs are evidently important for embryo growth and development in humans. Riboflavin supply during fetal development seems also to have a rather specific role in skeletal development. There are several reports of a range of skeletal abnormalities in rats and mice born to dams fed a riboflavin deficient diet, including shortening of the digits and defects of the axial skeleton (Noback and Kupperman 1944; Nelson et al. 1956). An interaction has been reported between the occurrence of cleft palate in genetically-susceptible chickens and maternal riboflavin deficiency. Maternal riboflavin deficiency enhanced the effects of the genetic trait and increased the frequency of cleft palate in the offspring (Juriloff and Roberts

1975). Furthermore, the teratogenic effects (including cleft palate) of the phenothiazine derivative T-82 in rats can be significantly mitigated by high dose riboflavin, administered intraperitoneally (Horvath et al. 1976). The human corollary of this is not known although Faron et al. (2001) have suggested that a case of recurrent cleft lip and palate observed in siblings of a patient with malabsorption syndrome may be associated with riboflavin deficiency.

The importance of riboflavin carrier protein(s) to riboflavin transport across cell membranes and intracellular trafficking in humans is not clear. The involvement of a carrier protein in the uptake of riboflavin by cells in culture was first alluded to by Said et al. (1998), who showed a carrier-mediated uptake of riboflavin by HepG2 cells in culture that was upregulated in response to substrate depletion and inhibited by the riboflavin analogues lumichrome and lumiflavin. More recent work reported a receptor-mediated endocytosis pathway for riboflavin uptake into cells (Huang and Swaan 2000; Huang et al. 2003) and this group has further elucidated a role for a riboflavin carrier protein in the uptake and trafficking of riboflavin in mammalian cells, apparently under cAMP regulation (Mason et al. 2006; D'Souza et al. 2006).

12.2.1 Riboflavin Deficiency and Development of the Gastrointestinal Tract

Riboflavin depletion during the weaning process in rats has been shown to have a rapid and reproducible effect on the development of the gastrointestinal tract. Gastrointestinal development is considered to follow a program of structural and functional change from early in fetal development until weaning, and is evidently subject to regulation by nutrient and non-nutrient factors such as hormones and growth factors. Moderate riboflavin depletion from around weaning has a profound impact on the structural and cytokinetic development of the rat small intestine. An early event is crypt hypertrophy, which is associated with increased crypt cellularity (Williams et al. 1996a, b). The rate of crypt bifurcation is also depressed in riboflavin-deficient weanling rats (Yates et al. 2001), which may be a factor in the subsequent failure to increase the size of the villus population. The fact that crypt hypertrophy is not associated with an increase in the size of the proliferative compartment, and neither is it associated with an increased crypt cell proliferation rate, suggests a role for riboflavin in the regulation of apoptosis (Yates et al. 2001). Furthermore, the morphological and cytokinetic effects in the gastrointestinal tract of feeding weanling rats a riboflavin-deficient diet can be reproduced in animals in which the gastrointestinal lumen is deprived of riboflavin whilst systemic riboflavin status is maintained at a normal level through regular intraperitoneal injections of a flavin (Yates et al. 2003). This suggests the presence of an apical receptor/transporter, ligation of which is central to gastrointestinal development. Riboflavin in the lumen may therefore behave as a signalling molecule, and the effects of luminal riboflavin depletion on gastrointestinal development may be partially independent of its role as an essential nutrient (Fig. 12.3).

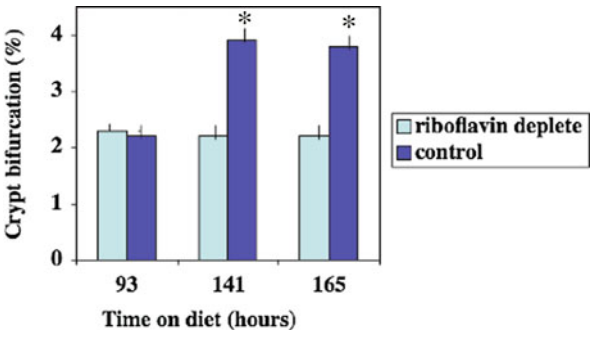


Fig. 12.3 Crypt bifurcation in the duodenum of rats fed a riboflavin deplete diet from weaning or in weight-matched controls fed a complete diet. Values are means with standard errors (Yates et al. 2001). *Significantly different from control (ANOVA and Scheffe test)

Given the evidence that riboflavin deficiency leads to developmental abnormalities during embryogenesis and the specific effects of riboflavin deficiency on cell growth and proliferation during gastrointestinal development, it is hypothesised that riboflavin is a determinant of cell fate, either through direct regulation of proteins of the cell cycle or through effects on biochemical pathways important for cellular proliferation or apoptosis. Such effects would be independent of effects of riboflavin on energy–generating steps of intermediary metabolism.

12.3 Nutrients and Regulation of Proteins of the Cell Cycle

Effects of riboflavin depletion on aspects of growth and development strongly suggest that this vitamin plays an important role in the determination of cell fate. There are plausible mechanisms for such a role. A key apoptosis-inducing protein, (apoptosis-inducing factor-homologous mitochondrion-associated inducer of death, AMID, or p53-responsive gene, PRG3) has 6-hydroxy-FAD as cofactor (Marshall et al. 2005). Accumulation of unfolded proteins and the associated stress response in riboflavin-deficient cells leads to a reduction in the proliferation rate of HepG2 cells, although it is not clear whether the association is causative (Manthey et al. 2005).

The normal cell progresses through a cycle of change comprising four distinct phases. These are, gap 1 (G1), DNA synthesis (S), gap 2 (G2), and mitosis (M). The progression of the cell through these phases is under the regulation of key proteins, the expression of which is influenced by cellular and extracellular factors such as DNA integrity and nutrient availability. Cyclin-dependent kinase complexes regulate the transition of the cell from one phase to another, and these cyclin-CDK complexes are in turn regulated by other gene families including the INK4 and CKI families (Johnson and Walker 1999). The expression of these regulatory proteins can be influenced by nutrients. Retinoic acid is active in regulation of the cell cycle, for example, through effects on the expression of cyclins and CDKs (Alisi et al. 2003). Importantly, the effect of retinoic acid on cell proliferation is specific to the cell type (Chen et al. 2002; Kosaka et al. 2001). During embryogenesis retinoic acid

inhibits proliferation of certain cell types, including hepatocytes and smooth muscle cells. In the embryonic mouse retinoic acid upregulates the cyclin-dependent kinase inhibitors, p21 and p27 in primordial endothelial cells, which leads to cell cycle arrest in G1 (Bohnsack and Hirschi 2004). In contrast, retinoic acid stimulates proliferation of other cell types, including cardiomyocytes (Stuckmann et al. 2003). The vitamin D metabolite 1,25(OH)2 has antiproliferative and pro-differentiation effects in different cell types, mediated by increased expression of P21, P27 and other regulatory proteins (Swami et al. 2003). Osteoblasts, haematopoietic cells and keratinocytes all show a depressed proliferation in response to 1,25(OH)2, and an upregulation of a number of cell cycle regulatory proteins, including p21 and p53 (Swami et al. 2003).

12.3.1 Riboflavin and Regulation of the Cell Cycle

Relatively little is known about the role of riboflavin in the regulation of cell proliferation, apoptosis and differentiation. Zempleni's research group have conducted some interesting studies of riboflavin depletion in HepG2 cells that show evidence of an altered cell cycle. They hypothesised that the reported abnormal folding of secretory proteins in riboflavin–deficient cells in culture (Manthey et al. 2005) would lead to a stress response characterised by cell cycle arrest (Werner et al. 2005). HepG2 cells were cultured in RPMI-1640 medium containing either a high concentration of riboflavin (532 nmol/l, control) or 3.1 nmol/l (riboflavin depleted), for 4 days. Media contained 10% dialysed bovine calf serum, which would have contributed little to the total riboflavin content of the medium, although the actual concentration is not reported. Riboflavin depletion was confirmed as a reduction in glutathione reductase activity. Riboflavin-depleted HepG2 cells showed an enhanced binding activity of transcription factors to endoplasmic reticulum stress elements, and an increase in the concentration of the stress protein GADD153, indicative of cell stress (Fig. 12.4). Flow cytometry indicated an alteration in cell cycle progression in riboflavin-depleted cells, with an increase in the proportion of cells in G1 and a reduction in the proportion of cells in S phase and G2 compared with cells incubated in medium containing high concentrations of riboflavin. Decreased cell proliferation rates were inferred from a reduction in 3H-thymidine uptake (Werner et al. 2005). The authors concluded that the accumulation of unfolded proteins in riboflavin-deficient HepG2 cells triggers the unfolded protein response (Sidrauski et al. 2002), with an associated cell cycle arrest. The same group further examined some possible mechanisms for the effects of riboflavin depletion on cell cycle progression in HepG2 cells in culture (Manthey et al. 2006). They tested the hypothesis that cell cycle effects were mediated by oxidative damage to DNA and proteins. They reported an increase in DNA strand breakage, measured using the Comet assay, in response to 4 days growth in riboflavin-deficient medium compared with a high concentration of riboflavin (300 nM). Protein carbonylation also increased in response

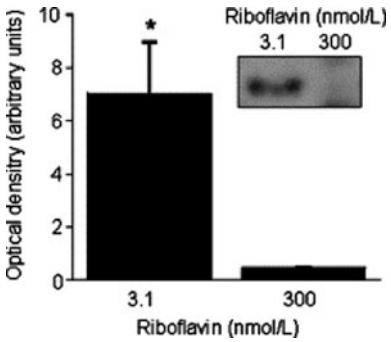


Fig. 12.4 Abundance of GADD153 increases in response to riboflavin deficiency in HepG2 cells. Cells were cultured in media containing 3.1 and 300 nmol/L riboflavin for 4 days. GADD153 was quantified using Western blot analysis and gel densitometry. $^{*}P<0.05$ vs. 300 nmol/L riboflavin ($n=4$). The *inset* depicts a representative image from Western blot analysis (reprinted from 'Riboflavin deficiency causes protein and DNA damage in HepG2 cells, triggering arrest in G1 phase of the cell cycle' Manthey et al. 2006, with permission from Elsevier)

to riboflavin-depletion, indicating oxidative damage. It is argued that these effects are a direct result of impaired antioxidant protection as glutathione reductase activity falls, with a likely fall in glutathione concentration in the riboflavin-depleted cells.

Thus riboflavin depletion in the HepG2 cell in culture does seem to be associated with an increase in oxidative stress, and downstream effects on cell cycle progression. In these experiments the authors chose to use very high concentrations of riboflavin as a 'control', and these cells may have behaved quite differently from a true physiologic control. The same group conducted gene expression analysis in riboflavin-depleted HepG2 cells, using the Affymetrix Human genome U133A Array. Riboflavin depletion was associated with up or down-regulation of genes, including those known to be important for cell cycle progression and proliferation, and apoptosis. Although genes from both of these functional clusters showed both increased and decreased transcription there was a preponderance of genes showing increased transcription. In contrast, genes important for protein synthesis and degradation were more likely to be down regulated. Taken together, results from this group suggest that riboflavin depletion of HepG2 cells in culture leads to dysregulation of the cell cycle, specifically arrest of cells in G1.

12.3.2 Effects of Riboflavin Depletion on an In Vitro Model of Enterocyte Proliferation/Differentiation

In the light of the profound effects of riboflavin deficiency on the development of the gastrointestinal tract in weaning rats we propose that riboflavin has multiple roles in the enterocyte associated with functions in cell fate determination in

addition to its accepted role in redox metabolism. In particular, riboflavin regulates the cell cycle so as to influence cell proliferation, differentiation and death of intestinal epithelial cells. In order to test this hypothesis work has been conducted using a human intestinal epithelial cell model (Caco-2 cell, obtained from the European Collection of Cell Cultures, ECACC). Cells were grown in specially prepared DMEM containing no riboflavin. Cells were cultured under three conditions:

(i) Riboflavin-rich medium, consisting of standard DMEM containing 10% foetal calf serum. The riboflavin content of this medium was 1.2 μM, which is higher than the concentration of riboflavin in human plasma following supplementation with moderate dose riboflavin (Powers et al. 2011).
(ii) Physiological medium, consisting of riboflavin-free DMEM containing 10% foetal calf serum. The riboflavin content of this medium was 16 nM. This approximates the riboflavin concentration in plasma from healthy people (Moat et al. 2003).
(iii) Riboflavin-depleted medium, consisting of riboflavin-free DMEM containing 10% dialysed foetal calf serum. The riboflavin content of this medium was 5.5 nM. This approximates the riboflavin concentration in human plasma in the lowest fifth centile of a healthy population (Hustad et al. 2000).

Flavin concentrations were measured in the fetal calf serum before and after dialysis, and in the final medium, by HPLC (Capo-chichi et al. 2000). Following transfer of cells from the riboflavin-rich medium to physiological riboflavin medium the intracellular concentration of FAD fell by about 10%, that of FMN fell by about 30% whilst the riboflavin concentration fell to 50% of that in the control medium, and concentrations were maintained as such over 6 passages. Cell proliferation was unaffected as were basal levels of apoptosis. Transferring cells to riboflavin-depleted medium (5.5 nM) elicited a further progressive decrease in intracellular flavin concentrations to about 50% of that in the physiological cells by 72 h. About 90% of the intracellular flavins were in the form of FAD by this stage. The cell doubling time increased from 0.88 days to 1.62 days during riboflavin depletion.

Riboflavin depletion of Caco-2 cells altered progression of cells through the cell cycle. Following growth in physiological and riboflavin-depleted medium for up to 72 h cells were trypsinised, washed in buffer, fixed in ethanol and incubated with propidium iodide to stain DNA, and analysed by flow cytometry. During growth in riboflavin-depleted medium there was a progressive fall in the proportion of cells in G1 and a progressive increase in the proportion of cells in G2, compared with those cells grown in physiological medium. By 72 h of growth in riboflavin-depleted medium there was a greater proportion of cells in G2 than in G1 with no significant change in S phase, suggesting impaired progression of cells through G2 or M (Fig. 12.5; Table 12.2). Further study has indicated that a more prolonged period of riboflavin depletion leads to accumulation of aneuploid cells, and that the cell cycle alteration is likely to be in M rather than G2 phase (Nakano et al. 2011).

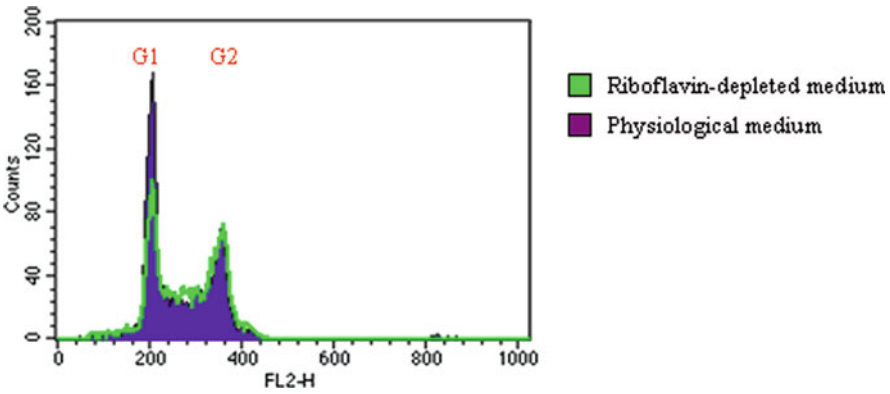


Fig. 12.5 Flow cytometry analysis of Caco-2 cells following growth for 72 h in physiological medium (16 nM riboflavin) and riboflavin-depleted (5.5 nM) medium. Data are shown for a single representative experiment at 72 h

Table 12.2 Effect of riboflavin depletion of CaCo-2 cells on cell cycle phase distribution CaCo-2 cells were grown for 72 h in riboflavin-depleted (5.5 nM riboflavin) or physiological (16 nM riboflavin) medium for 72 h. Values represent the percentage of cells in different stages of the cell cycle, measured using flow cytometry (mean (SEM) of data from 3 experiments). Riboflavin depletion was associated with a significantly greater percentage of cells in G2 ($P = 0.008$) and significantly fewer cells in G1 ($P = 0.011$) compared with cells grown in physiological medium (Wilcoxon non-parametric test)

		Time (hours) in experimental medium		
Growth conditions	Phase of cell cycle	24	48	72
Physiological	G1	44.4 (1.14)	43.4 (0.12)	50.5 (2.41)
Riboflavin-depleted	G1	45.4 (0.12)	37.0 (1.76)	29.6 (0.76)
Physiological	S	26.2 (0.64)	26.9 (1.12)	20.7 (3.36)
Riboflavin-depleted	S	20.2 (0.73)	25.6 (1.27)	29.2 (0.32)
Physiological	G2	25.2 (0.91)	26.2 (1.21)	25.0 (0.81)
Riboflavin-depleted	G2	31.5 (0.86)	31.7 (0.61)	35.4 (0.15)

12.3.3 Apoptosis

Using direct depletion methods we have not been able to detect any change in the basal levels of apoptosis in Caco2 cells. This suggests that riboflavin depletion alone is insufficient to trigger apoptosis. However, when the riboflavin uptake competitor lumiflavin was used to drive down intracellular levels of riboflavin, a marked decrease in attached cell count was identified, at a 4 fold molar excess of lumiflavin (Fig. 12.6). This cell death occurred within 72 h of lumiflavin treatment. This finding seems to correlate with the observation that luminal depletion of riboflavin in systemically riboflavin replete rats drives alterations in crypt morphology attributable

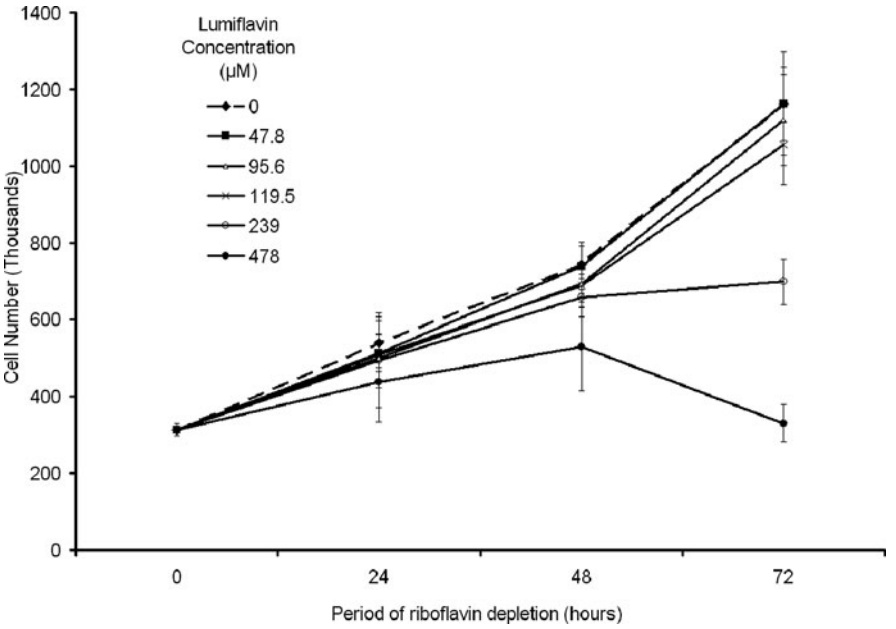


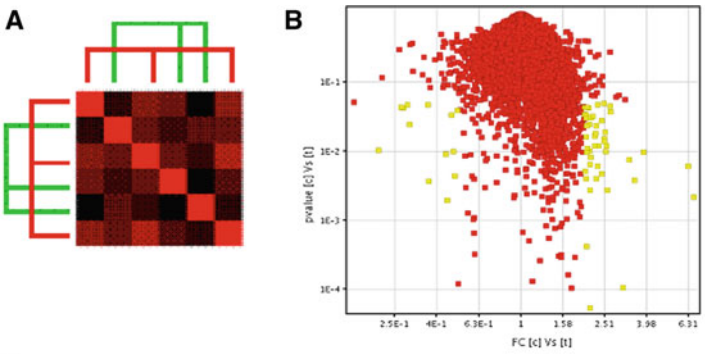
Fig. 12.6 Effects of riboflavin depletion on cell number. Caco-2 cells were grown in the presence of different concentrations of lumiflavin. Data are the mean and SD of cell numbers at 72 h for three independent experiments

to changes in levels of apoptosis. We hypothesize that in this particular context, riboflavin is acting as a specific signal to cells via a hypothetical apical receptor. Ligation of the receptor would be required to maintain cellular viability in vitro and tissue homeostasis in vivo.

The particular effects of riboflavin depletion on progression through the cell cycle appear to be dependent on the cell type. This observation has been made for other nutrients; retinoic acid for example will inhibit proliferation and promote differentiation in embryonic hepatocytes and endothelial cells whilst promoting proliferation in embryonic cardiomyocytes (Stuckmann et al. 2003).

12.3.4 Transcriptional Consequences of Riboflavin Depletion

In order to understand the mechanisms by which riboflavin depletion might inhibit cell cycle progression DNA microarray analysis was undertaken on RNA extracted from cells cultured in riboflavin depleted and physiological medium for up to 72 h. Following 72 h of growth in riboflavin depleted medium 60 genes showed at least a two-fold change and 11 genes showed at least a three-fold change in mRNA concentration (Fig. 12.7). A cluster of these genes is known to



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	P (All)	P (<0.05)	P (<0.01)	P(<0.005)	P (<0.0001)
FC All	22.283	761	167	83	21
FC>1.1	5.522	665	152	75	19
FC>1.5	808	283	85	47	14
FC>2.0	123	60	23	13	3
FC>3.0	18	11	6	3	1

Fig. 12.7 Alterations in gene expression after riboflavin depletion. Caco2 cells were grown in riboflavin-depleted DMEM (5.5 nM riboflavin) or in medium supplemented with riboflavin to physiological levels (16 nM). RNA was extracted and expression analysed using Affymetrix U133 arrays. **Panel A**: Heat map showing overall similarity between arrays, where *redness* indicates similarity, cluster indicated with a *red* dendogram are the physiological group, the *green* dendogram indicates the depleted group. **Panel B**: Fold change versus probability (volcano plot) or all data, spots in *yellow* are considered as significant. **Panel C**: Numbers of genes reaching increasing levels of probability and increasing levels of fold change, 60 genes reached a *P* value of 0.05 for a FC of >2.0

play a role in the regulation of the cell cycle, including CEACAM1 and cyclin A2. Effects of riboflavin depletion were also seen in genes associated with the stress response (STIP1) and energy generation (AK2, ATP5G1) (Nakano et al. 2011).

In addition to effects of riboflavin depletion on mRNA for particular genes it is possible that some effects occur at the translational or post-translational level. Retinoic acid for example induced the ubiquitination of cyclin D1 in immortalised human bronchial epithelial cells, leading to proteolysis, and subsequent cell cycle arrest, even though mRNA levels for this protein are not influenced (Spinella et al. 1999). Studies are in progress to characterise changes in the expressed proteome in CaCo-2 cells depleted of riboflavin. Preliminary analysis confirms that riboflavin depletion leads to an alteration in the cellular proteome (Fig. 12.8).

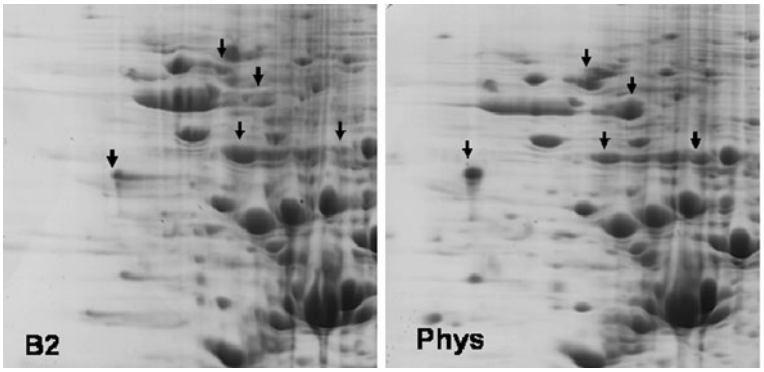

Fig. 12.8 2-D gels of proteins expressed in cells grown in riboflavin–depleted medium (B2) or in a physiological concentration of riboflavin (Phys). The *arrows* indicate proteins that appear to show an altered expression in response to riboflavin depletion. Further analysis will reveal the identity of these proteins

12.4 Conclusions

The requirement for dietary riboflavin to human health has been appreciated for decades. The metabolic basis for this has been hitherto attributed to the well-defined role of flavins in redox metabolism and energy generation. It is becoming apparent that flavins can modulate cell cycle progression, probably through effects on the expression of regulatory genes. There are numerous mechanisms by which flavins may exert such effects, at the transcriptional, translational and post-translational levels, including direct binding to RNA (resulting in a reduction in translational fidelity), and histone modification. It is also possible that influences of flavin concentrations on gene expression are mediated by effects on cellular redox status, bringing us neatly back to classic flavin biochemistry.

The challenge now is to characterise fully the role of dietary riboflavin in the regulation of the cell cycle, to understand the underpinning mechanisms and to evaluate the functional implications in terms of human health.

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Chapter 13
Vitamins B6 and Cancer

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Abstract Epidemiologic and laboratory animal studies have suggested that the availability of vitamin B6 modulates cancer risk. The means by which B6 mediates this effect is not known with any surety but it has been reported that high dietary vitamin B6 attenuates and low dietary vitamin B6 increases the risk of cancer. In fact vitamin B6 is widely distributed in foods and overt deficiency of this vitamin is not common. Nevertheless, marginal or secondary vitamin B6 deficiency, which might have an adverse effect on carcinogenesis, is rather common especially among old adults and alcoholics. This chapter addressed currently available information regarding the relationship between vitamin B6 and cancer.

Keywords Cancer · Chemoprevention · One-carbon metabolism · PLP · Supplementation · Vitamin B6

Abbreviations

MTHFR	methylenetetrahydrofolate reductase gene
iNOS	inducible NO synthase
NO	nitric oxide
PLP	pyridoxal-5′-phosphatase
THF	tetrahydrofolate

13.1 Introduction

Vitamin B6, which is the collective term for the family of 2-methyl-3-hydroxy-5-hydroxymethylpyridine compounds, is widely distributed in foods; foods of animal origin such as meat, fish, poultry, eggs, and diary products as well as foods of plant

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origin such as whole grain cereal products, white potato and other starchy vegetables, and noncitrus fruits. There are six major derivatives; pyridoxine, pyridoxal and pyridoxamine, and their phosphorylated derivatives, pyridoxine-5′-phosphatase, pyridoxal-5′-phosphatase (PLP) and pyridoxamine 5′-phosphatase. Vitamin B6 in human breast milk is present as pyridoxal and pyridoxal phosphate. In animal-derived foods it is also present largely as pyridoxal phosphate and pyridoxamine phosphate, while in plant derived foods vitamin B6 is present mainly as pyridoxine, pyridoxine phosphate, and pyridoxine glucoside (Clayton 2006). PLP, the major coenzymatic form of vitamin B6, involves in 112 enzyme reactions (3%) among 3,870 enzymes catalogued in the ENZYME data base (Ames et al. 2002), especially for amino acid metabolism, one-carbon metabolism, lipid metabolism, gluconeogenesis, heme biosynthesis, and neurotransmitter biosynthesis. In the U.S. an estimated average requirement of 1.1 mg/day and an RDA of 1.3 mg/day for men and women (19–50 years) were established in 1998. For adults over 50 years, the RDA is 1.5 mg/day for women and 1.7 mg/day for men.

Nowadays the classical symptoms of vitamin B6 deficiencies such as seborrheic dermatitis, microcytic hypochromic anemia, epileptiform convulsions, depression, and confusion are extremely rare, whereas 10–20% of the population and 51% of elderly hospitalized patients have marginal or inadequate vitamin B6 status (Joosten et al. 1993; Selhub et al. 1993; Bender 2003). Secondary deficiencies of vitamin B6 may result from malabsorption, uremia, cancer, heart failure, cirrhosis, and increased metabolic activity associated with alcohol or the use of some medication such as isoniazid, penicillamine, cycloserine, and theophylline. Current interest in vitamin B6 focuses on its role in decreasing circulating homocysteine, a risk factor for vascular disease, or on its role in the risk of cancers.

13.2 Evidence Indicating the Relationship Between Vitamin B6 and Cancer

13.2.1 Epidemiologic Evidence

Old observational studies have found a low circulating vitamin B6 concentration in cancer patients. In 1960 Wachstein et al. reported that PLP concentrations in plasma and leucocytes were decreased in patients with leukemia compared with those in normal subjects (Wachstein et al. 1960). In 1977 Potera et al. reported that plasma PLP was normal in early breast cancer but reduced both in cases of local recurrence ($p < 0.01$) and systemic metastasis ($p < 0.001$) and the difference in plasma PLP between the early breast cancer patients and those with wide-spread disease was also significant ($p < 0.01$), indicating that decrement of plasma PLP concentration is in parallel with cancer progress (Potera et al. 1977). Thereafter, epidemiologic studies have suggested associations of vitamin B6 level in diet or blood with cancer risk, even though results were not always consistent.

13.2.1.1 Colorectal Cancer

In a most recent large Scottish case-control study (2,028 cases and 2,722 controls) an inverse and dose-dependent association was found between the risk of colorectal cancer and the intake of dietary vitamin B6 (OR = 0.77, 95% CI = 0.61–0.98; p for trend = 0.03) (Theodoratou et al. 2008). In two other recent epidemiologic studies conducted in the U.S. (Slattery et al. 1997) and in Europe (Jansen et al. 1999), which investigated the protective effect of high intakes of plant foods against colorectal cancer, high dietary intake of vitamin B6 reduced the risk of colorectal cancer in both men and women (p for trend <0.01) as well as the dietary intake of vitamin B6 was inversely associated with the colorectal cancer mortality. In a population based cohort study Harnack et al. (2002) found that the incidence of proximal colon cancer was lower among those with high dietary intake of vitamin B6 and folate (RR = 0.65, 95% CI = 0.5–0.84) than among those with the lowest intake of these vitamins. In a prospective nested case-control study from the Nurses' Health Study cohort Wei et al. (2005) provided more convincing evidence that both plasma PLP concentration and total vitamin B6 intake were inversely associated with the risk of colon cancer (RR = 0.42, 95% CI = 0.21–0.85 and RR = 0.51, 95% CI = 0.27–0.97, respectively). They also reported a trend of inverse association between plasma PLP concentration and advanced distal colorectal adenoma (RR = 0.65, 95% CI = 0.37–1.11, p for trend = 0.08).

A few studies, which investigated the effect of intake of B vitamins and methylenetetrahydrofolate reductase (MTHFR) genotype on the risk of colorectal cancer, have found an interaction between vitamin B6 intake and MTHFR gene in colonic carcinogenesis. MTHFR gene, which regulates one-carbon pools for DNA synthesis and biological methylation, has a common polymorphism 677 C to T and homozygous variants (677TT) have reduced enzyme activity in vivo and in vitro (Kang et al. 1988; Friso et al. 2002). Le Marchand et al. (Le Marchand et al. 2002) reported the two-way interactions: an apparent direct association between colorectal cancer risk and vitamin B6 intake in subjects with the CC wild-type and a strong inverse association with vitamin B6 intake in subjects with the TT genotype (p for interaction = 0.02) in a population-based case-control study. Slattery et al. (1999) also reported that high levels of intake of folate, vitamin B6, and vitamin B12 were associated with a 30–40% reduction in risk of colon cancer among those with the TT (homozygous variant) relative to those with low levels of intake who were CC wild genotype. Ulrich et al. (Ulrich et al. 1999) also reported that high dietary vitamin B6 intake reduced the risk of colorectal adenoma, a precursor of colorectal cancer, in TT genotype (p for trend = 0.03). In a most recent population based case control study conducted in the UK, significant interactions were found between total vitamin B6 intake and MTHFR 677 C to T polymorphism as well as another common polymorphism, MTHFR 1298 A to C (C677T, p interaction = 0.016; A1298C, p interaction = 0.003) (Sharp et al. 2008). However, the results are not always reproducible. In the other case-control study conducted in Japan neither vitamin B6 nor any genetic polymorphisms in one-carbon metabolism was significantly associated with colorectal cancer (Otani et al. 2005).

Chronic alcohol intake interferes with one-carbon metabolism (Stickel et al. 2000), in which vitamin B6 serves as a coenzyme. Choi et al. (1999) reported that alcohol fed rats had a 33% lower plasma PLP concentrations ($p = 0.02$) and a 16% lower hepatic PLP concentration ($p = 0.03$) than controls along with decreased genomic DNA methylation in the colon ($p < 0.05$). Cravo et al. (Cravo et al. 1996) reported that in chronic alcoholics serum PLP concentration was significantly decreased compared with control ($p < 0.001$). Interestingly, in a population-based cohort study, a long-term intake of dietary vitamin B6 was inversely associated with the risk of colorectal cancer (p for trend = 0.002) and the association was most evident among women with moderate to high alcohol consumption. The multivariate RR for colorectal cancer comparing extreme quintiles of vitamin B6 intake was 0.28 (95% CI = 0.13–0.59) among women who consumed > or = 30 g/week of alcohol (Larsson et al. 2005). These observations suggest that vitamin B6 may play an important role in the prevention of alcohol associated colorectal carcinogenesis.

13.2.1.2 Lung Cancer

In a nested case-control study conducted within the α-tocopherol β-carotene cancer prevention study cohort Hartman et al. (Hartman et al. 2001) reported a significantly lower risk for lung cancer among men who had higher serum vitamin B6 levels. Compared with men with the lowest vitamin B6 concentration, men in the fifth quintile had about one half of the risk of lung cancer (OR = 0.51, 95% CI = 0.23–0.93, p for trend = 0.02). In the most recent case-control study high intake of vitamin B6 reduced the risk of lung cancer among women with MTHFR TT genotype compared with women with MTHFR CC genotype (OR = 0.29, 95% CI = 0.15–0.56) (Shi et al. 2005).

13.2.1.3 Breast Cancer

Compared with colorectal cancer, less convincing is an association between vitamin B6 and breast cancer risk. In a case-control study from the Nurses' Health Study cohort the multivariable RR comparing women in the highest quintile of plasma vitamin B6 levels with those in the lowest quintile was 0.70 (95% CI = 0.48–1.02, p for trend = 0.09) (Zhang et al. 2003). In a population study, which investigated the dietary folate intake and breast cancer risk, a minimal effect of vitamin B6 was found: among women who consumed a low level of vitamin B6, dietary folate was weakly associated with a reduced risk of breast cancer, whereas a striking inverse association was observed for dietary folate intake among those whose intake levels of vitamin B6 were high (Shrubsole et al. 2001), indicating that the protective effect of folate against breast cancer may be modified by dietary intake of vitamin B6, even though dietary intake of vitamin B6 was not independently related to the risk of breast cancer. On the other hand, in a population-based case-control study daily vitamin B6 intake level in breast cancer cases was rather significantly higher than that in controls ($p = 0.03$) (Shrubsole et al. 2004) and two other population-based

case-control studies have found no relation of breast caner risk with vitamin B6 intake (Lajous et al. 2006) as well as plasma PLP concentration (Wu et al. 1999). The most recent case control study demonstrated an inverse association between plasma PLP and the risk of breast cancer among postmenopausal women (p for trend = 0.04) (Lin et al. 2008), while in the Nurses' Health Study II vitamin B6 intake was not associated with the risk of breast cancer among premenopausal women ages 26–46 years (Cho et al. 2007).

13.2.1.4 Prostate Cancer

In a case-control study, which investigated the effect of fat intake on the risk of prostate cancer, high dietary intake of vitamin B6 has a tendency to reduce the risk of prostate cancer (OR = 0.77, 95% CI = 0.53–1.12, p for trend = 0.077) and total intake of vitamin B6 including supplements has a reduced risk of this cancer (OR = 0.70, 95% CI = 0.48–1.03, p for trend = 0.029) (Key et al. 1997). In the α-tocopherol β-carotene cancer prevention study conducted in Finland vitamin B6 intake was inversely, but modestly, associated with prostate cancer risk (RR for highest versus lowest quintile = 0.88, 95% CI = 0.72–1.07, p for trend = 0.045) (Weinstein et al. 2006), even though the same group previously reported no association between vitamin B6 and prostate cancer risk in the same cohort (Weinstein et al. 2003). In a recent case-control study conducted in Italy vitamin B6 intake had no relation with this cancer risk (Pelucchi et al. 2005).

13.2.1.5 Pancreas Cancer

In a nested case-control study serum PLP concentrations showed an inverse dose-response relationship with pancreatic cancer risk (OR for highest versus lowest tertile = 0.48, 95% CI = 0.26–0.88, p for trend = 0.02) (Stolzenberg-Solomon et al. 1999). In this study an increased pancreatic cancer risk was also observed with high exposure to cigarettes, which is known to reduce circulating plasma PLP (Vermaak et al. 1990; O'Callaghan et al. 2002), suggesting that maintaining adequate vitamin B6 status may reduce the risk of pancreatic cancer associated with cigarette smoking. However, the same group found no association between vitamin B6 intake and pancreas cancer risk from the α-tocopherol β-carotene cancer prevention study cohort, even though in this cohort cigarette smoking was still associated with an increased risk of this cancer (Stolzenberg-Solomon et al. 2001). Most recently in a prospective nested case-control study using four large prospective cohorts in the US, the plasma concentration of vitamin B6 was not associated with a reduced risk of pancreas cancer (OR for highest versus lowest quartile = 0.91, 95% CI = 0.57–1.46) (Schernhammer et al. 2007).

13.2.1.6 Non-Hodgkin's Lymphoma

In a population-based case-control study of non-African-American adult women and men from four US Surveillance, Epidemiology, and End Results study centers,

Lim et al. (2005) demonstrated that higher intake of vitamin B6 was associated with the reduced risk of non-Hodgkin's lymphoma (OR for highest versus lowest quartile = 0.57, 95% CI = 0.34–0.95), suggesting that vitamin B6 may be protective against this cancer. They further demonstrated that interactions between dietary vitamin B6 and genes involved in one-carbon metabolism were also associated with the reduced risk of non-Hodgkin's lymphoma (Lim et al. 2006). However, from the α-tocopherol β-carotene cancer prevention study cohort same authors found no association between vitamin B6 and the risk of overall non-Hodgkin's lymphoma (Lim et al. 2007).

13.2.2 Evidence from Cultured Cell Studies

High concentrations of vitamin B6 have been reported to suppress growth of animal and human cancer cells; (1) pyridoxine supplemented medium (5 mM) retarded and eventually killed Fu5-5 rat hepatoma cells (DiSorbo and Litwack 1982), (2) pyridoxal supplemented medium (0.5 mM) inhibited the growth of a human malignant melanoma cell line (DiSorbo and Nathanson 1983), (3) either pyridoxine (5 mM) or pyridoxal (0.5 mM) supplemented medium showed an 80% reduction in B16 melanoma cells (DiSorbo and Litwack 1982), and (4) pyridoxine supplemented medium (5 mM) suppressed growth of human hepatoma cell line HepG2 (Molina et al. 1997).

Most recently Shimada et al. (2006) demonstrated that growth of the feline mammary tumor cell line FRM was inhibited by the addition of pyridoxine in a dose dependent manner (1–5 mM) and use of 5 mM pyridoxine caused an almost complete arrest of cell growth. Ren and Melmed (Ren and Melmed 2006) demonstrated that PLP also inhibited cell proliferation in non cancer cell line such as rat pituitary MMQ and GH3 cells in a dose dependent manner (0.01–1 mM). Collectively, vitamin B6 inhibits the growth of cultured cells originated from both cancer and non-cancer tissue.

13.2.3 Evidence from Animal Studies

Even though previous animal studies have suggested a relation between vitamin B6 and carcinogenesis, the most convincing are data from a series of animal studies conducted in a chemical carcinogen model of colon cancer in mice, evaluating the effect of dietary vitamin B6 on colonic carcinogenesis. Komatsu et al. (2001) reported that dietary supplementation with vitamin B6 suppressed colonic tumorigenesis by reducing cell proliferation. Mice were fed diets containing 1, 7, 14 or 35 mg pyridoxine HCl/kg diet. The recommended AIN-93 dietary level is 7 mg pyridoxine HCl/kg diet. After injections of azoxymethane, an alkylating chemical carcinogen, 7, 14, and 35 mg pyridoxine HCl/kg diets significantly suppressed the incidence and number of colon tumor, colonic cell proliferation and expression of c-myc and c-fos proteins in a dose dependent manner. Using the same animal model, Komatsu

et al. (2002) then provided observations which suggest that the preventive effect of vitamin B6 against colon tumorigenesis is, in part, mediated by reducing oxidative stress and nitric oxide production. Most recently similar results were reproduced by the same group to determine the high-fat diet effect, indicating that dietary supplementation of vitamin B6 reduces cell proliferation from the early stage of colonic carcinogenesis and this inhibitory effect is markedly enhanced by a high-fat diet (Komatsu et al. 2005).

Shimada et al. (2005) also found that high dietary pyridoxine (35 mg pyridoxine HCl/kg diet) reduced tumor incidence compared with low dietary pyridoxine (7 mg pyridoxine HCl/kg diet) in the chemical carcinogen model of mammary tumor. The number of proliferating cells was also significantly reduced by high dietary pyridoxine.

Animal studies also suggested that tumor tissue might have a different vitamin B6 metabolism compared with normal tissue. In an animal model using highly differentiated transplantable Morris hepatomas and Buffalo strain rats, inoculated tumors in each hind leg were apparently incapable of the complete synthesis of co-enzymatically active PLP from inactive precursor forms, indicating that the metabolism of vitamin B6 was markedly different in the hepatomas than control livers (Thanassi et al. 1981). In the same animal model, compared with pair-fed controls the growth of inoculated Morris hepatomas was significantly decreased when inoculated at 31 days, but not 0, 7 and 15 days, after initiation of vitamin B6 deficient diet. It suggests that impairment in general animal metabolism as a result of vitamin B6 deficiency reduces the tumor growth rather than vitamin B6 deficiency by itself (Tryfiates and Morris 1976).

In an animal study BALB/c mice were fed diets containing pyridoxine at 0.2, 1.2, 7.7 or 74.3 mg/kg. After 4 weeks of diet, mice received a subcutaneous injection of herpes simplex virus type 2-transformed cells to induce tumors. Mice fed 7.7 and 74.3 mg pyridoxine had 14 and 32% less tumor volumes compared with those fed 1.2 mg pyridoxine, respectively and significant inverse associations between tumor volume and tumor PLP concentrations were also found in 1.2, 7.7, and 74.3 mg pyridoxine diet groups (Gridley et al. 1987). Interestingly, mice fed a diet with 0.2 mg pyridoxine had the lowest tumor volume.

13.2.4 Evidence from Clinical Trials

Since animal and human studies have suggested that abnormal tryptophan metabolism can cause bladder cancer, it evoked that supplementation with vitamin B6, which involves in tryptophan metabolism, might reverse the abnormality or prevent recurrences of superficial bladder cancer (Koss and Lavin 1971; Byar and Blackard 1977). In a double blind randomized clinical trial that enrolled 65 patients with biopsy confirmed transitional cell carcinoma of urinary bladder, Lamm et al. demonstrated that a high dose vitamin B6 supplementation (100 mg) with supplementation of vitamin A, C, and E plus Zinc reduced the recurrence rate of bladder tumor (Lamm et al. 1994). However, the other double blind randomized

phase III trial demonstrated no effect of supplementation with vitamin B6 alone on the recurrence of transitional cell tumor of the bladder (Newling et al. 1995). To date there is no clinical trial which indicates that vitamin B6 supplementation per se is effective for cancer prevention or reduces the recurrence of cancer.

Zhang et al. (2006) reported an association between intake of vitamin B6 and colorectal cancer risk among women enrolled in a randomized trial on aspirin and vitamin E in disease prevention. Total vitamin B6 intakes were not significantly associated with the risk of colorectal cancer. However, dietary intakes of vitamin B6 were significantly inversely associated with the risk of colorectal cancer among women who were not taking supplements containing vitamin B6. Lim et al. (2005) also reported that total vitamin intake (food plus supplement) is not associated with the risk of non-Hodgkin's lymphoma, while the risk of this cancer is inversely associated with dietary vitamin B6. These observations are consistent with a folate supplementation trial, which demonstrates that the risk of lung cancer is inversely associated with dietary folate but not with intake of fortified folate, supplemented folate, or total folate intake. These observations collectively suggest that supplementation with vitamin B6 in a single synthetic chemical form might not be as effective as high vitamin B6 diet, which contains different forms of vitamin B6 and other nutrients needed for vitamin B6 metabolism.

13.3 Candidate Mechanisms

In 1985 Prior (1985) proposed a theoretical involvement of vitamin B6 in tumor initiation, hypothesizing that vitamin B6 deficiency occurring at the same time as contact with carcinogen could well lead to tumor initiation and subsequent cancer development, because vitamin B6 is a co-enzyme involved in the biosynthesis of thymidine, deficiency of which has been reported to increase replication errors and hence increase mutagenesis. Thereafter, several candidate mechanisms have been suggested but the exact mechanism by which vitamin B6 modulates carcinogenesis is not yet known (Table 13.1).

13.3.1 Antioxidant Defense Mechanism

DNA is continuously exposed to various endogenous and exogenous mutagens. Among them, oxidation is one of the most common vulnerabilities to genetic stability and oxidative DNA damage that have been suggested as an important mechanism in carcinogenesis. By the same token reduced cancer incidence has been found in populations with high dietary antioxidant intake (Poulsen 2005).

In a diabetes cell culture model pyridoxine and pyridoxamine inhibited oxygen radical production (Jain and Lim 2001) and in a rodent study vitamin B6 deficiency caused a decrease in antioxidant defense system and an increase in oxidant stress in liver tissue (Taysi 2005). Moreover, in a chemical carcinogene model of colon cancer Komatsu et al. (2002) demonstrated that vitamin B6 supplementation

Table 13.1 Candidate mechanisms by which vitamin B-6 modulate carcinogenesis

Mechanisms		References
Antioxidant defense mechanism	Inhibition of oxygen radical production	Komatsu et al. (2002), Jain and Lim (2001), and Taysi (2005)
Anti-inflammatory effect	Nitric oxide production	Komatsu et al. (2002), Kojima et al. (1999), Jaiswal et al. (2001), and Yanaka et al. (2005)
Cell proliferation	Expression of *c-myc* and *c-fos*	DiSorbo et al. (1985), Komatsu et al. (2001), Shultz et al. (1988), and Oka (2001)
Angiogenesis	Inhibition of microvessel outgrowth	Matsubara et al. (2001, 2004)
One-carbon metabolism	DNA methylation and synthesis	Le Marchand et al. (2002), Slattery et al. (1999), Ulrich et al. (1999), Shi et al. (2005), and Lim et al. (2007)
Immune function	Humoral and cell-mediated immune responses	Rall and Meydani (1993), Cheng et al. (2006), and Gebhard et al. (1990)
Regulation of steroid hormone	Estrogen-induced gene expression	Allgood and Cidlowski (1992)
Niacin deficiency	DNA repair and genomic stability	Shibata et al. (1995), Kirkland (2003), and Ames (2001)
Inhibition of DNA replication	Replicative DNA polymerases activity	Matsubara et al. (2003) and Mizushina et al. (2003)

suppressed the oxidative stress markers, colonic 8-hydroxyguanosine and 4-hydroxy-2-nonenal, compared with low vitamin B6 diet as well as these two markers were significantly associated with the expression of c-myc and c-fos, indicating that reduced oxidative stress by vitamin B6 supplementation might be in part involved in the mechanism of its preventive effect on tumorigenesis.

13.3.2 Anti-inflammatory Effect

Nitric oxide (NO), which is produced by various cell types such as macrophages, endothelial cells, and certain cancer cells, has been thought to play a role in carcinogenesis (Kojima et al. 1999). NO production is dependent on NO synthase and increased expression of inducible NO synthase (iNOS) has been reported in several types of tumors originated from head and neck, brain, breast, and colon. Upregulation of iNOS activity, which is associated with production of large amounts of NO that can cause tissue damage, is a cardinal feature of inflamed tissues as well as a plausible link between inflammation and cancer initiation, progression, and promotion (Jaiswal et al. 2001). Early on Komatsu et al. (2002) demonstrated that high dietary vitamin B6 significantly reduces the expression of colonic iNOS protein in a chemical carcinogen model, suggesting that the lower NO production by vitamin B6 might have partial responsibility for the reduced tumorigenesis by vitamin B6 supplementation. Most recently in a cultured cell model Yanaka et al. (2005)

demonstrated that pretreatment of RAW cells with vitamin B6 (pyridoxal) inhibits not only lipopolysaccharide-induced expression of iNOS and COX-2 at the mRNA and protein levels but also nuclear translocation of the nuclear factor-kappaB, the proinflammatory transcription factor. It suggests that the anti-inflammatory effect of vitamin B6 is also mediated by suppression of nuclear factor-kappaB activation.

13.3.3 Cell Proliferation

In a human malignant melanoma cell line Shultz et al. (1988) found that pharmacologic concentrations of pyridoxal (0.25–0.5 mM) resulted in significant reductions in cell proliferation, even though physiologic level of pyridoxal (0.005 mM) did not retard cell growth and pyridoxine rather stimulated the growth. On the other hand, the growth of human HepG2 cells was completely inhibited in medium supplemented with pyridoxine in the millimolar range. The growth inhibition of HepG2 cells was accompanied by a marked inhibition of gene expression, protein synthesis, and secretion of serum proteins, particularly albumin. The expression of oncogenes, such as c-fos and c-myc, was considerably reduced in the tumor cells, probably by a similar mechanism to the inhibition of albumin gene expression by vitamin B6 (Oka 2001). Antiproliferative effect of vitamin B6 on cultured cells is also described at the Section 13.2.2.

DiSorbo et al. (1985) reported that mice pretreated with pyridoxal and then injected with B16 melanoma cells had a 62% reduction in tumor weight. Even when tumors were first established in mice and then treated with pyridoxal, a 39% reduction in tumor growth was observed. In a chemical carcinogen model of colon cancer Komatsu et al. (2001) reported that vitamin B6 supplemented diets suppressed tumorigenesis by reducing cell proliferation compared with a low vitamin B6 diet. Interestingly, cell proliferation in all of the colonic epithelium was significantly correlated with the number of tumors and expression of c-myc and c-fos proteins in the colonic crypts were significantly reduced by supplemented vitamin B6. This observation suggests that suppression of cell proliferation by vitamin B6 might be conveyed through reduced expression of cell cycle control genes.

13.3.4 Angiogenesis

In an ex vivo serum-free matrix culture model using rat aortic ring, Matsubara et al. (2001) found that high concentrations of PLP (2.5 and 5 mM) and pyridoxine (5 mM) completely inhibited microvessel outgrowth. Inhibition of PLP on microvessel outgrowth was in a dose-dependent manner within the range of 25–500 μM. These results suggested that the anti-tumor effect of high levels of vitamin B6 might be mediated through suppression of angiogenesis. Most recently same group reported that vitamin B6 suppresses endothelial progenitor cells in circulation, which is associated with tumor induced angiogenesis (Matsubara et al. 2004). Further studies are needed to clarify whether vitamin B6 suppresses carcinogenesis by reducing angiogenesis.

13.3.5 One-Carbon Metabolism

One-carbon metabolism is a network of interrelated biochemical reactions which regulate transfer of the one-carbon moiety into biochemical pathways essential for DNA synthesis (thymidylate and purine synthesis) and biological methylation including DNA methylation (Fig. 13.1) (MacKenzie 1984). Serine hydroxymethyltransferase, a PLP containing enzyme, catalyzes the reversible transfer of methyl group from serine to tetrahydrofolate (THF) to generate glycine and 5,10 methyleneTHF, and this reaction plays a central role in one-carbon metabolism for nucleotide synthesis and remethylation of homocysteine (Shane 1989; Townsend et al. 2004). Homocysteine, a metabolite of one-carbon metabolism, also condenses with serine to form cystathionine in an irreversible reaction catalyzed by a PLP-containing enzyme, cystathionine-β-synthase (transsulfuration) (Craig 2004). Cystathionine is then converted to cysteine, a precursor of glutathione and taurine, catalyzed by another PLP containing enzyme, cystathionine-γ-lyase.

Animal studies demonstrated that vitamin B6 deficiency reduces activities of hepatic serine hydroxymethyltransferase and cystathionine-β-synthase (Martinez et al. 2000), while high dietary vitamin B6 enhances hepatic serine hydroxymethyltransferase enzyme activity (Scheer et al. 2005). Human studies have demonstrated that vitamin B6 deficiency causes a postprandial hyperhomocysteinemia, especially after oral methionine loading, by retarding transsulfuration pathway (Bleie et al. 2004). Taken together, vitamin B6 depletion substantially affects one-carbon metabolism that controls both nucleotide synthesis and DNA methylation, alterations of which have been regarded as molecular mechanisms for colonic carcinogenesis.

Evidence suggests that vitamin B6 modulates colorectal carcinogenesis through one-carbon metabolism. As described at the Section 13.2.1.1 recent epidemiologic

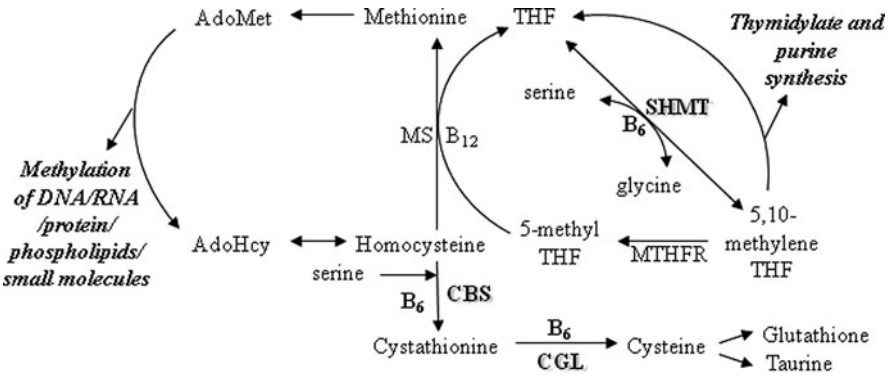

Fig. 13.1 Vitamin B6 in one-carbon metabolism. SHMT: serine hydroxymethyltransferase, CBS: cystathionine-β-synthase, CGL: cystathionine-γ- lyase, MTHFR: methylenetetrahydrofolate reductase, MS: methionine synthase, AdoMet: S-adenosylmethionine, AdoHcy: S-adenosylhomocysteine, THF: tetrahydrofolate

studies have demonstrated that an interaction between vitamin B6 and MTHFR gene can affect the risk of colorectal cancer, revealing the importance of one-carbon metabolism in the vitamin B6 associated carcinogenesis.

13.3.6 Other Mechanisms

13.3.6.1 Immune Function

Even though there is no direct evidence, it emerges that vitamin B6 supplementation might reduce cancer risk by enhancing immune function, because in animal and human studies vitamin B6 deficiency altered both humoral and cell-mediated immune responses (Rall and Meydani 1993) and vitamin B6 supplementation increased total lymphocyte count, T-helper and T-suppressor cell numbers, and the percentage of T-lymphocyte cells and T-suppressors in critically ill patients (Cheng et al. 2006). In an athymic nude mouse model injected with human malignant melanoma xenografts, Gebhard et al. (1990) found that tumor-bearing mice fed a diet containing 61.6 mg pyridoxine/kg had significantly greater lipopolysaccharide-induced spleen cell proliferation compared with mice fed the control diet containing pyridoxine 4.1 mg/kg. However, tumor incidence and progression was not modified by a high level of dietary vitamin B6.

13.3.6.2 Regulation of Steroid Hormone

Vitamin B6 is known to modulate transcriptional activation by multiple members of the steroid hormone receptor super family, which play a critical role in the development of breast cancer. In a cultured cell study estrogen-induced gene expression was reduced by 30% under conditions of elevated intracellular vitamin B6 and enhanced by 85% in vitamin deficiency (Allgood and Cidlowski 1992).

13.3.6.3 Niacin Deficiency

Niacin is largely obtained from the diet but a certain portion is manufactured from tryptophan. Since vitamin B6 involves in this pathway, vitamin B6 deficiency reduces the conversion from tryptophan to niacin (Shibata et al. 1995) and thereby can induce niacin deficiency, which has a potential to influence DNA repair, genomic stability (single- and double-strand breaks, oxidative lesions, or both), and the immune system, eventually having an impact on cancer risk (Kirkland 2003; Ames 2001).

13.3.6.4 Inhibition of DNA Replication

Vitamin B6 has a physiologic role in maintaining the proper structure of the chromosome by controlling the activities of replicative DNA polymerase and DNA topoisomerases I and II (Matsubara et al. 2003). In a cultured cell study PLP

inhibited eukaryotic DNA polymerases, DNA polymerase α and ε, and cell proliferation (Mizushina et al. 2003). These effects may relate to the anti-cancer effect of vitamin B6.

13.4 Clinical Implication

In contrast to case-control or prospective cohort studies, most of intervention trials with individual B-vitamins or other nutrients have frequently failed to reach the significant chemopreventive effect, lending that an isolated nutrient in pill form may not have the same biologic effect as a nutrient in the food. The most plausible explanation is that food sources have more physiologic forms as well as other nutrients which can add synergistic or stabilizing effects. It also seems that supplementation with a high-dose single form of vitamin B6 might alter the normal metabolism of other nutrients or target enzyme reactions, because; (1) oral pyridoxine supplementation with 120 mg/day for 5 weeks reduced serum folate concentration as well as total plasma homocysteine concentration in healthy subjects (Mansoor et al. 1999), (2) old studies suggested that supplementation with PLP can suppress thymidylate synthesis which is critical for DNA replication and DNA repair (Huang et al. 1992; Chen et al. 1989). In this regard vitamin B6 supplementation with an isolate form such as pyridoxine might not be sufficient enough to meet the goal of chemoprevention without co-supplementation of substrates for the target enzyme or other nutrients for its metabolism. To clarify whether vitamin B6 supplementation is clinically useful for cancer chemoprevention, it needs well-designed clinical trials considering the optimal dose, timing, and supplement formula as well as subjects' age and gene polymorphisms.

13.5 Conclusion and Future Perspectives

A body of evidence suggests that low dietary vitamin B6 increases and high dietary vitamin B6 reduces the risk of cancer. However, epidemiologic observations are not always consistent and despite decades of studies the exact mechanism(s) by which vitamin B6 conveys the modulation effect on carcinogenesis is not yet known. Since vitamin B6 involves in more than 100 biological enzyme reactions and thereby directly or indirectly affects multiple cellular and molecular pathways implicated in carcinogenesis, it appears that vitamin B6 influences carcinogenesis in a complex fashion. Studies for nutrient and gene interactions in cancer also suggest that common gene polymorphisms modulate the effect of vitamin B6 on carcinogenesis.

Even though current intervention trials do not support the clinical use of vitamin B6 supplementation for cancer chemoprevention, this vitamin still has a high potential to prevent or delay the onset of cancer because many evidences suggest that high dietary vitamin B6 reduces the risk of cancer. Future studies are needed to find the concrete evidence and to define the exact mechanism for vitamin B6 associated carcinogenesis.

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Chapter 14
Vitamin B6 and Cardiovascular Disease

Simonetta Friso, V. Lotto, R. Corrocher, and Sang Woon Choi

Abstract While overt vitamin B6 deficiency is not a frequent finding nowadays in medical practice, evidence suggests that insufficiency of this vitamin is rather widespread in a quite large portion of the population such as the elderly or in not unusual conditions such as that of alcohol addiction. Moreover, a mild deficiency in B6 vitamin is a state that may be associated with an increased risk of cardiovascular disease. Epidemiologic evidence from case control and prospective studies have suggested that low dietary intake or reduced blood concentrations of vitamin B6 is associated with an increased risk of cardiovascular disease, although most recent trials demonstrated the ineffectiveness of vitamin B6 supplementation on the prevention of cardiovascular events recurrence. Due to limited and somewhat inconsistent data together with the ample variety of critical functions in which vitamin B6 is involved in the human body, it is very challenging to attempt at establishing a cause and effect relationship between vitamin B6 and risk of cardiovascular disease as it is to delineate the exact mechanism(s) by which vitamin B6 may modulate such risk. In the present chapter we review the currently available knowledge deriving from both epidemiological and mechanistic studies designed to define potential candidate mechanisms for the association of vitamin B6 impairment and risk of cardiovascular disease development.

Keywords Vitamin B6 · Coronary artery disease · Stroke peripheral Vascular disease · Atherosclerosis · Supplementation · One-carbon metabolism

Abbreviations

CAD	coronary artery disease
CHD	coronary heart disease
CI	confidence interval
MI	myocardial infarction

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OR odds ratio
PLP pyridoxal-5′-phosphate

14.1 Introduction

Following the observation of the presence of atherosclerotic lesions in monkeys fed a pyridoxine-deficient diet (Rinehart and Greenberg 1949; Rinehart and Greenberg 1956), the hypothesis regarding the role of vitamin B6 in atherogenesis has been tested by several subsequent human studies which addressed the significance of this vitamin as an independent risk factor for atherothrombotic disease (Chasan-Taber et al. 1996; Dalery et al. 1995; Folsom et al. 1998; Friso et al. 2004, Kok et al. 1989, Rimm et al. 1998; Robinson et al. 1996, 1998; Verhoef et al. 1996). Recent reports also highlighted the possible relationship of vitamin B6 with cerebrovascular disease and, more specifically, with ischemic stroke and transient ischemic attack (Kelly et al. 2003, 2004).

Vitamin B6 is a coenzyme necessary for a large number of enzymes which are mainly involved in amino acid metabolism but also lipid and carbohydrate metabolic pathways (Shane 2000).

Because vitamin B6 is one of the major determinants of total plasma homocysteine concentrations (Dalery et al. 1995; Miller et al. 1992; Selhub et al. 1993, 1995), a well-known predictor for atherosclerotic disease (Eikelboom et al. 1999; Refsum and Ueland 1998; Stanger et al. 2003), the role of vitamin B6 in coronary artery disease (CAD) risk has been mostly addressed through its coenzymatic function for the degradation of homocystiene via the transsulfuration pathway in one-carbon metabolism (Fig. 14.1) (Selhub and Miller 1992). Measurements of total plasma homocysteine were not available in a number of the early studies which claimed a correlation of vitamin B6 with CAD (Kok et al. 1989; Labadarios et al. 1987; Serfontein et al. 1985; Vermaak et al. 1986, 1987). Therefore, the association of low plasma pyridoxal-5′-phosphate (PLP, the active form of vitamin B6) and coronary artery disease was not adjusted for this factor. However, most recent studies, in which measurement of total plasma homocysteine concentration was available, have clearly demonstrated that the association between vitamin B6 and the risk of CAD is independent from the homocysteine pathway at least for a significant part (Folsom et al. 1998; Friso et al. 2004; Robinson et al. 1995).

The magnitude of the association between low plasma vitamin B6 and CAD is rather mild in most studies (Folsom et al. 1998; Friso et al. 2004; Robinson et al. 1995), although this is not surprising but, rather, it is absolutely compatible with the multifactorial pathogenesis of atherosclerosis, in which there is an interplay of several genetic, nutritional and life style-related factors. Atherosclerosis is a complex disease that represents the final outcome of the interaction of many different contributory agents. Furthermore, the penetrance of different risk factors is variable in each individual. This idea of risk factor is important because it provides the concept of individually-tailored definition of risk and may also help to design specific and more effective intervention strategies.

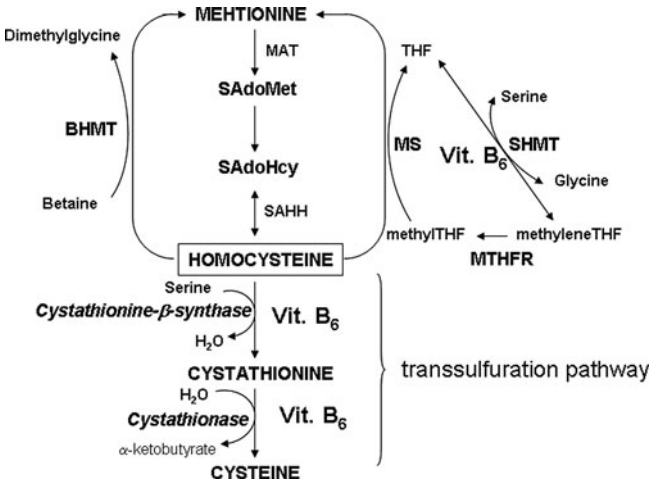

Fig. 14.1 A schematic representation of one-carbon metabolism in which vitamin B6 acts as a major player for its functions as a coenzyme in a number of enzymatic reactions. In particular, in the transsulfuration pathway, vitamin B6 acts as a coenzyme of cystathionine-β-synthase for the conversion of homocysteine to cystathionine and of cystathionase for the synthesis of cysteine from cystathionine. Vitamin B6 functions as a coenzyme for SHMT (serine hydroxymethyltransferase) which catalyzes the conversion of tetrahysdrofolate (THF) to methylenetetrahydrofolate (MTHF). Other abbreviations: MTHFR: methylenetetrahydrofolate reductase; BHMT: betaine hydroxymethyltransferase; MS: methionine synthase; SAdoMet. S-adenosylmethionine; SAdoHcy: S-adenosylhomocysteine; MAT: methionine adenosyl transferase; SAHH: S-adenosylhomocysteine hydrolase

The mechanisms for a vitamin B6-related atherogenesis are still poorly understood. Nevertheless, considering the epidemiologic evidence and understanding some of the potentially implicated mechanisms for the relationship between low vitamin B6 and atherosclerotic disease may facilitate, by correcting the vitamin status, the design of potentially easy-to-perform preventive and therapeutic measures.

In the present chapter, we address the currently available knowledge concerning epidemiologic evidence and propose candidate mechanisms for the association between vitamin B6 and cardiovascular disease risk.

14.2 Epidemiologic Evidence for Vitamin B6 as a Risk Factor for Cardiovascular Disease

14.2.1 Vitamin B6 and Coronary Artery Disease

Several studies report an association between low plasma vitamin B6 and higher risk of coronary artery disease (CAD) (Table 14.1) as well as its major thrombotic complication, myocardial infarction (Dalery et al. 1995; Folsom et al. 1998; Friso

Table 14.1 Epidemiologic studies which evaluated low vitamin B6 as a risk factor for cardiovascular disease

Author and References	Study design	Main findings
Kok FJ et al., Am J Cardiol 1989, Rotterdam, The Netherlands	Case-control	Multivariate adjusted relative odds of MI for subjects in the lowest plasma PLP quartile (< 20 nmol/L) – 5.2 (95% CI 1.4–18.9)
Dalery K et al., Am J Cardiol 1995, Québec, Canada	Case-control	Lower PLP levels in CAD (angiographically documented) than in controls ($P < 0.005$)
Robinson K et al., Circulation 1995, Cleveland, OH, USA	Case-control	In patients with low vitamin B6, OR for CAD adjusted for multiple risk factors including tHcy – 4.3 (95% CI 1.1–16.9, $P < 0.05$)
Chasan-Taber L et al., J Am Coll Nutr 1996, Boston, MA, USA	Case-control	RR of MI for lowest 20th percentile of PLP (< 28.9 nmol/L) in a multivariate model – 1.5 (95% CI 1.0–2.2); if adjusted for folate: RR 1.3 (95% CI 0.9–2.1)
Verhoef P et al., Am J Epidemiol 1996, Boston Area Health Study; Boston, MA, USA	Case-control	Dietary and plasma vitamin B6 lower in cases than controls. OR for the highest quintile of plasma PLP (>88.9 nmol/L) – 0.32 (0.11–0.59) when age and sex adjusted; 0.51 (0.19–1.36) after multiple adjustments
Verhoef P et al., Arterioscler Thromb Vasc Biol 1997, Rotterdam, The Netherlands	Case-control	CAD patients (angiographically documented) had plasma PLP lower than controls but no statistical significance ($P = 0.08$)
Folsom AR et al., Circulation 1998, ARIC Study, Minneapolis, MN, USA	Prospective case-cohort	RR between extreme quintiles of PLP – 0.28 (95% CI 0.1–0.7, $P = 0.001$)
Robinson K et al., Circulation 1998, European COMAC Group	Case-control	PLP< 23.3 nmol/L (20th percentile) associated to increase risk for atherosclerosis, independently of tHcy. (CAD + PAD + CVD: clinic and/or angiography documented)
Siri PW et al., J Am Coll Nutr 1998, Rotterdam, The Netherlands	Case-control	OR for low vitamin B6 – 0.86 (95% CI 0.33–2.22) (patients with severe angiography documented CAD)
Rimm EB et al., JAMA 1998, NHS, Boston, MA, USA	Prospective case-cohort	RR for vitamin B6 intake between extreme quintiles, adjusted for CVD risk factors – 0.67 (95% CI 0.53–0.85, $P = 0.002$). Reduced CAD risk among women who regularly used multiple vitamins (RR = 0.76, 95% CI 0.65–0.90)

Table 14.1 (continued)

Nahlawi M, Am J Cardiol 2002, Cleveland, OH, USA	Follow-up	Vitamin B6 deficiency in 21% of heart transplant recipients with -, and in 9% without CVD complications/death ($P = 0.05$). RR in low vitamin B6 ($\leq$20 nmol/L) for CVD events, including death, increased 2.7 times (95% CI 1.2–5.9, $P = 0.02$)
Friso S et al., Am J Clin Nutr 2004, Verona, Italy	Case-control	Significant, inverse, graded relation between PLP and both hs-CRP and fibrinogen ($P < 0.001$). OR for CAD-risk related to low PLP concentration: 1.89 (95% CI 1.18–3.03; $P = 0.008$)
Tavani A, Eur J Nutr 2004, Milan, Italy	Case-control	OR for acute MI risk for vitamin B6 intake – 0.34 (95% CI 0.19–0.60) for the highest versus the lowest tertile
Folsom AR et al., Arch Int Med 2006	Prospective case-cohort	Together with other markers of risk, also vitamin B6 added the most to the AUC (range 0.006–0.011)

MI myocardial infarction; *tHcy* total plasma homocysteine concentrations; *CVD* cardiovascular disease; *PLP* plasma-5′-phosphate; *CAD* coronary artery disease; *PAD* peripheral artery disease; *OR* odds ratio; *RR* relative risk; *AUC* area under the ROC (receiver operating characteristic) curve

et al. 2004; Kok et al. 1989; Labadarios et al. 1987; Nahlawi et al. 2002; Robinson et al. 1995, 1998; Serfontein et al. 1985; Siri et al. 1998; Vermaak et al. 1987). Among the earlier clinical studies conducted on patients affected by CAD, Dalery et al. described in a case-control study conducted in the province of Québec, Canada, that pyridoxal-5′-phosphate (PLP) levels, the active form of vitamin B6, were significantly lower in CAD subjects (150 angiographically documented CAD patients) than controls (584 healthy subjects) (Dalery et al. 1995). In the same year, Robinson et al. reported that plasma concentrations of PLP were lower in CAD patients compared to controls (Robinson et al. 1995). That study was initially designed to evaluate the interrelations between plasma homocysteine, a known risk factor for CAD, and vitamins of the B group which are the major determinants of plasma homocysteine levels. Interestingly, also when plasma levels of homocysteine were included in the multivariate analysis model, an odds ratio of 4.3 persisted for those subjects with low PLP (95% C.I. 1.1–13.7, $P = 0.04$). Robinson et al. concluded that low PLP is a risk factor for CAD even independently of plasma homocysteine levels (Robinson et al. 1995). In this study in which 304 patients with CAD identified by angiographic documentation were compared to 231 controls, the authors reported that vitamin B6 deficiency, defined for PLP levels lower that 20 nmol/L, was seen in about 10% of CAD patients compared to only 2% of controls subjects ($P < 0.01$) and yielded an odds ratio of coronary artery disease of 3.8 (C.I. 1.1–13.7, P=0.04) even after adjustments for all traditional risk factors for cardiovascular disease.

Additional prospective evidence on the role of vitamin B6 in the increase incidence of coronary heart disease (CHD) was then provided from findings of a nested case-cohort design within the ARIC (The Atherosclerosis Risk in Communities) study. In an analysis primarily intended to determine whether homocysteine-related factors including B6 vitamin are associated with the incidence of CHD over an average of 3.3 years of follow-up, the authors point quite strongly to an independent role of vitamin B6 as a protective factor in case of adequate levels of the vitamin (Folsom et al. 1998). Age, -race, and sex-adjusted geometric mean levels of PLP were significantly lower in incident CHD cases ($N = 232$) compared to a reference cohort sample of 537 (of whom 10 were also CHD cases) (19.0 nmol/L vs. 31.5 nmol/L; $P < 0.01$). The incidence of CHD was negatively associated with PLP in both genders ($P < 0.05$). The relative risk for the highest versus lowest quintile of plasma PLP was 0.28 (95% CI = 0.1–0.7; $P = 0.001$). Remarkably, PLP was associated with CHD incidence even after multiple adjustments for other traditional risk-factors including total and HDL cholesterol, hypertension, diabetes and smoking status.

In a multicenter case-control study in Europe, 750 patients with documented vascular disease and 800 controls frequency-matched for age and sex were compared to identify that concentrations of vitamin B6 were significantly lower in case than in control subjects (Robinson et al. 1998). Concentrations of plasma PLP below the lowest 20th percentile for control subjects (corresponding to plasma PLP < 23.3 nmol/L) was associated with an increased risk for atherosclerosis and the relationship between vitamin B6 and atherosclerosis was independent of traditional risk factors including plasma total homocysteine levels (Robinson et al. 1998). The risk associated with low vitamin B6 status (odds ratio 1.84; 95% C.I. 1.39–2.42, $P < 0.001$) persisted when adjusted for the concentrations of fasting (1.76; 95% C.I. 1.33–2.34, $P < 0.001$) as well as post-load total plasma homocysteine concentrations (1.79; 95% C.I. 1.35–2.37, $P < 0.001$).

One of the main issues to be considered in the hypothesis of an independent relationship between low PLP and coronary atherosclerotic disease, pertains to the uncertainty on the possible mechanisms involved in such an association. The role of potential candidate mechanisms for a vitamin B6 related atherogenesis have been investigate through cell culture and animal model studies as described below in the present chapter. However, based on the hypothesis of a possible role of vitamin B6 in inflammatory processes some mechanistic hypothesis for the link between low vitamin B6 and coronary heart disease was also done using clinical studies. Indeed, a growing body of evidence supports the theory of atherosclerosis as an inflammatory disease (Libby et al. 2002; Pasceri et al. 2000; Ross 1999). Systemic markers of inflammation, such as fibrinogen and high-sensitivity C-reactive protein have been proposed to be solid predictors of CAD risk (Liuzzo et al. 1994; Ridker et al. 2000). The current understanding is that inflammation plays an essential role at all stages of the atherosclerotic process (Ross 1999) and acute-phase reactants have been proven to be strong and independent risk factors for CAD (Grundy et al. 1999; Harris et al. 2006; Ridker et al. 2004). In patients with rheumatoid arthritis, a disease that share many similarities with coronary atherosclerosis, plasma PLP levels are inversely related to tumor necrosis factor alpha production (Roubenoff et al.

1995) and other major markers of inflammation (Chiang et al. 2003). Most recently, a cross-sectional survey observed also an inverse association between levels of vitamin B6 and plasma fibrinogen concentrations (James et al. 2000; Ji et al. 2003).

Data from 891 participants of the population-based Framingham Heart Study cohort were analyzed precisely to explore a possible relationship between vitamin B6 and a major marker of acute phase. This study highlighted an inverse correlation between plasma PLP and values of C-reactive protein (Friso et al. 2001). This association appeared to be strong and independent of other biomarkers related to vitamin B6 metabolism and suggested a possible function of plasma PLP in inflammatory processes (Friso et al. 2001).

Taking into account that systemic markers of inflammation are solid predictors of CAD risk, a subsequent study was designed to investigate a cohort of angiography-defined severe multivessel CAD patients ($N = 475$) compared to 267 subjects free from coronary atherosclerosis with the primary aim of evaluating the relation between plasma PLP and major markers of acute-phase reaction in affecting CAD risk (Friso et al. 2004). That study conducted in an Italian population confirmed the inverse relationship between plasma PLP and both hs-CRP and fibrinogen ($P < 0.001$). The prevalence of low PLP (considered as < 50th percentile in control population, i.e. 36.3 nmol/L) was significantly higher among CAD patients compared to controls ($P < 0.001$). That levels of plasma PLP could be referred to a mild PLP impairment even compared to previous studies which defined a risk for CAD for PLP values as low as 20 nmol/L (Robinson et al. 1995). Yet such moderate impairment is sufficient to confer a higher risk for CAD. Furthermore, the odds ratio for CAD risk related to low PLP was 1.89 (95% CI: 1.18–3.03, $P = 0.008$) even in a multivariate model in which hs-CRP, fibrinogen, total plasma homocysteine as well as all major CAD risk factors were included. However, it is to be noted that the magnitude of such association is quite bland but this is not surprising if one considers that atherosclerosis is a very complex disease in which a number of genetic, environmental and life-style factors are involved. Yet, it is interesting to note that low plasma PLP concentrations when considered in combination with hs-CRP or with an increased LDL to HDL-cholesterol ratio, significantly enhanced the risk of disease (Friso et al. 2004).

The association of low PLP and CAD, however, has not been confirmed in all studies. In a study designed to evaluate the role of plasma total homocysteine and B vitamins in the risk of coronary atherosclerotic disease, Verhoef et al. (1997) observed that cases had slightly lower PLP levels compared to controls but this difference was not statistically significant (26.9 vs. 27 nmol/L, $P = 0.08$). It is to be noted that population control subjects had lower mean levels of plasma PLP (23.3 nmol/L). The authors considered such finding potentially related to the healthier dietary habits adopted by cases after the cardiology treatment. In such case-control study in which subjects were recruited from men and women undergoing coronary angiography in a Hospital in Rotterdam, the Netherlands, cases ($N = 131$) were defined when at least one coronary vessel had an occlusion $\geq 90\%$ and $\leq 40\%$ in a second coronary artery and controls ($N = 88$) when there was $\leq 50\%$ vessel occlusion in only one epicardial coronary vessel.

In the same case-control study, conducted in angiography defined patients, Siri et al. (1998) subsequently evaluated whether the association between vitamins of the B group and CAD is mediated by the effect of rising plasma homocysteine levels, observed that there was no difference on plasma PLP levels between cases ($N = 131$) and controls ($N = 88$). The ORs for low vitamin B6 was 1.23 (95% CI: 0.53–2.83) when age and gender adjustments were performed. However, after multiple adjustments (for age, gender, heavy smoking, diastolic blood pressure, total HDL cholesterol, alcohol intake, body mass index, serum creatinine, vitamin B12 and folate, fasting total homocysteine), the OR was estimated as 0.60 (95% CI: 0.22–1.63) in the lowest quartile (cut-off point for vitamin B6 concentrations was 20.6 nmol/L) and 0.88 (95% CI: 0.34–2.31) for the highest quartile (vitamin B6 concentrations >36.7 nmol/L). The authors, therefore, concluded that vitamin B6 was not clearly related, in their study, to higher risk of atherosclerosis.

As for a more specific association between low PLP and myocardial infarction (MI), the main thrombotic complication of CAD, after a number of early and rather heterogeneous studies that defined an association between low vitamin B6 and MI (Iqbal et al. 2005; Kok et al. 1989; Labadarios et al. 1987; Serfontein et al. 1985; Tavani et al. 2004; Vermaak et al. 1987), further support for such hypothesis was suggested from additional case-control and prospective studies.

In a study conducted among Dutch patients, Kok et al., observed lower vitamin B6 status in patients with acute MI ($N = 84$) compared to healthy control subjects ($N = 84$) (Kok et al. 1989). The multivariate adjusted relative odds of acute MI (in which homocysteine values were not included) for subjects with plasma PLP levels < 20 nmol/L (lowest quartile) was five times higher as compared with those in the highest quartile (relative odds = 5.2, 95% CI: 1.4–18.9).

Data from a nested case-control study using prospectively collected samples from the large male Physician Health Study (14,916 subjects) (Chasan-Taber et al. 1996) showed that for the lowest 20% of vitamin B6 values (PLP levels < 28.9 nmol/L), the multivariate relative risk of MI was 1.5 (95% CI: 1.0–2.2). The inclusion of homocysteine in the multivariate model attenuate only slightly the relative risk to 1.3 (95% CI: 0.8–2.1). Adding total homocysteine levels to that model did not add significant predictive value. As stated by the authors, these prospective data from US physicians, although not statistically significant were compatible with the hypothesis that lower dietary intake of vitamin B6 contribute to risk of MI (Chasan-Taber et al. 1996).

In the retrospective setting of the Boston Area Health Study, conducted among men and women at first MI event admitted to the coronary or intensive care units, Verhoef at al., observed that both dietary and plasma levels of vitamin B6 were lower in cases ($N = 130$) than population controls ($N = 118$) and that there was an inverse association with the risk of MI that was also independent of other known risk factors in a multivariate analysis adjusted for multiple confounders (Verhoef et al. 1996). For plasma PLP, the highest two quintiles (≤88.9 and >88.9 nmol/L, respectively) showed a trend toward an inverse relationship with the risk of MI even when, among other classical risk factors, also strong confounders such as total/high density lipoprotein cholesterol ratio, cigarette smoking, body mass index or family history

for diabetes, hypertension or ischemic heart disease were included in the model (OR = 0.32 for PLP levels >88.9 nmol/L). The association between pyridoxal-5′-phosphate and myocardial infarction risk was independent also of plasma folate and homocysteine, suggesting a potential protective effect of vitamin B6 through mechanisms other than those related to homocysteine metabolism.

Some authors reported early on that lower pyridoxal-5′-phosphate levels during the acute phase of myocardial infarction (Kok et al. 1989; Serfontein et al. 1985; Vermaak et al. 1986, 1987), concentrations that returned to baseline after 3–4 days (Vermaak et al. 1988). This observation may need further substantiation from other clinical and mechanistic investigation for a potential part of vitamin B6 in the acute phase of the disease.

In the prospective cohort of the Nurses' Health Study, Rimm et al. in 1998, observed that the relative risk of coronary heart disease between extreme quintiles for vitamin B6 (median intake 4.6 mg/die vs. 1.1 mg/die) was 0.67 (95% CI: 0.53–0.85, $P = 0.002$) after controlling for traditional cardiovascular risk factors including hypertension, smoking, intake of alcohol, fiber, vitamin E and saturated, polyunsaturated and trans fat (Rimm et al. 1998). During 14 years of follow-up of this large (80,082 subjects) prospective cohort study of women, Rimm et al., found graded associations between higher intakes of vitamin B6 and lower risk of CHD where the risk was lowest with higher intake of food and supplement sources combined (relative risk = 0.71; 95% CI: 0.56–0.90). Results from this population of women showed a lower risk for CHD in individuals with higher vitamin B6 levels. Remarkably, in this population of US women average intakes of vitamin B6 (3 mg/day; median = 1.7 mg/d) was well above those from national average (1.51 mg/day) but still a graded reduction in risk was detected with higher intake. The lowest risk was among women with intake of vitamin B6 above 3 mg/d. Therefore, it could be speculated that levels of vitamins that are currently defined sufficient to avoid deficiency of the vitamin among non pregnant women (current RDA 1.6 mg/day), could not be adequate for coronary heart disease prevention.

The prospective ARIC Study, in a recent evaluation, assessed the association of novel markers of risk (Folsom et al. 2006). Of the 19 markers studied, vitamin B6, together with other risk factors such as lipoprotein-associated phospholipase A2, interleukin-6 and soluble thrombomodulin added the most to the area under the receiver operating characteristic curve (Folsom et al. 2006).

In summary, vitamin B6 impairment was reported to be associated to higher risk for CAD (Robinson et al. 1995) in patients with coronary atherosclerosis established by angiographic documentation (Dalery et al. 1995; Friso et al. 2004; Robinson et al. 1995, 1998; Verhoef et al. 1996, 1997). This role for vitamin B6, which emerged as a potentially relevant risk factor for CAD, was also observed in prospective study settings (Chasan-Taber et al. 1996; Folsom et al. 1998, 2006). This association was observed to be independent of plasma homocysteine levels (Folsom et al. 1998, 2006; Friso et al. 2004; Robinson et al. 1995, 1998), a well-recognized CAD risk factor whose plasma concentrations vitamin B6 is a major determinant. The risk for CAD conferred by a mild impairment in plasma pyridoxal-5′-phosphate, the active

form of vitamin B6 is rather mild (Folsom et al. 1998, 2006; Friso et al. 2004; Robinson et al. 1995, 1998; Siri et al. 1998, Verhoef et al. 1997) as expected by the multifactorial genesis of this complex disease. Remarkably, in the view of the importance of the concomitant presence of several risk factors in determining an amplification in disease risk, the role of a modest vitamin B6 impairment may have a significant part in atherosclerosis, especially considering that, if not evaluated, it could remain unknown for the absence of the clinically manifested signs of an overt deficiency. The relationship between low PLP and CAD was found to be independent also of inflammatory indexes (Friso et al. 2001; Kelly et al. 2004) which have been reported to be related to both CAD risk and associated to vitamin B6 levels (Friso et al. 2001).

14.2.2 Vitamin B6 and Peripheral Artery Disease

Data on a relationship between vitamin B6 and peripheral artery disease are few (Wilmink et al. 2004). Daily folate and vitamin B6 intakes were found to be lower in cases than in controls and they were independent predictors of peripheral artery occlusive disease. An increase daily vitamin B6 intake by one standard deviation, decreased the risk of peripheral artery disease by 29% (Wilmink et al. 2004).

14.2.3 Vitamin B6 and Stroke

In a study evaluating the association between plasma homocysteine and extracranial carotid artery stenosis, Selhub et al. described a relationship between lower vitamin B6 levels and carotid atherosclerotic disease which diminished when adjusted for homocysteine levels (Selhub et al. 1995). Recent reports from case-control studies highlighted a possible relationship between low plasma levels of vitamin B6 with the onset of cerebrovascular disease and, more specifically, with ischemic stroke and transient ischemic attack (Kelly et al. 2003, 2004; Khatami et al. 1988). Plasma PLP levels were found to be significantly lower in cases ($N = 180$) than controls ($N = 140$) with the evidence of a strong association between stroke/TIA and low PLP (considered as < 20 nmol/L), with an OR of 4.6 (95% CI: 1.4–15.1; $P < 0.001$). This association was found to be independent of other vascular risk factors, vitamin supplement use, and also total plasma homocysteine levels. Furthermore, for the highest PLP quintile compared with the lowest, the OR was 0.1 (95% CI: 0.03–0.38; $P < 0.001$), therefore, defining a possible protective influence for higher PLP levels (Kelly et al. 2003; Khatami et al. 1988). The same case-control study was subsequently evaluated for a possible relationship among vitamin B6 status, total plasma homocysteine levels and C-reactive protein (Kelly et al. 2004). The authors observed an adjusted OR of 16.6 (95%CI: 2–139.9; $P = 0.01$) for low PLP in the highest compared with the lowest CRP quartile. In the same year, another group of researcher published a follow-up study (He et al. 2004) which evaluated intakes of

vitamin B6, folate and vitamin B12 in relation to risk of ischemic and hemorrhagic stroke. A number of 43,732 men were enrolled, free of cardiovascular disease and diabetes at baseline, and were followed for 4 years and evaluated every 4 years by a semiquantitative food frequency questionnaire. The authors documented 725 incident strokes (455 ischemic, 125 hemorrhagic and 145 of unknown types) during the follow-up of 14 years. Intakes of folate and vitamin B12, adjusted for lifestyle and dietary factors, were associated with a significantly lower risk of ischemic stroke. On the contrary, vitamin B6 was not associated with risk of ischemic stroke (P for trend, not significant) (He et al. 2004).

In another study designed to assess the association of dietary folate, vitamin B6, and vitamin B12 intake with cardiovascular mortality using data from the Spanish National Nutrition Survey, vitamin B6 failed to obtain a definitive result in regard of a possible association of vitamin B6 impairment with cardiovascular mortality, although evinced a limited protective effect that reached statistical significance only with respect to cerebrovascular mortality in men, after multiple adjustments for age, income, total energy and established risk factors (Medrano et al. 2000).

14.3 Candidate Mechanisms for a Vitamin B6 Related Atherogenesis

Since vitamin B6 is an easily available vitamin from both natural and fortified foods, an overt vitamin B6 deficiency is currently a rare condition that is usually secondary to severe malnutrition, alcoholism, or pharmacological therapy such as that with isoniazide (Snider 1980) which may induce a vitamin B6 impairment. Nevertheless, a marginal or frank inadequate vitamin B6 status is nowadays common in the elderly. Recent epidemiologic studies have suggested that low vitamin B6 status is associated with the increased risk of cardiovascular disease but, to date, the exact mechanism is not yet known, even though many candidate mechanisms have been suggested. It appears that a cardioprotective effect of vitamin B6 might be conveyed in a complex fashion because vitamin B6 serves as a coenzyme for a large number of reactions which primarily include enzymes involved in the amino acid but also in the lipid and carbohydrate metabolic pathways.

Indeed, PLP is a coenzyme necessary for a large number of metabolic reactions, and an impaired PLP status have been related likewise to inflammation (Roubenoff et al. 1995), immune function (Meydani et al. 1992; Rall and Meydani 1993), and thrombosis (Packham et al. 1981; Schoene et al. 1986), all pivotal mechanisms throughout all stages of atherosclerotic process. The main candidate mechanisms invoked in the vitamin B6 related atherosclerosis are summarized in Table 14.2. Figure 14.2 shows a scheme for the major mechanisms potentially implicated in a vitamin B6 related atherogenesis.

The two major derivates of vitamin B6 compounds in animal tissues are the coenzyme species pyridoxamine-5′-phosphate (PMP) and pyridoxal-5′-phosphate (PLP) (Shane 2000) which represents the major coenzymatic form in plasma. PLP

Table 14.2 Candidate mechanisms for vitamin B6 related atherogenesis

Mechanism	Description	References
Homocysteine metabolism	Vitamin B6 deficiency induces hyperhomocysteinemia through inhibition of cystathionine-β-synthase	Clarke and Lewington (2002), D'Angelo and Selhub (1997), Eikelboom et al. (1999), Finkelstein (1998), McCully (1970), McCully and Ragsdale (1970), McCully and Wilson (1975), Refsum and Ueland (1998), Refsum et al. (2006), Selhub et al. (1993, 1995), Welch and Loscalzo (1998)
Coagulation pathway	Vitamin B6 affects platelet aggregation by inhibiting ADP receptors and prolong bleeding time, occupancy of glycoprotein IIb/IIIa receptor or down-regulation of glycoprotein IIb gene	Brattstrom et al. (1990), Brownlee et al. (1984), Cattaneo et al. (2001), Chang et al. (1999, 2002), Packham et al. (1981), Khatami et al. (1988), Palareti et al. (1986), Saareks et al. (2002), Schoene et al. (1986), Undas et al. (1999), van Wyk et al. (1992)
Arterial wall degeneration	Impairment of lysyl oxidase activity by vitamin B6 deficiency induces arterial wall degeneration. Vitamin B6 also inhibits endothelial cell proliferation and induce endothelial dysfunction	Bruel et al. (1998), Ji et al. (2003), Levene and Murray (1977), Matsubara et al. (2003, 2004), Murray and Levene (1977), Rodriguez et al. (2002), Woessner (1991)
Cholesterol metabolism	Inhibition of advanced glycation and lipoxidation end-products by Vitamin B6 deficiency induces hypercholesterolemia	Brattstrom et al. (1990), de Gomez Dumm et al. (2003), Degenhardt et al. (2002), Kirsten et al. (1988), Knipscheer et al. (1997), Metz et al. (2003), Onorato et al. (2000), Schuitemaker et al. (2001), Vijayammal and Kurup (1978), Yamada (1961)
Fatty acids composition	Vitamin B6 deficiency impairs conversion of linoleic acid to arachidonic acid and the metabolism of n-3 PUFA from alpha-linoleic acid to eicosapentaenoic and docosahexaenoic acid	Bordoni et al. (1998), Cabrini et al. (2001), Cunnane et al. (1984), Harris et al. (2006), Kirschman and Coniglio (1961), Tsuge et al. (2000)
Inflammation	Low plasma levels of PLP are inversely related to inflammatory biomarkers such as high-sensitivity C-reactive protein and fibrinogen	Folsom et al. (2003), Friso et al. (2001, 2004), James et al. (2000), Roubenoff et al. (1995)
Immunological function	Vitamin B6 deficiency impairs T lymphocyte and macrophage differentiation and interleukins production	Meydani et al. (1992), Rall and Meydani (1993), Roubenoff et al. (1995)

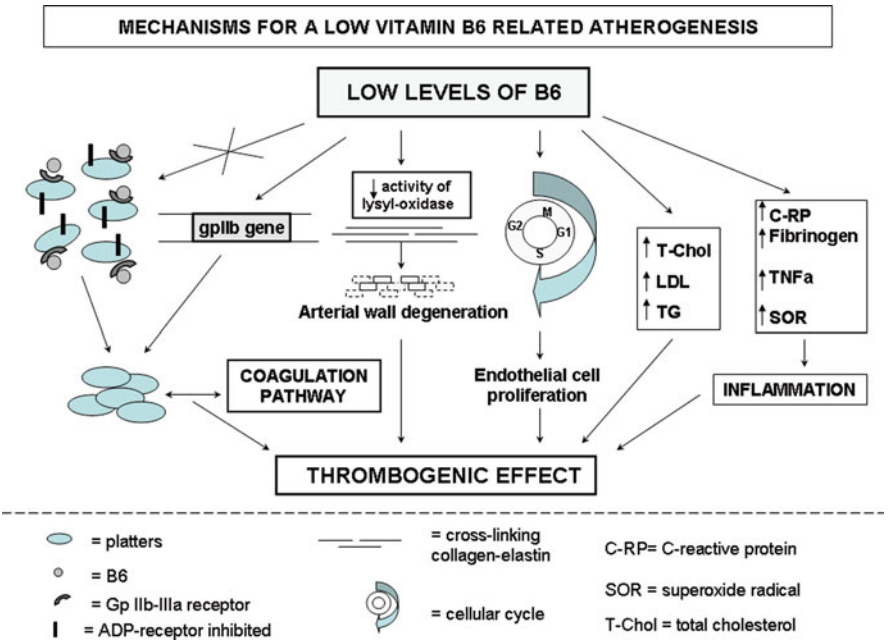

Fig. 14.2 Cartoon showing mechanisms involved in the vitamin B6 related atherogenetic process

is the metabolically active coenzyme produced by the phosphorylation of pyridoxal compound after the oxidation of other vitamin B6 vitamers in the liver. Because it reflects liver PLP levels and stores, plasma PLP has been used, both clinically and experimentally, as a sensitive indicator of tissue vitamin B6 status (Sampson and O'Connor 1989).

14.3.1 Vitamin B6 and Cholesterol Metabolism

Disturbances of cholesterol metabolism is a major risk factor for CAD and it has been suggested that low plasma concentrations of the active vitamin B6 form, PLP, may increase the risk of CAD by affecting cholesterol metabolism. An advanced degree of hypercholesterolemia has been reported in a vitamin B6 deficient animal model, while vitamin B6 supplementation has been shown to decrease plasma cholesterol concentrations (Vijayammal and Kurup 1978) and to protect against the development of atherosclerotic lesions (Yamada 1961). Pyridoxine treatment has also been observed to reduce plasma total cholesterol and low-density lipoprotein cholesterol in atherosclerotic patients (Brattstrom et al. 1990). In patients with chronic renal failure, treatment with pyridoxine induced a significant decrease in total cholesterol and triglycerides levels (de Gomez Dumm et al. 2003; Kirsten et al. 1988) and this effect has been attributed, at least in part, to its inhibitory effect on

advanced glycation and lipoxidation end-products (Degenhardt et al. 2002; Metz et al. 2003; Onorato et al. 2000).

However, not all of the evidence supports the lipid lowering effect of vitamin B6. Daily supplementation with 200 mg pyridoxine hydrochloride for 28 days had no significant effect on either total cholesterol or VLDL and LDL cholesterol content among chronic hemodialysis subjects. In a double-blind, randomized, placebo-controlled study, which was conducted in patients with familial hypercholesterolaemia, magnesium-pyridoxal-5′-phosphate-glutamate (MPPG), a derivative of vitamin B6, had no effect on the lipid profile including total cholesterol level (Knipscheer et al. 1997). In a recent placebo-controlled, double-blind, randomized trial, conducted in subjects with serum cholesterol levels between 7.0 and 9.9 mmol/l, 12 months treatment of MMPG was also not effective to reduce total cholesterol level with the exception of a modest effect on reducing serum LDL cholesterol ($P = 0.04$) (Schuitemaker et al. 2001), indicating that vitamin B6 supplementation is not effective to control hypercholesterolemia.

Collectively, evidence suggests that vitamin B6 status is associated with cholesterol metabolism but to date it is not know yet how vitamin B6 affects cholesterol metabolism as well as whether the effect of vitamin B6 on CAD is indeed conveyed through cholesterol metabolism.

14.3.2 Vitamin B6 and Fatty Acids Composition

The metabolic balance among different classes of fatty acids is necessary to support optimal cardiovascular function and tissue levels of these fatty acids have been proposed as risk markers for cardiovascular disease and coronary artery disease (Harris et al. 2006). It has also been described that vitamin B6 affects lipid metabolism by modifying fatty acids composition (Kirschman and Coniglio 1961) particularly in the metabolism of essential fatty acids since vitamin B6 seems particularly involved in the conversion of linoleic acid to arachidonic acid (Cunnane et al. 1984; She et al. 1994) and to impair the metabolism of n-3 PUFA from alpha–linoleic acid to eicosapentaenoic and docosahexaenoic acid with the most significant reduction in the production of docosahexaenoic acid (Tsuge et al. 2000), therefore suggesting an impaired activity of the delta-6-desaturase activity, the enzyme which catalyzes the rate-limiting step in the conversion of linoleic acid to longer and more unsaturated metabolites (Bordoni et al. 1998; Cabrini et al. 2001).

14.3.3 Vitamin B6 and Inflammation

An alternative hypothesis is that the relationship between low PLP and increased CAD risk is due to the common relationships with inflammatory markers. Indeed, a growing body of evidence sustains the theory of inflammation as a major player underlying the pathogenesis of atherosclerosis (Libby et al. 2002; Pasceri et al. 2000; Ross 1999). The current knowledge recognizes that inflammation exerts an essential role at all stages of the atherosclerotic process (Grundy et al. 1999; Libby

et al. 2002; Ross 1999) and acute-phase reactants have been proven to be solid and independent risk factors for CAD (Grundy et al. 1999, Ridker 2001; Ridker et al. 2000). Systemic markers of inflammation such as fibrinogen and, particularly, high-sensitivity C-reactive protein (hs-CRP), have been proposed to be strong predictors of CAD risk (Grundy et al. 2004; Kannel et al. 1987; Liuzzo et al. 1994; Ridker 2001; Ridker et al. 2000). In a prospective cohort from the Nurses' Health Study and the Health Professionals Follow-up Study elevated levels of inflammatory markers, particularly CRP, indicates an increased risk of coronary heart disease (Pai et al. 2004).

It has been reported that low plasma levels of PLP were inversely related to C-reactive protein in the population-based Framingham Heart Study cohort (Friso et al. 2001) and this association appeared to be strong and independent of other major biomarkers related to vitamin B6 metabolism (Friso et al. 2001). In healthy middle-aged adults among the Atherosclerosis Risk in Communities (ARIC) Study, an inverse association of plasma PLP with white blood cell count was demonstrated, although in that study there was no association with C-reactive protein (Folsom et al. 2003). In a most recent case-control study with subjects who were characterized by angiography for severe coronary atherosclerosis or CAD-free, a significant, inverse, graded relation was observed between PLP and both hs-CRP and fibrinogen ($P < 0.001$), indicating that vitamin B6 status may affect the risk of CAD by modulating inflammatory process.

14.3.4 Vitamin B6 and Immunological Function

There is a bulk of data showing that immune mechanisms are strongly involved in atherogenesis (Binder et al. 2002). Although much remains still to be learned on whether an imbalanced immune system function may be acknowledged as the ultimate trigger behind inflammation in atherogenesis (Libby 2002), several findings have proven the impact of immune signaling system in modulating inflammatory response for the progression of atheroma (Greaves and Channon 2002; Libby and Aikawa 2002). Low concentrations of PLP have been related to alteration of immunological function, including impaired T lymphocyte and macrophage differentiation and interleukins production (Meydani et al. 1992; Rall and Meydani 1993). The inverse relationship between PLP and markers of inflammation together with the finding of an association of low PLP with higher CAD risk may be explained with a role of PLP in affecting the early steps of atherogenesis, in which recruitment of monocytes, T cells, macrophages, and other cells acting for the immune function regulation, promote the cytokine-induced mechanisms for initiating the inflammatory response (Binder et al. 2002; Libby 2002).

14.3.5 Vitamin B6 and Coagulation Pathway

Thrombosis underlies most acute manifestations of coronary atherosclerotic disease (Girelli et al. 2000) and platelet-rich thrombi that form at sites of rupture

or fissure of atherosclerotic plaque can cause myocardial infarction (Fuster et al. 1992). PLP has been reported to affect platelet aggregation by inhibiting ADP receptors (Packham et al. 1981; 94) and prolong bleeding time (van Wyk et al. 1992), the occupancy of glycoprotein IIb/IIIa receptor (Chang et al. 2002) or the down-regulation of the expression of glycoprotein IIb gene (Chang et al. 1999). Pyridoxine supplementation has been also described to increase antithrombin III activity (Brattstrom et al. 1990) possibly due to the competitive inhibitory effect of non-enzymatic glycosylation by PLP of lysine residues (Khatami et al. 1988) at the heparin-binding site of antithrombin III (Brattstrom et al. 1990; Brownlee et al. 1984). Another indirect demonstration of a potential thrombogenic effect of low vitamin B6 is the independent association of low PLP with higher risk for deep vein thrombosis (Cattaneo et al. 2001; Lussana et al. 2003).

Other recent studies have shown that treatment with vitamin B6 restores blood coagulation abnormalities in homocystinuric subjects (Palareti et al. 1986) and attenuates the formation of thrombin-antithrombin III complexes and prothrombin fragment 1+2 in subjects treated for mild hyperhomocysteinemia (Undas et al. 1999). The increase in prostacyclin production and the inhibition of thromboxane and leukotriene synthesis have been also described (Saareks et al. 2002).

14.3.6 Vitamin B6 and Arterial Wall Degeneration

Arterial wall degeneration has been proposed as a possible mechanism for cardiovascular disease. Once arterial wall degeneration is initiated, a positive feed back loop may be established whereby degeneration leads to pressure increases, ultimately leading to further vascular degeneration (Zion et al. 2003). The impaired activity of lysyl oxidase, another vitamin B6 – dependent enzyme which is involved in the cross-linking of collagen and elastin, may favor arterial wall degeneration even in conditions of moderate vitamin B6 deficiency (Levene and Murray 1977; Murray and Levene 1977). Reduced activity levels of this enzyme which is also down-regulated by LDL (Rodriguez et al. 2002) result in lower degree of elastin and/or collagen cross-linking, affecting the biochemical properties of extracellular matrixes (Bruel et al. 1998) as well as the susceptibility of collagen and elastin to degradation by proteinases and, thereby, enhances endothelial permeability (Woessner 1991). PLP has been also described to have an inhibitory effect on endothelial cell proliferation (Matsubara et al. 2003, 2004) and reverse endothelial dysfunction induced low-density lipoprotein (Ji et al. 2003).

14.3.7 Vitamin B6 and Anti-oxidant Function

Accumulating evidence suggests that increased oxidant stress, which results from an imbalance between oxidant production and antioxidant defenses, is involved in the pathogenesis of cardiovascular diseases by impairing endothelial function (Heitzer et al. 2001). Several studies have demonstrated that plasma markers of oxidative

stress are increased in coronary heart disease or in the presence of its classic risk factors (Stephens et al. 2006) and, furthermore, vitamin B6 has been shown to have antioxidant effect by inhibiting superoxide radical generation, reducing lipid peroxidation (Cabrini et al. 1998; Maranesi et al. 2004; Selvam and Devaraj 1991; Selvam and Ravichandran 1991), and by preventing damage to mitochondrial membrane integrity in a cell culture model (Kannan and Jain 2004). These observations jointly suggest that vitamin B6 may modulate the risk of cardiovascular diseases through its antioxidant effect.

14.3.8 Vitamin B6 and Homocysteine

Vitamin B6 is a cofactor for cystathionine-β-synthase and γ-cystathionase, both of which are enzymes involved in the transsulfuration pathway of homocysteine metabolism as well as for serine hydroxymethyl transferase, which catalyzes the methyl transfer for homocysteine remethylation and for nucleotide synthesis pathway (Fig. 14.1, Chapter 7). Cystathionine-β-synthase catalyzes, in an irreversible reaction, the condensation of homocysteine and serine to form cystathionine. The cystathionine is then hydrolyzed by cystathionase to form cysteine and α-ketobutyrate (Fig. 14.1). Thus, by reducing the function of cystathionine-β-synthase vitamin B6 deficiency causes accumulation of S-adenosylhomocysteine as well as export of homocysteine from the cell, leading to hyperhomocysteinemia. This biochemical change induced by vitamin B6 deficiency is quite similar to what is brought about by a well-known genetic disorder which is related to a reduced cystathionine-β-synthase activity and provokes premature atherosclerosis and thrombosis (McCully 1970; McCully and Ragsdale 1970; McCully and Wilson 1975).

Precisely because of the role of PLP as a cofactor in the transsulfuration as well as remethylation pathway of homocysteine (Selhub and Miller 1992), low plasma PLP levels represent a determinant of higher plasma total homocysteine (Selhub et al. 1993), an independent risk factor for occlusive vascular disease (Clarke and Lewington 2002; D'Angelo and Selhub 1997; Eikelboom et al. 1999; Finkelstein 1998; Refsum and Ueland 1998; Refsum et al. 2006; Stanger et al. 2003; Welch and Loscalzo 1998). Therefore, it has been suggested that the role of PLP in atherosclerosis is conveyed through its effect on total plasma homocysteine concentrations (Selhub et al. 1995).

14.4 Vitamin B6 Supplementation and Cardiovascular Disease Prevention

Epidemiologic studies conducted over the past three decades have supported the hypothesis of an association of mild hyperhomocysteinemia with an elevated risk of cardiovascular disease. Subsequently, hyperhomocysteinemia and its association

with a deficiency of B-vitamins have offered a unique target for the preventive approach with inexpensive and innocuous naturally occurring B vitamins including vitamin B6.

The most convincing data regarding vitamin B6 supplementation and cardiovascular disease prevention are from the Heart Outcomes Prevention Evaluation (HOPE) 2 Study (Lonn et al. 2006) and the Norwegian Vitamin (NORVIT) trial (Bonaa et al. 2006), both of which are prospective, randomized, double-blind, placebo-controlled multi-center trials. In the (HOPE) 2 study, daily supplementation with 50 mg of vitamin B6 in association with 2.5 mg of folic acid and 1 mg of vitamin B12 for 5 years did not reduce the risk of major cardiovascular events in patients with vascular disease (Lonn et al. 2006). In the NORVIT study daily supplementation with 40 mg of vitamin B6 was not associated with any significant benefit for the recurrent myocardial infarction, stroke, and sudden death attributed to coronary artery disease during 40 months follow up (Bonaa et al. 2006). Combined B vitamin supplementation of 40 mg of vitamin B6 with 0.8 mg folic acid and 0.4 mg of vitamin B12 even tended to be harmful (RR = 1.22, 95%CI = 1.00–1.50, $p = 0.05$). Furthermore, the Vitamins and Thrombosis (VITRO) study, a randomized, double blind, and placebo-controlled, which was designed to investigate the effect of daily supplementation of B vitamins on the secondary prevention of deep vein thrombosis and pulmonary embolism in patients with a first event of venous thrombosis, found that supplementation of 50 mg pyridoxine along with 5 mg folate and 0.4 mg vitamin B12 also did not prevent recurrent venous thrombosis (den Heijer et al. 2007). Collectively, those studies consistently demonstrated that vitamin B6 supplementation is not unequivocally effective for the prevention of recurrence of cardiovascular events including coronary artery disease, stroke, and deep vein thrombosis. Further studies specifically designed to evaluate an effect of vitamin B6 supplementation by considering specific time and dose factors may be needed.

A few small trials, a primary endpoint of which is the progression of atherosclerosis, demonstrated the effectiveness of vitamin B6 supplementation with folate and vitamin B12 on carotid intima-media thickness in hyperhomcysteineic renal transplant or patients at risk to cerebral ischemia (Marcucci et al. 2003; Till et al. 2005). However, results of two double blind, placebo-controlled, randomized trial after coronary angioplasty or stent were inconsistent. Supplementation of vitamin B6 together with folate and vitamin B12 after coronary angioplasty decreased the rate of restenosis and need for revascularization (Schnyder et al. 2001), while supplementation after coronary stenting increased the risk of in-stent restenosis and the need for target-vessel revascularization (Lange et al. 2004).

The negative result of vitamin B6 trials cannot preclude a protective effect in primary prevention as well as of more physiologic doses and forms of B vitamins. However, it could be difficult to demonstrate that vitamin B supplementation that does not work in patients who have had a clinical vascular event would work well in those without a clinical event or with subclinical atherosclerosis. In contrast to the clinical trials that determine the effect of vitamin B6 supplementation on the secondary prevention, clinical trials for the primary prevention needs longer duration as

well as larger population to answer whether vitamin B6 supplementation is indeed effective to prevent cardiovascular disease before the first vascular event or during the younger life. Studies are needed to find the optimal dose and combination of vitamin B6 forms that maximize efficacy and minimize adverse effects, to identify subjects who will get the most beneficial effect from interventions with vitamin B6, even on the basis of genetic susceptibility and taking into account other environmental factors to adequately assess the role of B vitamins in the primary prevention of cardiovascular disease.

14.5 Conclusions and Future Perspectives

As illustrated in the present chapter, many observations emphasize the importance of vitamin B6 as a significant, independent risk factor for cardiovascular disease and in some (Robinson et al. 1998), but not all, case-control studies vitamin B6 deficiency is clearly associated with higher risk for cardiovascular disease (Friso et al. 2004; Robinson et al. 1995, 1998), even though clinical trials testified the ineffectiveness of vitamin B6 supplementation on recurrence of vascular events.

Since vitamin B6 is involved in a very large number of physiologic reactions, is likely that vitamin B6 may affect cardiovascular disease in a complex fashion of inter-relationships among several factors and thereby it could be very hard to define the exact mechanism(s) as well as the optimal endpoints for clinical trials.

Current clinical trials indicate that vitamin B6 supplementation is not effective for the prevention of recurrence of cardiovascular disease. However, it is still open to determine whether supplementation with vitamin B6 is effective for the primary prevention of cardiovascular disease. It is also to be evoked that supplementation with vitamin B6 in a single high-dose synthetic form might not be as effective as adequate dietary vitamin B6 intake, since in the latter case, the vitamin is made available through many different physiologic forms and in combination with a number of other nutrients and, particularly, of other B vitamins which are involved in the complex vitamin B6 metabolism.

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Chapter 15
Vitamin B6: Beyond Coenzyme Functions

Georg T. Wondrak and Elaine L. Jacobson

Abstract Endogenous reactive intermediates such as photoexcited states of tissue chromophores, reactive oxygen species (ROS), reactive carbonyl species (RCS), and transition metal ions are mediators of tissue damage involved in initiation and progression of human pathologies including tumorigenesis, atherosclerosis, diabetes, and neurodegenerative disease. A large body of evidence now suggests that B6 vitamers antagonize the harmful activity of endogenous reactive intermediates fulfilling a very different role than that established as a cofactor for numerous enzymes. In this chapter, the structural basis of vitamin B6 activity as a potent antioxidant, metal chelator, carbonyl scavenger, and photosensitizer is presented and the physiological relevance is discussed.

Keywords Atherosclerosis · Carbonyl scavenger · Diabetes · Glycation · Metal chelator · Ntioxidant · Vitamin B6

15.1 Introduction

The role of endogenous reactive intermediates including photoexcited states of tissue chromophores, reactive oxygen species (ROS), reactive carbonyl species (RCS), transition metal ions, in various human pathologies is now firmly established. These intermediates have been implicated in the initiation and progression of diverse human pathologies including tumorigenesis, atherosclerosis, diabetes, and neurodegenerative disease (Baynes and Thorpe, 2000a; Sasaki et al. 1993; Schumacker 2006; Ulrich and Cerami 2001; Valko et al. 2007; Wondrak et al. 2002a, 2006b). The chemical nature of endogeous reactive species suggests the feasibility of therapeutic intervention by specific molecular antagonists.

In contrast to most other vitamins, vitamin B6 occurs as a family of closely related chemical derivatives comprising six vitamers as shown in Fig. 15.1:

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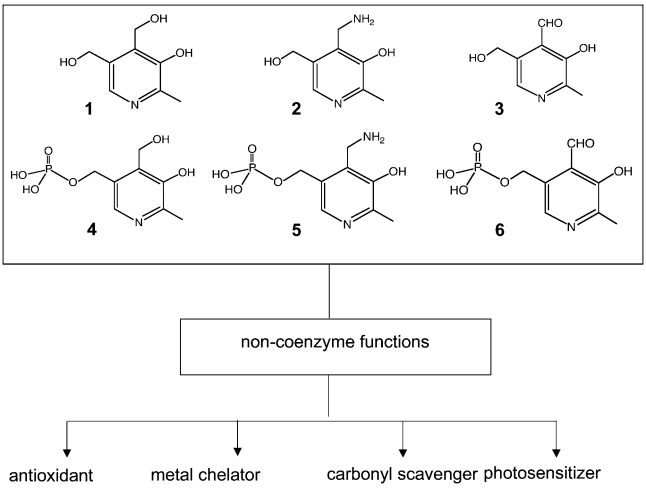

Fig. 15.1 Non-coenzyme functions of vitamin B6. Non-coenzyme functions of B6-vitamers can result from reactivity as antioxidant, metal chelator, carbonyl scavenger, and photosensitizer

pyridoxine (1), pyridoxamine (2), and pyridoxal (3), all of which can be phosphorylated to the corresponding 5′-phosphates (4–6) (McCormick and Chen 1999). The chemical versatility of B6-vitamers is remarkable and has been the subject of many detailed studies. Early findings obtained in the 1940s in the laboratory of Esmond Snell indicated that pyridoxal phosphate dependent enzyme reactions could be catalyzed by pyridoxal in the complete absence of any enzyme, demonstrating the potential of B6-vitamers to participate in spontaneous reactions under physiological conditions (Metzler et al. 1954). More recent studies have confirmed that B6-reactivity is not only essential for coenzyme function during enzymatic transformations including transamination, decarboxylation, racemization, and -elimination, but also allows participation in various non-enzymatic reactions as antioxidants, carbonyl scavengers, metal chelators, and photosensitizers as summarized in Fig. 15.1 (Voziyan and Hudson 2005a).

15.2 B6-Derived Antioxidants

B6 vitamers display spontaneous redox reactivities that are mainly attributable to the presence of a phenolic hydroxyl group in the 3-position of the pyridine ring (Culbertson et al. 2003). The reactions between pyridoxine and several oxygen radicals [hydroxyl (.OH), hydroperoxyl (.OOH), and superoxide (O_2.–)] have been investigated in much detail recently (Matxain et al. 2006). Based on addition reactions to the aromatic ring and H-atom abstraction by radical mechanisms the following reactions with .OH, .OOH, and O_2.– can occur leading to the intermediate formation of B6 free radicals (.B6):

$$B6 + .OH \rightarrow B6 + H_2O \quad (15.1)$$

$$B6 + .OOH \rightarrow B6 + H_2O_2 \quad (15.2)$$

$$B6 + .O_2 \rightarrow B6 + HOO \quad (15.3)$$

Pyridoxine is a highly effective antioxidant in chemical and cellular assays. Using a biological method that allows evaluation of human plasma and red blood cell resistance against free radical-induced hemolysis, potent antioxidant activity of pyridoxine was demonstrated (Stocker et al. 2003). Pyridoxine also exhibited antioxidant activity by suppressing free radical enhanced luminol chemiluminescence. In human erythrocytes, both pyridoxine and pyridoxamine interfere with hyperglycemia-induced superoxide radical formation and prevent lipid peroxidation (Jain and Lim 2001). In another study, pyridoxine and pyridoxamine suppressed hyperglycemia-induced crystalline oxidation in cultured lens cells (Jain et al. 2002). These findings suggest an antioxidant mechanism of action for the beneficial effects of pyridoxine supplementation on clinical symptoms of neuropathy and retinopathy in diabetic patients. In addition to providing protection against ROS including .OH and H_2O_2, B6-vitamers are also effective singlet oxygen ($1O_2$) antagonists.

$1O_2$, a highly reactive form of molecular oxygen that forms by energy transfer upon photoexcitation of organic sensitizer molecules, oxidizes cellular targets with diffusion controlled reaction rates. Importantly, $1O_2$ is a crucial reactive intermediate involved in cellular photodamage that results from photosensitization reactions in skin solar damage and photocarcinogenesis (Wondrak et al. 2006b).

Interestingly, pyridoxine-dependent resistance against self-sensitization and $1O_2$ damage has been demonstrated in the parasitic fungus cercospora keidi (Bilski et al. 2000). Plant cell lysis by this microorganism involves secretion of cercosporin, a perylenequinone photosensitizer that generates $1O_2$ in the presence of ground state $3O_2$ and solar light. Pyridoxine has been identified as an endogenous $1O_2$-antagonist that protects this pathogenic organism against self-sensitization. $1O_2$-antagonism by B6-vitamers seems to involve sacrificial reaction leading to chemical destruction, a mechanism of action that combined with the known activity of B6-vitamers as weak UVA-photosensitizers excludes their use as skin photoprotective agents as discussed below.

15.3 B6-Derived Metal Chelators

Pyridoxamine has long been known to be a moderately potent metal chelator that preferentially forms complexes with Cu^{2+} and Fe^{3+} ions (Williams and Neilands 1954). The bidentate structural feature essential for metal ion binding involves the 4aminomethyl- and the 3-hydroxyl-substituents of the pyridine ring. Chelation

involves the phenolate form of the 3-hydroxyl-substituent that displays a high degree of acidity (pKa 3.54) and therefore occurs in the anionic form under physiological conditions, where formation of a 2:1 complex between pyridoxamine and Cu2+ has been observed. Free and inadequately chelated transition metal ions are reactive species that contribute to cellular damage by participation in metal-catalyzed peroxide decay (Fenton reaction), lipid peroxidation, and other redox processes. Accumulating evidence suggests that free iron and copper ion-catalyzed reactions are important in the pathogenesis of various pathologies including ischemia reperfusion damage, neurodegenerative disease, secondary complications of diabetes, and atherosclerosis (Brewer 2007; Tabner et al. 2005). The pathological role of free transition metal ions suggests the feasibility of therapeutic intervention using small molecule transition metal ion chelators including pyridoxamine that bind and neutralize these reactive species. The preventive and therapeutic efficacy of metal chelators against various human pathologies has been documented in many preclinical and clinical studies. Indeed, metal chelation with subsequent suppression of metal ion-catalyzed oxidative and glycoxidative tissue damage seems to contribute to the efficacy of using high doses of pyridoxamine in the prevention and treatment of type II diabetic nephropathy in mouse models of the disease, a therapeutic application currently being evaluated in human phase II clinical studies as discussed below (Voziyan and Hudson 2005b).

15.4 Pyridoxamine as a Carbonyl Scavenger

Among the various B6-vitamers, only pyridoxamine contains the bis-nucleophilic 3hydroxy-4-aminomethyl-pyridine pharmacophore known to impart activity as a potent carbonyl scavenger and anti-glycation agent (Amarnath et al. 2004; Metz et al. 2003b; Onorato et al. 2000; Voziyan and Hudson 2005b). Carbonyl stress mediated by reactive carbonyl species (RCS) is an established source of endogenous tissue damage that occurs in various systemic pathologies, such as diabetes, atherosclerosis, Alzheimer's disease, and chronological aging (Baynes 2001; Baynes and Thorpe 2000b; Ulrich and Cerami 2001; Wondrak et al. 2002b, 2006b). Pyridoxamine is now in advanced clinical studies to test its therapeutic potential as a small molecule inhibitor of tissue carbonyl stress (Giannoukakis 2005; Khalifah et al. 2005). Chemical damage by RCS occurs by spontaneous aminocarbonyl reactions (referred to as 'glycation') between RCS and protein-bound amino groups leading to covalent protein adducts, crosslinking, and formation of protein-epitopes called advanced glycation endproducts (AGEs). The chemistry of AGE-accumulation in human tissue proteins is complex and depends on the crucial involvement of various RCS including mono-, di-, and oligo-carbonyl intermediates (e.g. glyoxal, methylglyoxal, 3-deoxyglucosone, glucosone) that originate from sugar metabolism, sugar autoxidation, and lipid peroxidation (Wondrak et al. 2002a).

AGEs accumulate on extracellular matrix proteins including collagen and elastin as a function of age inducing structural and functional alterations. In addition to protein crosslinking and matrix dysfunction, AGEs can stimulate pathological signaling through a receptor called RAGE involved in chronic inflammation and carcinogenesis (Bierhaus et al. 2000; Wondrak et al. 2005). Apart from the roles of AGE-epitopes as protein crosslinkers and RAGE-ligands, some AGEs with extended heterocyclic chromophores are now established skin photosensitizers that produce ROS upon UVA-irradiation adding to the photooxidative burden of chronologically aged skin and thereby contributing to photocarcinogenesis (Wondrak et al. 2006b). The role of carbonyl stress in the pathology of atherosclerosis, secondary diabetic complications associated with extracellular matrix protein damage including neuropathy, retinopathy, and nephropathy, and skin actinic and chronological damage is now well established (Baynes 2001; Baynes and Thorpe 2000b; Bierhaus et al. 2000, 2005; Ulrich and Cerami 2001; Wondrak et al. 2002b, 2006b). RCS that mediate carbonyl stress are therefore potential molecular targets for therapeutic intervention, and experimental evidence suggests the possibility of therapeutic intervention by rational design of novel RCS-antagonists, called carbonyl scavengers (Reddy and Beyaz 2006; Wondrak et al. 2002a). Carbonyl scavengers are experimental small molecule nucleophilic agents that inactivate RCS by sacrificial covalent adduction. Screening assays for the identification of nucleophilic carbonyl scavengers have been described (Wondrak et al. 2002a), prototype agents have been identified, and their efficacy has been tested in cellular and animal models of carbonyl stress (Giannoukakis 2005; Khalifah et al. 2005; Roberts et al. 2003; Williams 2004; Wondrak et al. 2006a).

Recently, pyridoxamine was identified as a prototype agent to suppress carbonyl stress and glycation reactions (Amarnath et al. 2004; Metz et al. 2003b; Onorato et al. 2000; Voziyan and Hudson 2005b). An abbreviated reaction scheme illustrating the chemistry of carbonyl scavenging by pyridoxamine directed against RCS is shown in Fig. 15.2. Numerous preclinical and some early clinical studies suggest that pyridoxamine is a potent inhibitor of carbonyl stress and tissue glycation damage in vivo (Giannoukakis 2005; Khalifah et al. 2005). Pyridoxamine inhibited the progression of renal disease, decreased hyperlipidemia, and protected against a range of pathological changes in the retinas of streptozotocin-diabetic rats (Degenhardt et al. 2002).

In the Zucker rat model of diabetes and hyperlipidemia, characterized by elevated levels of plasma alpha-dicarbonyl compounds and protein AGEs, pyridoxamine treatment had significant renoprotective effects and effectively decreased plasma levels of RCS including glyoxal and methylglyoxal (Nagaraj et al. 2002). Moreover, RCS-adducts of pyridoxamine were detected in urine of pyridoxamine-treated animals, indicating that the carbonyl-trapping mechanism is operative in vivo (Metz et al. 2003a). In human clinical studies, pyridoxamine has shown a favorable safety profile, and phase 2 clinical studies of this agent (Pyridorin^{TM}, Biostratum Inc.) in patients with diabetic nephropathy have been conducted (Giannoukakis 2005).

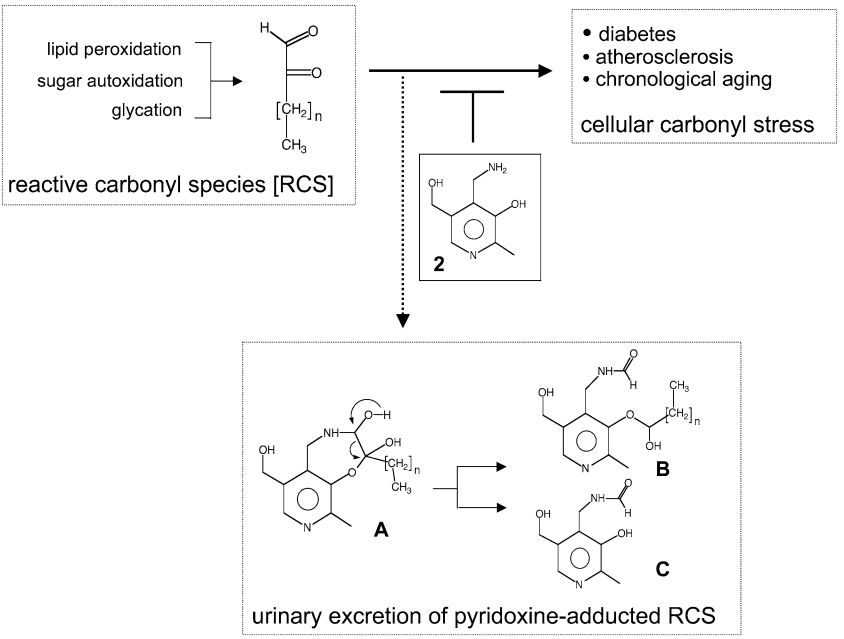

Fig. 15.2 Pyridoxamine as an inhibitor of cellular carbonyl stress. Pyridoxamine traps RCS by covalent adduction thereby interfering with tissue carbonyl stress involved in various human pathologies. RCS-trapping by pyridoxamine involves bis-nucleophilic adduction via the 4-aminomethyl- and 3-hydroxyl-functionalities with formation of products A-C. N-formylpyridoxamine (FAPM, product C) is the major pyridoxamine-adduct detected in urine of diabetic and obese rats treated with pyridoxamine (Metz et al. 2003b)

15.5 B6-Vitamers as Potential Mediators of Skin UVA-Photosensitization

All B6-vitamers share the 3-hydroxypyridine (3-HP) core moiety known to have potent photodynamic activity as recently revealed by a detailed structure activity relationship study of skin cell photosensitization by various endogenous pyridinium derivatives including B6-vitamers (Wondrak et al. 2004). Photosensitization occurs as a consequence of initial formation of excited states of chromophores and their subsequent interaction with substrate molecules (type I photoreaction) or molecular oxygen (type II photoreaction) through energy and/or electron transfer (Foote 1991; Wondrak et al. 2006b). Members of the extended 3-HP class of endogenous skin photosensitizers comprise all B6 vitamers and other 3-HP containing skin chromophores including the collagen crosslink pyridinoline, certain AGEs, and protein adducts of lipid peroxidation products as summarized in Fig. 15.3 (Wondrak et al. 2003, 2006b). Indeed, UVA photosensitization of human skin cells by B6-vitamers (pyridoxine, pyridoxamine, pyridoxal, pyridoxal-5′-phosphate) occurred in the lower micromolar range with dose-dependent inhibition of proliferation, cell

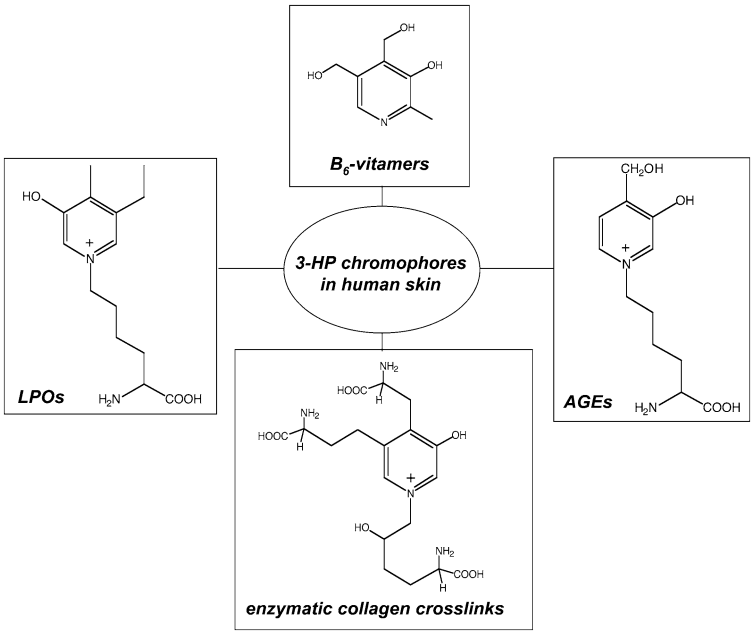

Fig. 15.3 The 3-hydroxypyridine family of potential skin photosensitizers. All B6 vitamers in human skin contain the phototoxic 3-HP moiety activated by solar UVB and UVA radiation. Moreover, 3-HP containing advanced glycation endproducts (AGEs) and lipid peroxidation (LPOs) products formed on skin proteins by reaction of lysine residues with RCS accumulate in skin during chronological and photoaging Paradoxically, 3-HP is also found in the enzymatic skin collagen crosslink pyridinoline, a constitutive component of the human dermal extracellular matrix

cycle arrest in G2/M, induction of apoptosis, intracellular oxidative stress, and p38 MAPkinase activation, all of which were reversible by thiol antioxidant intervention (Wondrak et al. 2004). Obviously, the presence of a phenolic 3-OH substituent is an essential structural requirement for sensitizer activity of B6-vitamers, since 2-HP and 4-HP, which occur predominantly as the tautomeric pyridone structures in aqueous solutions at neutral pH (Wang 1996), display no photosensitizer activity. This is consistent with a free radical mechanism of phototoxicity of biogenic 3-HP-derivatives observed in a recent study using cultured human skin cells (Wondrak et al. 2004). In simple in vitro models of protein photooxidation, B6-vitamers were moderately potent photosensitizers, effecting protein photo-crosslinking and photo-oxidation with incorporation of molecular oxygen. Based on these results, it was concluded that B6-vitamers constitute a novel class of UVA-photosensitizers capable of mediating skin photooxidative damage. Indeed, increased photosensitivity is a known consequence of B6-overdosing in humans (Murata et al. 1998), and phototoxicity of UV-irradiated pyridoxamine was reported as early as 1947 by Shwartzman and Fisher (1947) followed by other reports thereafter (Maeda et al. 2000; Sato et al. 1993).

B6-vitamers may therefore act as endogenous skin photosensitizers with relevance to human skin in vivo, since human skin contains various B6-vitamer forms in significant amounts (approximately 100 nmol/g protein (Coburn et al. 2003)), with pyridoxal-5′-phosphate and pyridoxal being the predominant vitamers in vivo. However, it is important to note that the potential phototoxic activity of B6-vitamers in human skin may be balanced in vivo by their antioxidant and singlet oxygen quenching activity above mentioned, suggesting a complex role in prevention and mediation of skin photodamage to be addressed by future studies.

15.6 Summary

Recent studies suggest that B6-vitamers can illicit therapeutic effects in a variety of human pathologies that depend on ROS, RCS, and transition metal ions including atherosclerosis, diabetes, and other age-related diseases. These non-coenzyme roles of B6-vitamers may therefore represent an opportunity for innovative therapeutic interventions.

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Chapter 16
Cobalamin Deficiency

Wolfgang Herrmann and Rima Obeid

Abstract Cobalamin (Cbl, vitamin B12) consists of a corrinoid structure with cobalt in the centre of the molecule. Neither humans nor animals are able to synthesize this vitamin. Foods of animal source are the only natural source of cobalamin in human diet. There are only two enzymatic reactions in mammalian cells that require cobalamin as cofactor. Methylcobolamin is a cofactor for methionine synthase. The enzyme methylmalonyl-CoA-mutase requires adenosylcobalamin as a cofactor. Therefore, serum concentrations of homocysteine (tHcy) and methylmalonic acid (MMA) will increase in cobalamin deficiency. The cobalamin absorption from diet is a complex process that involves different proteins: haptocorrin, intrinsic factor and transcobalamin (TC). Cobalamin that is bound to TC is called holotranscobalamin (holoTC) which is the metabolically active vitamin B12 fraction. HoloTC consists 6 and 20% of total cobalamin whereas 80% of total serum cobalamin is bound to another binding protein, haptocorrin. Cobalamin deficiency is common worldwide. Cobalamin malabsorption is common in elderly subjects which might explain low vitamin status. Subjects who ingest low amount of cobalamin like vegetarians develop vitamin deficiency. No single parameter can be used to diagnose cobalamin deficiency. Total serum cobalamin is neither sensitive nor it is specific for cobalamin deficiency. This might explain why many deficient subjects would be overlooked by utilizing total cobalamin as status marker. Concentration of holotranscobalamin (holoTC) in serum is an earlier marker that becomes decreased before total serum cobalamin. Concentrations of MMA and tHcy increase in blood of cobalamin deficient subjects. Despite limitations of these markers in patients with renal dysfunction, concentrations of MMA and tHcy are useful functional markers of cobalamin status. The combined use of holoTC and MMA assays may better indicate cobalamin status than either of them. Because Cbl deficiency is a risk factor for neurodegenerative diseases an early diagnosis of a low B12 status is required which should be followed by an effective treatment in order to prevent irreversible damages.

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16.1 Cobalamin and Its Metabolism

The discovery of cobalamin (Cbl; vitamin B12) was after several years of intensive studies on pernicious anemia, a previously fatal disease. In the early 1920s Minot and Murphy demonstrated that they were able to cure pernicious anaemia by whole liver extract. Later, it was shown that liver is an important source of cobalamin. Cobalamin was then isolated and the crystallized molecule was identified. Cobalamin belongs to a group of compounds of similar chemical structure but completely different biological functions. Cbl consists of a corrinoid molecule with cobalt in the centre of this molecule. The synthetic forms of cobalamin are cyanocobalamin and hydroxycobalamin. There are only two forms of Cbl that have biological activity as cofactors in enzyme reactions (Fig. 16.1). These are adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) (Herzlich and Herbert 1988). In mammalian metabolism, Cbl is required for only two key enzymatic reactions. The first reaction occurs in the cytosol and involves synthesis of methionine from homocysteine. Methionine synthase and its cofactor MeCbl catalyze this reaction. The second pathway takes place in the mitochondria and involves isomerisation of methylmalonyl-CoA to succinyl-CoA. This reaction is catalyzed by methylmalonyl-CoA mutase with AdoCbl as a cofactor (Stroinsky and Schneider

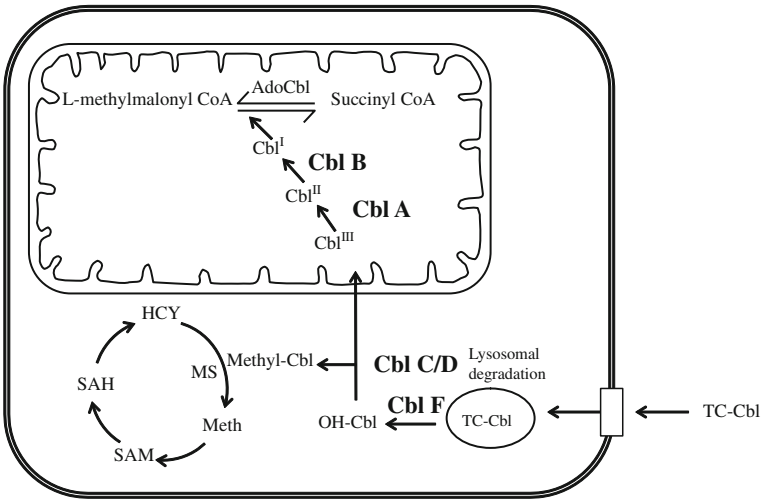

Fig. 16.1 The metabolic pathways enhanced by cobalamin and inherited disorders of cobalamin (see text for details). Hcy; homocysteine, Met; methionine, SAH; S-adenosylhomocysteine, SAM; S-adenosylmethionin, methyl-Cbl; methyl cobalamin, Ado-Cbl; adenosyl cobalamin

1987). The latter reaction is part of the catabolism of odd-chained fatty acids, cholesterol and several amino acids. The excess of methymalonyl-CoA is converted into methylmalonic acid (MMA). Therefore, cobalamin deficiency leads to MMA elevation that is considered a sensitive marker for this deficiency. A reduced flux through the methylmalonyl-CoA mutase reaction is discussed to contribute to subsequent neurological tissue damage.

16.1.1 Sources of Cobalamin

Cobalamin synthesis is very complex and restricted to a certain stain of bacteria. Neither humans, nor animals are able to synthesize this complex molecule. Plants and fungi are thought to neither synthesize, nor to use the vitamin as well. Animals can get cobalamin by consuming foods contaminated with synthesizing bacteria and then incorporate the vitamin into their body organs. Foods of animal source are the only natural source of cobalamin in human diet. The main sources of cobalamin in humans diet are meet (2–5 μg/100 g), fish (2–8 μg/100 g), milk (1.5 μg/100 ml), cheese (1–2 μg/100 g) and eggs (2 μg/100 g) (Scott 1997). Cobalamin is essential for normal maturation and development of all DNA synthesizing cells including blood cells and cells of the central nervous system. The ultimate source of cobalamin in human diet is from foods contaminated with B12-synthesizing bacteria. Not all forms of cobalamin formed by microbes are metabolically active for mammalian cells. Some naturally occurring forms of corrinoids have similar structure but no biological roles in humans. These are produced by some algae (spirulina) and are termed analogous. Cobalamin-analogous may even block the normal metabolism of the vitamin (Herbert and Drivas 1982).

16.1.2 Absorption, Excretion and Haemostasis of Cobalamin

Cobalamin is bound to food proteins and must be released before that the vitamin can be absorbed. This is achieved by the action of gastric acid and proteolytic enzymes in the stomach. In the stomach cobalamin is captured by haptocorrin, an R-binder protein made in the saliva and stomach. In the upper small intestine, pancreatic enzymes and an alkaline pH degrade the haptocorrin-cobalamin complex. The free vitamin is then captured by intrinsic factor, another B12-binding protein. The intrinsic factor-cobalamin complex is transported to the terminal ileum where the complex is recognised and internalised by specific membrane receptors of the enterocytes (intrinsic factor receptor). The receptor-mediated absorption of cobalamin is a saturable process and a maximal amount of 3 μg of the vitamin per meal can be internalized via this pathway. After absorption of cobalamin-intrinsic factor complex into the enterocytes, the complex is degraded and cobalamin is transferred to a third binding protein, transcobalamin (TC).

Transcobalamin is synthesized within the enterocytes and is the only binder that can deliver cobalamin into cells via TC-receptor. The TC-cobalamin complex is

released into the portal circulation and is subsequently recognised by TC-receptors that are expressed by all cell types. The part of cobalamin which is bound to TC is named holotranscobalamin (holoTC) (Carmel 1985). Only 6–20% of total plasma cobalamin is present as holoTC (Hall 1977). The remaining part of cobalamin is bound to haptocorrin and is called holohaptocorrin (holoHC) (England et al. 1976). Despite that haptocorrin binds almost 80% of total plasma cobalamin, the functions of this protein are not well investigated.

The bioavailability of cobalamin from vitamin preparations is greater than that from foods. In contrast to the receptor mediated absorption, about 1% of free cobalamin is absorbed by passive diffusion even in the absence of intrinsic factor. Therefore, even patients with pernicious anemia, or those with disturbed gastrointestinal pH or atrophic gastritis may benefit from relatively high doses of oral cobalamin treatment. Furthermore, a considerable amount of cobalamin is secreted into the bile. Two thirds of the secreted cobalamin in the bile is reabsorbed in the ileum. The liver contains most of the body's cobalamin. It is calculated that 2–3 mg of cobalamin are stored in the liver (Markle 1996). The kidney and the brain are also two important organs that accumulate cobalamin. The kidney can release cobalamin in case of short term depletion of the vitamin. Cobalamin is excreted into urine and this can be reabsorbed in the proximal tubules via a specific receptor (megalin) (Moestrup et al. 1996). The major route by which cobalamin is lost from the body is through the feces.

16.2 Cobalamin Deficiency

Frank cobalamin deficiency is common worldwide (Morris et al. 2002; Obeid et al. 2002). In developed countries, cobalamin deficiency is restricted to patients with malabsorption, intestinal resection, or those who do not ingest a sufficient amount of the vitamin (Herrmann et al. 2003; Carmel 1997). Cobalamin deficiency is common in elderly people and in those who ingest a strict vegetarian diet. In contrast, cobalamin deficiency has been reported in children, middle age, and elderly people from developing countries.

Pre-clinical cobalamin deficiency is the state in which metabolic evidence of insufficiency exists without symptoms of anemia or neurological complications. Measurement of serum concentrations of metabolic markers allow detection of a large number of asymptomatic subjects who are depleted of the vitamin.

16.2.1 Cobalamin Deficiency in the Elderly

16.2.1.1 Factors Contributing to Low Vitamin Status with Aging

Cobalamin deficiency in geriatric population has gained a particular importance in recent years (Baik and Russell 1999; Obeid et al. 2004). Serum concentration of cobalamin decreases with age and that of MMA and tHcy increases. On the one

hand, the decline in cobalamin status with age could not be explained by a low dietary intake of the vitamin. On the other hand, it is well recognized that low cobalamin status in the elderly is associated with an increased incidence of pernicious anemia (type A atrophic gastritis), and type B atrophic gastritis. In western countries, approximately 2% to 3% of free living elderly people (>60 years) had undiagnosed pernicious anemia (Carmel 1996; Krasinski et al. 1986). Ethnic differences in the incidence of pernicious anemia and the age of onset have been reported (Carmel and Johnson 1978).

16.2.2 Causes of Cobalamin Deficiency

16.2.2.1 Inherited Defects

The most important inherited disorders of cobalamin absorption, transport, metabolism, or utilization are presented in Table 16.1.

Congenital transcobalamin deficiency: This congenital recessive defect causes a lack of the binding protein, TC. Several cases have been described in the literature. Although babies are born asymptomatic, severe haematological and neurological

Table 16.1 Hereditary disorders of cobalamin absorption, transport, metabolism or utilization

Defect	Affected enzyme or step	Metabolic abnormalities	Therapeutic strategies
Congenital pernicious anemia (Intrinsic factor deficiency)	Cobalamin absorption	↑HCY, ↑MMA	Cobalamin + intrinsic factor
Imerslund-Gräsbeck syndrome	Selective cobalamin malabsorption	Proteinurea, ↑HCY, ↑MMA	Cobalamin injection
Transcobalamin II deficiency	Defective transport of cobalamin into the blood stream and into the cells	↑HCY, ↑MMA	High doses of systemic cobalamin
Methylmalonic aciduria	Methylmalonyl CoA mutase deficiency (mut^0, mut^-)	↑MMA, acidosis	Protein restriction (limiting the amino acids that use the propionate pathway)
Cbl A and Cbl B diseases	Failure to synthesize adenosylcobalamin	↑MMA	Cobalamin injections
Cbl E and Cbl G diseases	Failure to synthesize methylcobalamin	↑tHcy, ↓methionine	Cobalamin injections + Betaine
Cbl C and Cbl D diseases	Failure to synthesize adenosylcobalamin and methylcobalamin	↑MMA, ↑tHcy	Cobalamin injections + Betaine
Cbl F disease	Failure to release cobalamin from the lysosome	↑MMA, ↑tHcy	Cobalamin injections

manifestations are expressed few days to weeks after birth. This case is characterized by irritation, failure to thrive, and severe elevation of methylmalonic acid in blood and urine of affected children. Skin fibroblasts or lymphocytes isolated from affected children can not take up cobalamin, in contrast to cells isolated from healthy subjects. In most cases, high doses of cobalamin can improve clinical symptoms (Bibi et al. 1999; Teplitsky et al. 2003; Cooper and Rosenblatt 1987; Hakami et al. 1971). Abnormal transcobalamin has also been reported and is associated with similar symptoms.

Imerslund-Grasbeck-syndrom: This disorder is caused by a defective transport of cobalamin by enterocytes. This disease is characterized by proteinuria and lose of cobalamin in urine (Ben-Ami et al. 1990). Cobalamin malabsorption in this case is not related to intrinsic factor deficiency or to an abnormal cobalain-intrinsic factor complex. The cause behind cobalamin malabsorption and lose is related to a defective cubulin, the receptor for IF-cobalamin that is expressed in the enterocytes and the renal tubulus.

Disorders of cobalamin utilization: There are several inherited defects in cobalamin utilization. These are summarized in Fig. 16.1. Methylmalonic aciduria is related to a functional defect or a deficiency of the mithochondrial enzyme, methylmalonyl CoA mutase. This defect is characterized by severe elevation of blood and urine concentrations of MMA. Cultures of patients fibroblasts have shown that some patients do not response to cobalamin treatment, and others have a residual enzyme activity and response to cobalamin treatment. Cobalamin mutant A and B are related to adenosylcobalamin deficiency and cause methylmalonic aciduria. Cobalamin A defect is related to a failure in a reduction step and the second disorder is caused by a failure to transfer adenosyl to cobalamin.

Cobalamin mutant C, D, and F diseases are associated with combined adenosylcobalamin and methylcobalain deficiencies. Therefore, patients have usually, homocysteinuria, hypomethioninemia, and methylmalonic aciduria. The C and D defects involve a defective reductase step; whereas the F mutant involves inability of releasing cobalamin from the lysosome. The last two disorders in cobalamin utilization are, cobalamin mutant E and G that are related to methylcobalamin deficiency.

Haptocorrin deficiency (deficiency of R binder): This case has been recently reported and is associated with a very low total serum cobalamin. Nevertheless, subjects who express this phenotype remain asymptomatic and concentrations of holotranscobalain remain within the normal range (Lin et al. 2001).

16.2.2.2 Acquired Causes of Cobalamin Deficiency

Food cobalamin malabsorption: Food cobalamin malabsorption is the most common cause of cobalamin deficiency in elderly people. There are several diseases that may cause cobalamin malabsorption. Pernicious anemia (anti IF antibodies) is the most famous disorder of cobalamin absorption. In this case, cobalamin can not be absorbed because of the lack of IF. Antiparietal cell antibodies can cause lack of intrinsic factor and thus cobalamin deficiency.

Type B chronic atrophic gastritis is related to Helicobacter pylori infection and is described to cause cobalamin malabsorption. This disorder results in a low acid-pepsin production and food cobalamin malabsorption. The release of cobalamin from food protein is decreased in case of lowered gastric acidity (Doscherholmen et al. 1977). Importantly, because the production of IF is not affected in Type B atrophic gastritis, those subjects may benefit from crystalline cobalamin. Oral cobalamin therapy (3–5 mg/week) was effective in treatment of food-cobalamin malabsorption (Andres et al. 2001, 2003).

Celiac disease and tropical spree are also associated with cobalamin deficiency. In both cases, recurrent diarrhea causes severe damage to the gastrointestinal tract and interferes with cobalamin absorption by the enterocytes. Importantly, several drugs are known to alter gastrointestinal pH thus causing cobalamin malabsorption. Table 16.2 summarizes the most important acquired causes of cobalamin deficiency.

Cobalamin deficiency related to a low intake of the vitamin: The Recommended Dietary Intake (RDI) of cobalamin for adults is 2.4 μg/day (Institute of Medicine 2000). Recent studies indicated that a total daily intake of 6 μg/day can be necessary for preventing metabolic signs of deficiency in older women (Bor et al. 2006). Chronic low intake of cobalamin can cause severe deficiency. Cobalamin deficiency takes years to develop, even when one stops to ingest the vitamin. This is related to the relatively large body stores of cobalamin, in addition to the effective enterohepatic circulation that ensures reabsorption of the vitamin. Subjects who adhere to a life long strict vegetarian diet develop cobalamin deficiency (Herrmann et al. 2003; Herbert 1994). Studies have shown that the severity of cobalamin deficiency is related to the degree of animal food restriction and to the duration of the vegetarian lifestyle (Herrmann et al. 2003; Herbert 1994). Because milk and eggs contain a low amount of cobalamin, the intake of cobalamin in a lacto- and lacto-ovo-vegetarian

Table 16.2 Acquired causes of cobalamin deficiency

Disease or condition	Mechanism
Restricted intake	Vegetarians, children of vegetarian mothers, poverty, malnutrition, anorexia nervosa
Increased demands	Bleeding, pregnancy, lactation
Medications	Changing gastrointestinal pH, interaction with vitamin absorption or metabolism
Anti H2 receptor	
Proton pump inhibitors	
Oral contraceptives	
Pernicious anemia (type A atrophic gastritis)	Anti IF antibodies
Antiparital cell antibodies	Lake of IF
Type B atrophic gastritis (H. Pylori)	Changing gastrointestinal pH
Other gastrointestinal morbidities	Interact with the vitamin absorption
Terminal ileal diseases	
Pancreatic insufficiency	
Ileal or gastric resection	
Celiac disease, tropical spree	
Colitis ulcerosa, Morbus Crohn	

diet might be sufficient. Nevertheless, studies have shown that cobalamin status (indicated by MMA, tHcy, holoTC) was lower in lacto- and lacto-ovo-vegetarians compared with omnivorous subjects (Herrmann et al. 2003). In addition, metabolic signs of cobalamin deficiency were more common in vegans compared with lacto- and lacto-ovo-vegetarians (Herrmann et al. 2003). Chronic low intake of cobalamin and its deficiency may be endemic in developing countries and is related to poverty and malnutrition in this case (Refsum et al. 2001).

The RDI for cobalamin is higher for pregnant and lactating women. Cobalamin deficiency may occur in women who do not ingest a sufficient amount of the vitamin. Metabolic and clinical signs of cobalamin deficiency have been reported in newborn babies from strict vegetarian mothers or in breast fed infants from deficient mothers (Bjorke et al. 2001; Schneede et al. 1994; Graham et al. 1992; Dagnelie et al. 1989). The content of cobalamin in mother milk was related to markers of cobalamin deficiency in the infants. These conditions were partly reversible after cobalamin supplementation. In a study that included adolescents who were fed a macrobiotic diet until the age of 6 years, metabolic signs of cobalamin deficiency were observed even several years after ingesting an omnivorous diet (Dhonukshe-Rutten et al. 2005). These data show that metabolic signs of cobalamin deficiency can not be resolved after mild modifications of the diet. Moreover, clinical signs might be irreversible.

16.2.3 Diagnosis of Cobalamin Malabsorption

Schilling test was the first test described for diagnosis of cobalamin malabsorption. This test depends on using an oral dose of radioactively-labeled cobalamin and following the radioactivity in urine or blood of patients. Schilling test can be applied with or without IF. Furthermore, the test has been improved to address the issue of malabsorption of protein-bound cobalamin (Nickoloff 1988; Doscherholmen et al. 1983). The use of radio-labeled cobalamin has limited the use of Schilling test in recent years.

Other approaches have been suggested recently to assess cobalamin absorption. One approach is to measure the response of holoTC (cobalamin saturated part) after a small oral dose of cobalamin (Bor et al. 2005). This test might be also applied with recombinant human IF. Preliminary results indicated that recombinant human IF promotes B12 absorption among patients with evident cobalamin deficiency (Hvas et al. 2001, 2006). Although early results are encouraging, studies that include patients with malabsorption disorders should be conducted before setting this test to the routine use.

16.2.4 Diagnosis of Cobalamin Deficiency

16.2.4.1 Determination of Total Serum Cobalamin

Despite its serious limitations, total serum cobalamin is the first parameter that is measured when cobalamin deficiency is suspected. As mentioned above, 80% of

total cobalamin is bound to haptocorrin. This part is metabolically inert and can not be delivered into the cells. Therefore, minor changes in cobalamin status in the blood can not be reflected by concentrations of haptocorrin. Total serum cobalamin has several major limitations. On the one hand, low serum concentrations of cobalamin were not observed in all subjects with clinically evident cobalamin deficiency (false negative). On the other hand, there are many subjects with low serum cobalamin who are not deficient (false positive). Therefore, the low sensitivity and specificity of total cobalamin should be considered, particularly when serum cobalamin concentrations range between 150 and 350 pmol/L (Green 1995; Herrmann et al. 2000, 2001a). Approximately 45% of cobalamin-deficient subjects (MMA > 300 nmol/L and holoTC < 40 pmol/L) would have been overlooked, using cobalamin as a screening parameter. Obviously, the insensitivity of the total cobalamin test can not be resolved by simply shifting the cut-off value towards a higher level, because more subjects with normal B12 status will be falsely diagnosed as deficient (Fig. 16.2).

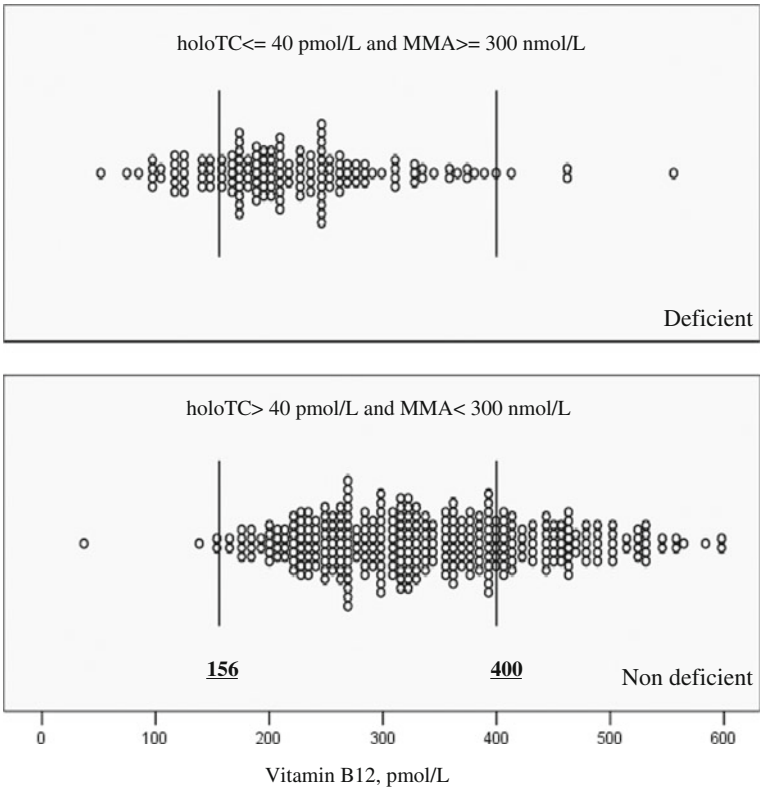

Fig. 16.2 Distribution of total serum cobalamin in deficient (*upper plot*) and non deficient (*lower plot*) subjects. cobalamin deficiency was defined as holoTC < 40 pmol/L and MMA ≥ 300 nmol/L

Because of its low costs compared to other available markers, cobalamin assay can be used as a first line parameter. Nevertheless, the results should be interpreted individually.

16.2.4.2 Determination of Holotranscobalamin

HoloTC represents the biologically active cobalamin fraction. Recent studies indicated that holoTC might be the earliest marker that indicate cobalamin status in blood (Bor et al. 2004).

Currently, the estimation of holoTC has been made possible by several methods (Ulleland et al. 2002; Refsum et al. 2006). Studies on cobalamin-deficient and non-deficient subjects have confirmed that holoTC has a better specificity and sensitivity compared with total B12 (Herrmann et al. 2005).

Limitations of holoTC should be also considered when interpreting the results of the test. Oral contraceptives have been reported to be associated with a 25% lower concentrations of holoTC without affecting serum concentrations of tHcy or MMA (Riedel et al. 2005). Moreover, renal insufficiency is associated with increased concentrations of holoTC in blood (Herrmann et al. 2005; Carmel et al. 2001). This metabolic condition is also associated with higher concentrations of MMA. Therefore, normal or elevated concentrations of holoTC in renal patients or in elderly people with mild renal insufficiency might not exclude cobalamin deficiency.

16.2.4.3 Metabolic Markers of Cobalamin Deficiency

The most important markers for cobalamin status are summarized in Table 16.3.

Plasma concentrations of methylmalonic acid (MMA): Because cobalamin is a cofactor for methylmalonyl-CoA mutase, cobalamin deficiency causes accumulation of methylmalonyl-CoA. The last compound is converted into methylmalonic acid (MMA). Serum concentration of MMA increase in case of cobalamin deficiency and thus MMA is considered a functional marker for cobalamin status. Concentrations of MMA (> 300 nmol/L) are more sensitive and specific for cobalamin deficiency than total serum cobalamin.

Limitations of this metabolic marker should be also kept in mind. Concentrations of MMA increase in renal dysfunction. Even sub-clinical decline in renal function (i.e., common in elderly people) may cause artificial increase in concentrations of MMA (Herrmann et al. 2001a). Intestinal bacterial overgrowth may also cause artificial elevation of MMA because of increased production of propionic acid, a precursor of MMA. Nevertheless, in these cases cobalamin deficiency can not be confirmed or excluded. A significant decrease in concentrations of MMA by cobalamin treatment indicates a pre-clinical deficiency.

Plasma concentrations of tHcy: Concentrations of tHcy increase in cobalamin deficiency because of the role of methylcobalamin as a cofactor for methionine synthase that converts tHcy into methionine. Nevertheless, concentrations of tHcy

Table 16.3 Available markers of B-vitamins deficiency

Parameter	Reference range	Material	Methods	Pre-analytical	Confounding factors
tHcy	< 12 μM	EDTA plasma, serum	GCMS, HPLC, (EIA) enzymatic immuno-assay, LC-MS/MS	Centrifuge and separate within 45 min preferably keep the sample on ice Several stabilizing agents are currently available (DZA)	Renal function (↑) Age (↑) Medications (↑) Hormone therapy (↑→)
MMA	< 0.300 μM	EDTA plasma, serum	GCMS LC_MS/MS	–	Renal function (↑) Age (↑)
Cobalamin	> 156 pmol/L	Serum	Chemiiluminescience, Microbiological assay	–	Renal function (↑)
holoTC	> 40 pmol/L	EDTA plasma, serum	RIA, ELISA	–	Renal function (↑)

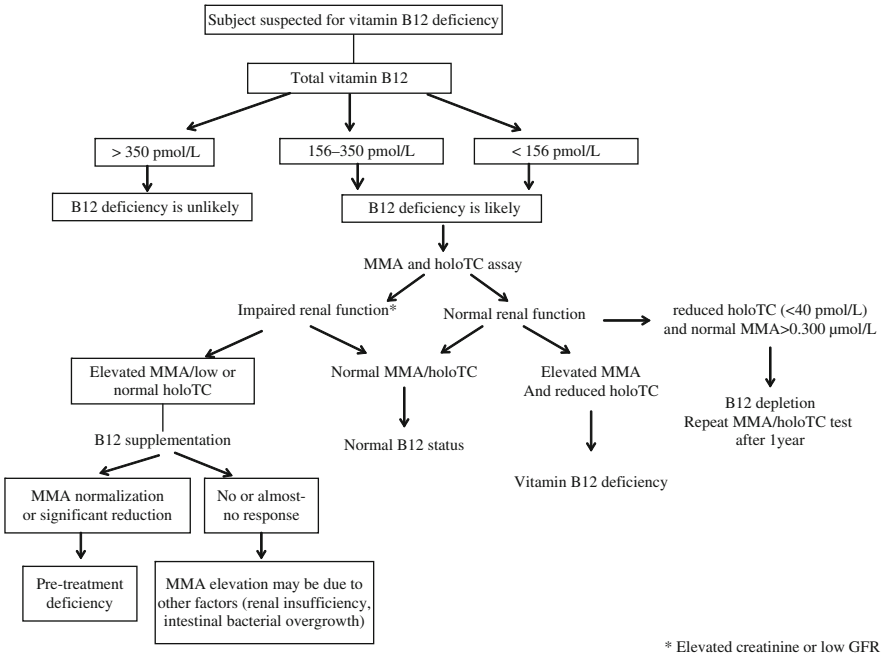

Fig. 16.3 Suggested algorithm for diagnosing cobalamin deficiency

increase also in folate and vitamin B6 deficiency or renal insufficiency (Herrmann et al. 2001b). Folate deficiency has more impact on tHcy compared with cobalamin deficiency. Unlike MMA elevation, tHcy elevation is not common in B12-deficient vegetarians, because of relatively high folate concentrations found in those subjects. High folate status partly compensates for the effect of cobalamin deficiency on tHcy elevation.

Figure 16.3 shows a diagnostic algorithm suggested for diagnosis of cobalamin deficiency.

16.2.4.4 Staging of Cobalamin Deficiency

Cobalamin deficiency is thought to be developed in four stages (Herbert 1994). In the early stages (I and II), the plasma and cell stores become depleted of the vitamin. This is reflected by a low plasma concentration of holoTC (Herbert et al. 1990; Lindgren et al. 1999). If the negative balance continues (stage III), metabolic markers of cobalamin deficiency become elevated (tHcy and MMA). This is explained by impairment of the B12-dependent enzymes (methionine synthase and methylmalonyl-CoA mutase). Clinical signs of cobalamin deficiency become obvious in stage IV (macrocytic anemia, neurological symptoms).

16.2.4.5 Diagnosis of Cobalamin Deficiency Utilizing Available Markers

There is no single parameter that can be reliably used to diagnose cobalamin deficiency. Megaloblastic anemia and neurological symptoms are neither sensitive nor they are specific for cobalamin deficiency. Metabolic signs (elevated MMA and tHcy) of deficiency were observed in the absence of haematological or clinical manifestations. Concentrations of total cobalamin in serum are also too insensitive (Herrmann et al. 2000, 2001b). Measurement of serum concentration of MMA alone or in conjunction with tHcy has partly resolved the demand for a sensitive and a specific test. In general, results of the metabolites should be interpreted with caution, because it is difficult to determine to which extent the impaired kidney function may participate in MMA and tHcy elevation. Subjects with serum cobalamin concentrations up to 350 pmol/L may have increased MMA and low holoTC indicating functional cobalamin deficiency. Studies on vegetarian subjects have shown that the majority of them had low holoTC in addition to metabolic signs indicating cobalamin deficiency (stage III) (elevated tHcy and MMA) (Herrmann et al. 2003).

16.2.4.6 Cobalamin Deficiency Causes Folate Trap

Because of the role of cobalamin in folate metabolism, cobalamin deficiency can cause a secondary folate deficiency. Cobalamin deficiency inhibits the activity of methionine synthase, and causes the retention of 5-MTHF. 5-MTHF becomes trapped because the transfer of the methyl group is inhibited (Fig. 16.1). The level of folate in serum or plasma of cobalamin deficient subjects may be normal to high normal, however, this is mostly as 5-MTHF. This phenomenon is called “folate

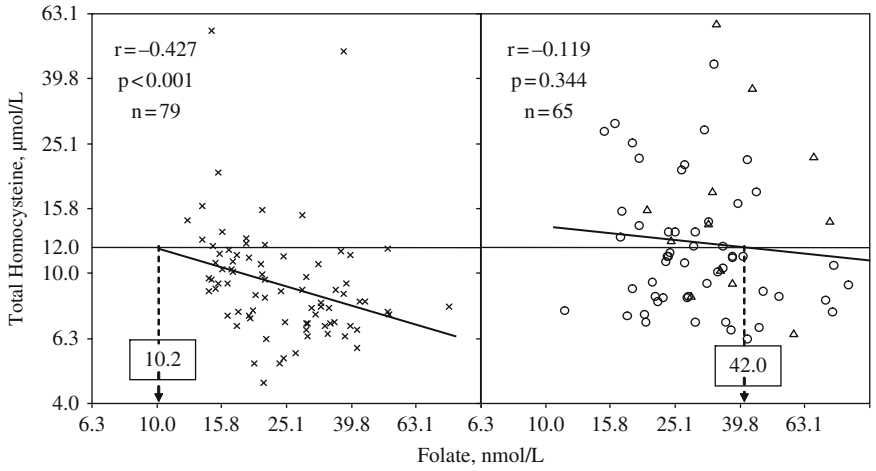

Fig. 16.4 Scatter plot showing concentrations of tHcy in relation to serum folate in omnivorous (*left plot*) and vegetarians (*right plot*). *Numbers* on the axis are anti-log

trap". Folate and cobalamin deficiency have similar clinical (megaloblastic red cells) and metabolic (elevation of tHcy) signs. This explains why cobalamin deficient subjects were frequently treated with folate. This treatment may even relief the hematological symptoms. Nevertheless, neurological signs of cobalamin deficiency may be worsen. Figure 16.4 shows this phenomenon in strict vegetarians compared with omnivorous subjects.

16.3 Cobalamin Deficiency in Renal Patients

Cobalamin deficiency is common in renal patients. Nevertheless, diagnosing cobalamin deficiency in renal patients remains a challenge. Renal insufficiency and hypovolemia cause elevation of MMA and tHcy (Herrmann et al. 2001b; Lindgren et al. 1999; Savage et al. 1994). Unexpectedly low serum concentrations of cobalamin or holoTC are uncommon in patients with renal insufficiency (Herrmann et al. 2005). A very important finding is that renal patients show significant metabolic improvements (reduction of MMA) after treatment with cobalamin (Obeid et al. 2005). The reasons for elevated serum cobalamins (total B12 and holoTC) in renal patients are not known. An abnormal distribution of holoTC (Carmel et al. 2001), a disturbed receptor activity for renal TC uptake and the possibility that TC is functionally altered by renal failure are possible explanations for accumulation of cobalamin in serum of renal patients.

16.3.1 Cobalamin Status and Vegetarian Diet

Cobalamin status is directly correlated with dietary intake and length of time following a vegetarian diet (Chanarin et al. 1985; Miller et al. 1991). According to the strictness of the diet, approximately 30–60% of vegetarian subjects might have metabolic evidence indicating cobalamin deficiency (Donaldson 2000; Rauma et al. 1995). Serum concentrations of MMA, holoTC, tHcy and total cobalamin are related to the type of the diet (Table 16.4). Vegan subjects had the lowest cobalamin status.

Table 16.4 Cobalamin status in vegetarian and non-vegetarian subjects

	Omnivorous $n = 79$	LOV/LV $n = 66$	Vegan $n = 29$
Cobalamin, pmol/L	287 (190/471)	192 (127/450)[a]	148 (99/314)[a,b]
HoloTC, pmol/L	54 (16/122)	23 (3/155)[a]	10 (2/78)[a,b]
MMA, nmol/L	161 (95/357)	355 (138/1948)[a]	708 (193/3470)[a,b]
tHcy, μmol/L	8.8 (5.5/16.1)	10.6 (6.4/27.7)[a]	12.8 (5.9/57.1)[a]
Folate, nmol/L	21.8 (14.5/51.5)	28.8 (16.1/77)[a]	31.8 (19.7/78.1)[a]
Creatinine, μmol/L	71 (53/93)	71 (53/88)	71 (49/88)

Data are median (5th/95th). $p < 0.05$: [a] compared to omnivorous controls; [b] comparing LOV/LV with vegans. LOV/LV; lacto-ovo/lacto-vegetarians

This is expected, because a vegan diet includes no kind animal foods. Although lacto- and lactoovo-vegetarians consume some animal foods (egg, milk, and milk products), metabolic signs of cobalamin deficiency were also common in this group (Herrmann et al. 2003) (Table 16.4). This group of vegetarians show intermediate cobalamin status compared with vegans and omnivorous (Herrmann et al. 2003). Approximately, 45% of subjects with low holoTC and elevated MMA had normal serum B12 (Fig. 16.2). It should be noticed that low doses of cobalamin such as the one usually found in the multivitamin preparations are not likely to prevent the depletion of the vitamin in individuals who ingest little or no animal products.

16.4 Cobalamin Status and Markers in Elderly People

Cobalamin deficiency is common in elderly people. A low dietary intake of cobalamin was not common in cobalamin deficient-elderly people. Nevertheless, several age-related physiological factors may influence negatively the absorption of the vitamin from the intestine. Cobalamin deficiency is associated with a poor cognitive performance and neurological damage. Approximately, 20% of elderly people were found to have a low concentration of holoTC and an elevated MMA indicating a relatively late stage of B12 deficiency. Importantly, a relatively large proportion of elderly subjects were found to have an elevated concentration of MMA and a normal holoTC, in addition to a higher concentration of serum creatinine. This typical profile associated with impaired renal function may delay the diagnosis of cobalamin deficiency. The relation between holoTC and MMA has been examined in relation to age and renal function (Obeid et al. 2004). In general, elderly people display higher MMA concentrations in ranges of holoTC comparable to that in younger subjects. This phenomenon was more pronounced in elderly with renal insufficiency. The last findings suggest that cellular-B12-delivery may be challenged in elderly with renal insufficiency, leading to MMA increment and holoTC retention.

Available evidence suggests that elderly people seem to be resistant to low oral doses of cobalamin (Rajan et al. 2002). Serum concentrations of MMA were not normalized in most B12-deficienct elderly people who received crystalline B12 in doses between 25 and 100 μg. The RDI of B12 for older adults (2.4 μg/day) is far below the dose likely to normalize serum concentrations of the metabolites in elderly people. Taken together, a low cobalamin status in elderly people with renal insufficiency may be overlooked by using holoTC as a sole marker. Moreover, the occurrence of cobalamin deficiency may be overestimated using MMA test. The presence of a low concentration of holoTC and an elevated MMA in elderly people may confirm B12 deficiency and the absence of this combined results may not exclude a deficiency of this vitamin. A significant reduction of MMA after cobalamin treatment might indicate a pre-treatment low status of the vitamin.

Cobalamin deficiency in elderly people was also associated with increased plasma SAH. This marker of methylation status was lowered by high oral dose of cobalamin (1 mg/day for 3 months) (Stabler et al. 1996, 2006).

16.5 Clinical Manifestations of Cobalamin Deficiency

16.5.1 Hematological Symptoms

Megaloblastic anemia is the classical finding of cobalamin deficiency. The underling mechanism of anemia in cobalamin deficiency is a slowed DNA synthesis in rapidly dividing blood cell. This will lead to large red cells (increased mean corpuscular volume, MCV). Nevertheless, megaloblastic anemia is neither sensitive, nor it is specific for cobalamin deficiency. Recent studies have shown that many cobalamin deficient subjects may develop neurological disorders without macrocytosis of red blood cells. Furthermore, because cobalamin and iron deficiencies might coincide in many cases, depending on MCV to diagnose cobalamin deficiency is not reliable. Microcytic anemia of iron deficiency can mask macrocytic anemia of cobalamin deficiency. The result is hypochromic red blood cells of normal size. In addition, the hematological manifestations of cobalamin deficiency can be mistaken by that of folate deficiency. Hypersegmented neutrophils are also common in cobalamin deficient subjects. Nevertheless, in general haematological indices are not reliable in diagnosing early stages of cobalamin deficiency.

16.5.2 Neurological Complications

Concentrations of tHcy > 12.0 μmol/L and that of MMA > 271 nmol/L are common in neuro-psychiatric population even in the absence of haematological manifestations (Lindenbaum et al. 1988). Cobalamin deficiency can cause lesions in spinal cord, peripheral nerves, and cerebrum and improvements have been reported after initiation of vitamin treatment (Masalha et al. 2001; Lorenzl et al. 2003). The most common symptoms are sensory disturbances in the extremities, memory loss, dementia, psychosis.

The association between cobalamin deficiency and depression has been documented in elderly women (Penninx et al. 2000). Metabolically significant cobalamin deficiency was present in approximately 15% of non-depressed women, 17% of mildly depressed women and in 27% of the severely depressed women (Penninx et al. 2000). Women with cobalamin deficiency were 2.05 times as likely to be severely depressed as were non-deficient women. Moreover, peripheral neuropathy occurred in 40% of cobalamin-deficient subjects (Shorvon et al. 1980).

Cobalamin deficiency can cause serious neurological symptoms in infants (Graham et al. 1992; Higginbottom et al. 1978; Kuhne et al. 1991). The most

common cause of vitamin deficiency at early age is being born or lactated by a vitamin deficient mother. A serious neurological syndrome and developmental disorders have been described in few exclusively breast-fed infants of strict vegetarian mothers who were cobalamin deficient (Graham et al. 1992; Higginbottom et al. 1978; Kuhne et al. 1991). Vitamin B12 deficiency in infants is associated with a marked developmental regression, a poor brain growth or a poor intellectual outcome. Other signs include impaired communicative reactions and fine and gross motor functions. Low vitamin B12 status had also negative influence on school achievement in schoolchildren.

Prolonged insufficient intake of the vitamin is also a common case of cobalamin deficiency in children. Adolescences previously consumed a macrobiotic diet had lower scores in some measures of cognitive performance as compared to adolescences consumed omnivorous diet from birth onward (Schneede et al. 1994; Dagnelie et al. 1989).

Available studies emphasize the need for early recognition and prevention or treatment of B-vitamins deficiency in children. Although, large scale screening programs are currently not recommended (Refsum et al. 2004), cases suspected for cobalamin deficiency should be individually judged. Cobalamin deficiency should be suspected in children with unexplained neurological symptoms, failure to thrive, or poor intellectual performance. A great attention should be paid to familial factors (maternal vitamin status), and predisposing environmental factors (poverty, vegetarian diet).

16.6 Treatment of Cobalamin Deficiency

A typical western diet provides an average intake of 2–6 μg/day of B12. An additional source of the vitamin is highly recommended for subjects with malabsorption or a low intake of the vitamin. Most elderly people who have already developed metabolic signs indicating cobalamin deficiency (i.e., elevated MMA) require more than 100 μg of oral B12 to normalize serum MMA, which is a larger dose than is available in most standard multivitamins and cobalamin supplements.

Folate treatment for cobalamin deficient subject may delay the diagnosis of B12 deficiency and cause irreversible neurological symptoms. Therefore, addition of cobalamin to folate treatment is strongly recommended. Doses and duration of vitamin supplementation should be individually determined, because malabsorption is a major limiting factor for the bioavailability of the vitamins in elderly people. Table 16.5 shows a few examples on cobalamin doses and responses to treatment in different clinical conditions.

Table 16.5 Examples about vitamin intervention trials

Study	Subjects	Dose, duration of B12	Metabolic effects	Other effects
Stabler et al. (2006)	39 elderly subjects mean age 76.3 y with elevated MMA	1000 μg cyanocobalamin/d, oral for 3 months	MMA (nmol/L); 434 → 240 tHcy (μmol/L); 12.7 → 9.6 SAH (nmol/L); 43 → 37	–
Hvas et al. (2001)	140 subjects with MMA (0.40–2.00 μmol/L)	Placebo-controlled study 1 mg im per week for 4 weeks follow up for 3 months	MMA –61% in treatment group (+4% in placebo) tHcy –23% in treatment group (vs. +2% in placebo)	Subjects with MMA > 0.60 μM showed improved neurological symptoms
Lewerin et al. (2003)	209 elderly mean age 76 y	Oral daily B12 0.5 mg (plus folic acid 0.8 mg and B6 3 mg) for 4 months, placebo controlled	Mean MMA; 0.22 → 0.19 in the treatment group 0.22 → 0.22 in the placebo group	–
Bolann et al. (2000)	51 subjects age 17–87 with baseline MMA > 0.26 μmol/L and a reduction of MMA by >=50% after cobalamin treatment	1 mg cyanocobalamin i.m. twice a week for 2.5 weeks	MMA (μmol/L); 0.88 → 0.14 tHcy (μmol/L); 25.8 → 13.4	–
Andres et al. (2003)	30 patients with food cobalamin malabsorption	250 –1000 μg of oral cyanocobalamin daily for at least 1 month	87% normalized their serum cobalamin 54% corrected their anemia	–
Kuzminski et al. (1998)	38 cobalamin deficient subjects	Either 1 mg i.m. on 9 occasions or 2 mg orally for 120 days Patients were tested after 4 months	In the oral group; B12 = 93 →1005 pg/ml MMA 3850 → 169 nmol/L tHcy 37.2 → 10.6 μmol/L In the i.m. group; B12 = 95 →325 pg/ml MMA 3630 → 265 nmol/L tHcy 40 → 12.2 μmol/L	–
Rajan et al. (2002)	23 elderly (age >64 y) with B12 ≤ 221 pmol/L and MMA > 271 nmol/L	Sequential daily treatment with oral cobalamin 25 μg, 100 μg, and 1000 μg for 6 weeks each	25 μg and 100 μg lowered but did not normalize MMA 1 mg was necessary for normalizing MMA in the majority of the subjects	–

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Chapter 17
Biochemistry of B12-Cofactors in Human Metabolism

Bernhard Kräutler

Abstract Vitamin B12, the "antipernicious anaemia factor", is a crystallisable cobalt-complex, which belongs to a group of unique "complete" corrinoids, named cobalamins (Cbl). In humans, instead of the "vitamin", two organometallic B12-forms are coenzymes in two metabolically important enzymes: Methylcobalamin, the cofactor of methionine synthase, and coenzyme B12 (adenosylcobalamin), the cofactor of methylmalonyl-CoA mutase. The cytoplasmatic methionine synthase catalyzes the transfer of a methyl group from N-methyl-tetrahydrofolate to homocysteine to yield methionine and to liberate tetrahydrofolate. In the mitochondrial methylmalonyl-CoA mutase a radical process transforms methylmalonyl-CoA (a remains e.g. from uneven numbered fatty acids) into succinyl-CoA, for further metabolic use. In addition, in the human mitochondria an adenosyl-transferase incorporates the organometallic group of coenzyme B12. In all these enzymes, the bound B12-derivatives engage (or are formed) in exceptional organometallic enzymatic reactions. This chapter recapitulates the physiological chemistry of vitamin B12, relevant in the context of the metabolic transformation of B12-derivatives into the relevant coenzyme forms and their use in B12-dependent enzymes.

Keywords 5′-adenosyl radical · Coenzyme B12 · Methionine synthase · Methylcobalamin · Methylgroup transfer · Methylmalonyl-CoA mutase · Radical reaction · Vitamin B12

Abbreviations and Symbols

Ado	5′-(deoxy)-adenosyl
AdoCbl	5′-deoxy-5′-adenosyl-cobalamin (coenzyme B12, also named adenosyl-cobalamin)
Ado-ImCba	5′-deoxy-5′-adenosyl-imidazolyl-cobamide
B12r	cob(II)alamin
B12s	cob(I)alamin

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BDE	(homolytic) bond dissociation energy
Cba	cobamide
Cbl	cobalamin (a DMB-cobamide)
CNCbl	cyano-cobalamin (vitamin B12)
CN-ImCba	cyano-imidazolyl-cobamide
DMB	5,6-dimethylbenzimidazole
H_2OCbl^+	aquo-cobalamin (B12a)
HOCbl	hydroxo-cobalamin
ImCba	imidazolyl-cobamide
MeCbl	methyl-cobalamin
Me-ImCba	methyl-imidazolyl-cobamide
MetH	methionine synthase (from *E. coli*)
MMCM	methylmalonyl-CoA mutase (from *Propionibacterium shermanii*)
NHE	normal hydrogen electrode
NMR	nuclear magnetic resonance
SAM	S-adenosyl-methionine (AdoMet)
UV/Vis	ultraviolet/visible absorbance spectrum

17.1 Introduction

B12-coenzymes are conceivably Nature's most broadly relevant and most complex organometallic cofactors (Eschenmoser 1988; Kräutler et al. 1998; Banerjee 1999). They are required (and are vitamins) in human metabolism, which depends on the binding and uptake of B12, on the controlled transport and metabolic transformation into the relevant B12-cofactors, and the catalysis by B12-dependent enzymes (Yamanishi et al. 2005). B12-derivatives (co)catalyze unique enzymatic reactions that directly depend upon the reactivity of the cobalt coordinated organic ligands (Kräutler and Ostermann 2003; Brown 2005).

About 60 years ago, the red cyanide-containing cobalt-complex vitamin B12 (cyano-cobalamin, CNCbl) was isolated as the (extrinsic) "anti-pernicious anaemia factor" (Fig. 17.1) (Rickes et al. 1948; Smith and Parker 1948). CNCbl is a relatively inert Co(III)-corrin, which crystallizes readily. It is the most important commercially available form of the naturally occurring B12-derivatives, although a direct physiological function of CNCbl appears not to exist (Ellenbogen and Cooper 1984). The physiologically relevant B12-derivatives are the light-sensitive and chemically more labile organometallic cofactors, coenzyme B12 (5′-deoxy-5′-adenosyl-cobalamin, AdoCbl) and methyl-cobalamin (MeCbl), as well as (in a formal sense) the "inorganic" and easily reducible B12-derivative aquo-cobalamin (H_2OCbl^+).

Important historic contributions to the biologically relevant chemistry of B12 have been reviewed earlier (Dolphin 1982; Friedrich 1988). Remarkable scientific advances contributing to the solution of the major "B12-mysteries" were achieved during the last decades (Kräutler et al. 1998; Banerjee 1999; Brown 2005), including the elucidation of the microbial B12-biosynthesis (Battersby 1998; Scott

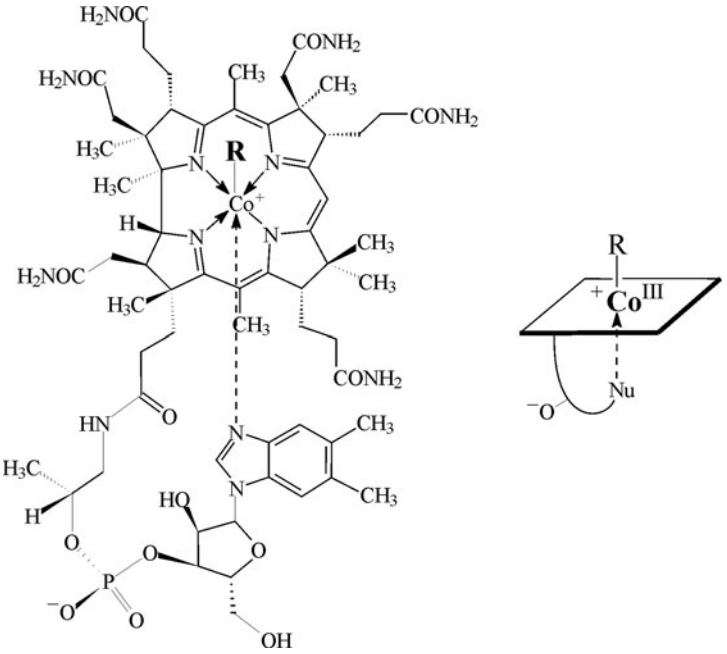

Fig. 17.1 *Left*: structural formulae of selected cobalamins (Cbl); *Right*: symbol used. Vitamin B12 (CNCbl), R = CN; coenzyme B12 (AdoCbl), R = 5′-Ado; methyl-cobalamin (MeCbl), R = CH3; aquo-cobalamin (H_2OCbl^+), R = H_2O^+; cob(II)alamin (B12r), R = e–

et al. 2003), important structural and mechanistic studies of B12-binding proteins (Drennan et al. 1994; Ludwig and Evans 1999; Gruber and Kratky 2002), as well as genetic studies of inborn errors of Cbl metabolism (Rosenblatt and Fenton 1999).

17.2 B12: Structure and Reactivity

17.2.1 B12-Structural Studies

Vitamin B12 (CNCbl), and other B12-derivatives (where the cyanide ligand of CNCbl is replaced by a different "upper" or β-ligand) are all cobalamins (Cbl, 5′,6′-dimethylbenzimidazolyl-cobamides), the B12-derivatives relevant in human metabolism. Pioneering X-ray crystallographic studies of Hodgkin et al. (Hodgkin et al. 1955; Lenhert and Hodgkin 1961) established the structure of CNCbl (and of its unique corrin core), as well as the amazing nature of the organometallic cofactor coenzyme B12 (AdoCbl). Since the time of these landmark analyses, accurate structural studies of a variety of crystalline B12-derivatives have become available, as presented in recent reviews (Kratky and Kräutler 1999; Randaccio et al. 2006).

The replacement of the cobalt coordinated 5,6-dimethylimidazole (DMB) base by a protein-derived imidazole in B12-dependent enzymes, such as methionine synthase (Drennan et al. 1994) and methylmalonyl-CoA mutase (Mancia et al. 1996) (see below), has been a puzzling discovery. To study the effect of an imidazole in B12-derivatives, unnatural imidazolyl-cobamides (ImCba) were made available via "guided" biosynthesis, such as 5′-deoxy-5′-adenosyl-imidazolyl-cobamide (Ado-ImCba) (Brown et al. 2004), cyano-imidazolyl-cobamide (CN-ImCba) and methyl-imidazolyl-cobamide (Me-ImCba), both analyzed by x-ray crystallography (Kräutler et al. 1994; Fasching et al. 2000).

Two structural characteristics of the corrin ligand are the saturated and direct trans-junction between two of its four five-membered rings and the non-planar nature of the corrin core in B12-derivatives. The bulky DMB-base was suggested to be a relevant enhancer to this latter "ligand-folding", which is a main factor also to the variability in the conformation of the corrin ligand (Kratky and Kräutler 1999). Indeed, the folding of the corrin ligand in CN-ImCba (11.3°) is less than half of that of vitamin B12 (CNCbl). This finding could be rationalized, as the less bulky and more nucleophilic imidazole showed less steric interaction with corrin ligand in CN-ImCba, than the DMB base in CNCbl. However, the crystallographic studies could not provide a pertinent structure-based rationalization of the observed displacement of the DMB-base by histidine in the enzymes. Crystallographic information on the

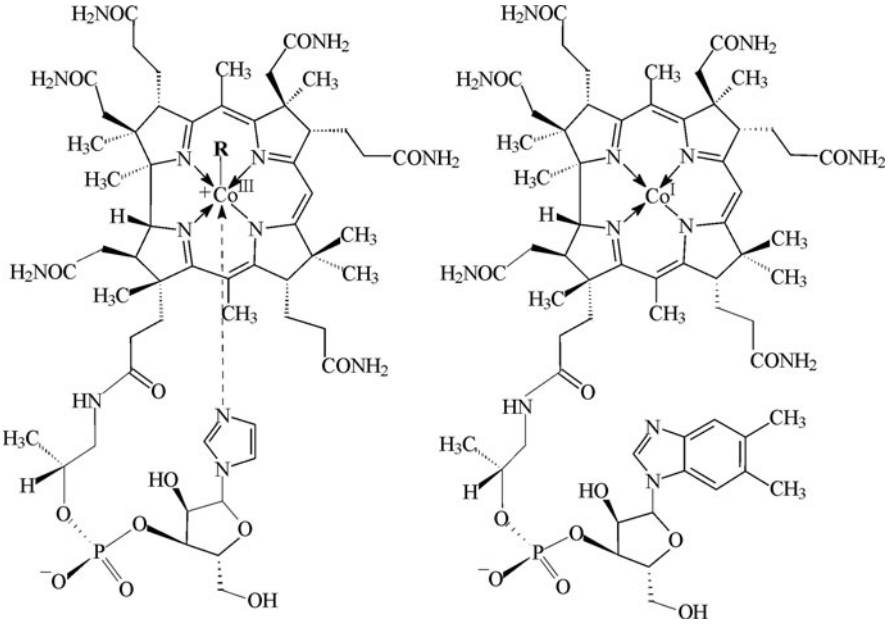

Fig. 17.2 Structural formulae. *Left*: of cyano-imidazolylcobamide (CN-ImCba, R = CN), methyl-imidazolylcobamide (Me-ImCba, R = methyl) and of adenosyl-imidazolylcobamide (Ado-ImCba, R = 5′-Ado); *Right*: of cob(I)alamin (B12s)

structure of cob(II)alamin (B12r) has been of particular interest (Kräutler et al. 1989), as it is the product of Co–C bond homolysis of coenzyme B12 (AdoCbl), relevant for the catalytic cycle of coenzyme B12-dependent enzymes (see below).

Nuclear Magnetic Resonance (NMR) spectroscopy has become an important methodology for the study of (diamagnetic) B12 derivatives, in (aqueous) solution. Frequently, it is complementary to crystal structure analysis and is particularly valuable in such cases, where crystals of the B12-derivative are not obtained (Konrat et al. 1998; Brown 1999). Natural corrinoids from bacteria were often characterized by NMR-spectroscopy (Hoffmann et al. 2000). By applying so called homo- and hetero-nuclear NMR-experiments and high field NMR-spectrometers, the structure of B12-derivatives in aqueous solution has been studied, and their behaviour as dynamic molecules could be analyzed (Summers et al. 1986; Konrat et al. 1998).

17.2.2 B12-Redoxchemistry

Under physiological conditions vitamin B12-derivatives have been observed in the oxidation states Co(III), Co(II), and Co(I), each possessing differing coordination properties and reactivities (Pratt 1972; Kräutler 1998).

Oxidation-reduction processes are of key importance in the metabolism of B12. Electrochemical methods have been crucial in analytical studies of B12-derivatives (Lexa and Savéant 1983) (Fig. 17.3). Axial coordination to the corrin-bound cobalt centre depends on the formal oxidation state of the cobalt ion (Kratky and Kräutler 1999): As a rule, the number of axial ligands decreases in parallel with the cobalt oxidation state. The diamagnetic Co(III) (coordination number 6) typically has two axial ligands bound, the paramagnetic (low spin) Co(II) (coordination number 5) has one axial ligand and the diamagnetic Co(I) (coordination number 4) has no axial ligands bound (Pratt 1972; Lexa and Savéant 1983). Redox reactions of B12-derivatives are, therefore, accompanied by a change in the number of axial ligands. In turn, the presence and the nature of axial ligands strongly influences the (thermodynamic and kinetic features of the) electron transfer of cobalt corrins (Lexa and Savéant 1983; Kräutler 1999).

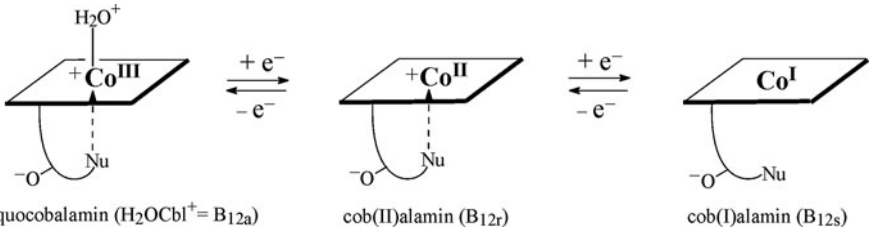

Fig. 17.3 Outline of the redox-transitions between the cob(III)alamin aquo-cobalamin (H_2OCbl^+, B12a), cob(II)alamin (B12r) and cob(I)alamin (B12s)

In Co(III)-corrins, such as vitamin B12 (CNCbl), coenzyme B12 (AdoCbl) and methyl-cobalamin (MeCbl), the corrin-bound cobalt centre is coordinatively saturated, when binding two axial ligands. In contrast, the metal centre in Co(I)-corrins, such as cob(I)alamin (B12s) is highly nucleophilic (with very low basicity) (Lexa and Savéant 1983). The intermediate oxidation level of the Co(II)-corrins, such as cob(II)alamin (B12r), provides a reactive metal-centred radicaloid species (Endicott and Netzel 1979; Kräutler et al. 1989). In neutral aqueous solution, cob(II)alamin (B12r) is present in its "base-on" form (i.e. the DMB-base is coordinating the Co(II)centre). B12r is converted into the "base-off" form, B12r-H^+, by protonation of the DMB-base, with pKa (B12r-H^+) = 2.9, and de-coordination from the Co(II)centre.

A standard potential vs. pH diagram has been established, which correlates the thermodynamics of the H_2OCbl^+-system (B12a /B12r /B12s) (Fig. 17.4) (Lexa and Savéant 1983). In the pH range 2.9–7.8, H2OCbl and (base-on) B12r represent the predominant Co(III)-/Co(II)-redox couple, with a standard potential of +0.20 V. For the Co(II)-/Co(I)-redox system there are two pH-independent standard potentials: at a pH less than 5.6 the Co(II)-/Co(I)-couple (base-off) B12r-H+/B12s-H predominates (standard potential of –0.50 V, at higher pH the redox couple (base-on) B12r / B12s exists with a more negative standard potential of –0.61 V.

The standard potential of the Co(III)-/Co(II)-redox pair of organometallic B12-derivatives, such as coenzyme B12 (AdoCbl) and methyl-cobalamin (MeCbl), is significantly more negative than that of B12r/B12s and out of the reach of biological reductants (Kräutler 1999). One-electron reduction of methyl-cobalamin to methyl-cob(II)alamin occurs at –1.36 V vs. NHE and demethylates MeCbl (at –30°C).

The thermodynamic trends of B12-redox systems can be summarized as:

(i) Intra-molecular coordination of the nucleotide base and/or strongly coordinating or nucleophilic ligands (such as cyanide ions) stabilize the cobalt centre against reduction and shift the Co(III)-/Co(II)-redox couples to more negative potentials.

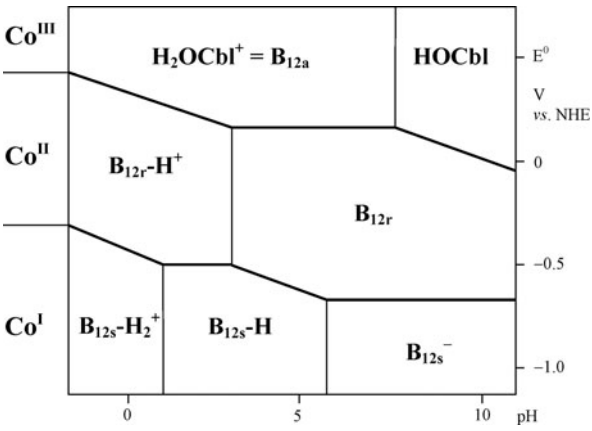

Fig. 17.4 Dependence of standard potentials of the redox system Co(III)-/Co(II)-/Co(I)-corrin (B12a/B12r/B12s) upon pH in aqueous solution (at 22°C), potentials are referenced to the normal hydrogen electrode (NHE) adapted from (Lexa and Savéant 1983)

(ii) The one-electron reduction of organometallic Co(III)-corrins typically occurs at more negative potentials than the Co(II)-/Co(I)-redox couple B12r/B12s and leads to loss of the organometallic ligand.

Electrochemistry is an excellent method for the selective production of reduced B12 forms. Since alkyl halides or alkyl tosylates react quickly and efficiently with Co(I)-corrins (Schrauzer and Deutsch 1969; Pratt 1972), which are cleanly generated at the electrodes, electrochemistry also is a method for the clean synthesis of most organometallic B12 derivatives (Kräutler 1999).

17.2.3 B12-Organometallic Reactivity

The reactivity of B12-derivatives in organometallic (redox-) reactions is the basis for their biological role as cofactors. Formation and cleavage of the Co–C bond in organometallic B12-cofactors are crucial steps in the reactions catalyzed by the B12-dependent enzymes (Kräutler et al. 1998; Banerjee 1999). In solution, cleavage and formation of the Co–C bond have been observed to occur in all of the basic oxidation levels for the cobalt centre of the corrin core (Kräutler and Ostermann 2003; Brown 2005).

Two mechanisms for these organometallic reactions have been found to be the basis for catalysis by the human cobalamin-dependent enzymes (Fig. 17.5):

(i) the homolytic mode, which involves the cleavage or formation of a single axial bond, as is typical of the reactivity of coenzyme B12 (AdoCbl): 5′-Ado-Co(III)-corrin $\rightleftarrows$ Co(II)-corrin + 5′-deoxy-5′-adenosyl radical
(ii) the nucleophile induced, heterolytic mode, which involves the cleavage or formation of two (trans-) axial bonds, as is typical of the reactivity of methylcobalamin (MeCbl): methyl-Co(III)-corrin + nucleophile $\rightleftarrows$ Co(I)-corrin + methylating agent

The homolytic mode of the cleavage of the Co–C bond of coenzyme B12 (AdoCbl) is of particular importance in its role as a cofactor. Indeed, thermal decomposition of AdoCbl in aqueous solution occurs readily at higher temperatures and leads predominantly to an adenosyl radical and cob(II)alamin (B12r) (Fig. 17.6). AdoCbl is thus considered to be a "reversible carrier of an alkyl radical" (or a reversibly functioning "radical source") (Halpern 1985). At room temperature (and in the dark), a neutral aqueous solution of AdoCbl is rather stable, with a calculated half life of 10^{10} s. The strength of the Co–C bond of AdoCbl (the homolytic Co–C bond dissociation energy = BDE) is about 30 kcal/mol (Halpern 1985; Hay and Finke 1986). The strength of the Co–C bond of AdoCbl is hardly influenced by the coordination of the nucleotide (Kräutler 1987, 1998).

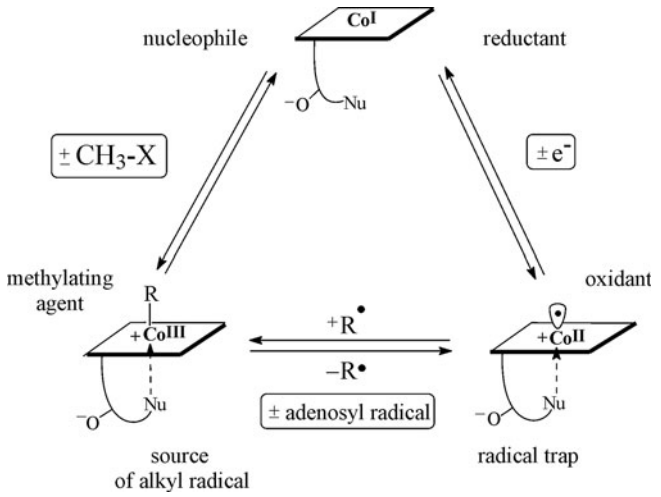

Fig. 17.5 Patterns of reactivity and formal analysis of elementary reaction steps of "complete" corrinoids in organometallic (redox-)transformations, relevant for their cofactor functions in B12-dependent enzymes

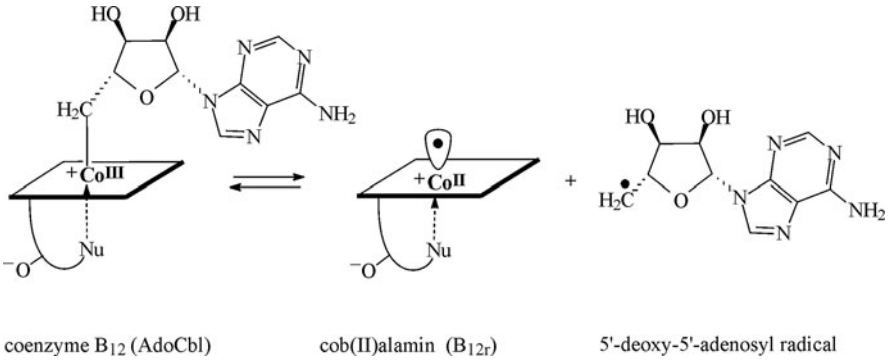

Fig. 17.6 Coenzyme B12 (AdoCbl), a reversibly functioning "radical source" (it provides a 5′-deoxy-5′-adenosly radical); in reverse, the "radical trap" B12r rapidly reacts with (5′-deoxy-5′-adenosyl) radicals to give organo-cob(III)alamins directly (such as AdoCbl)

The (crystal) structure of B12r is surprisingly similar to that of the cobalamin moiety of AdoCbl (Kräutler et al. 1989). The radicaloid B12r has a penta-coordinated Co(II)-centre, so that reactions with alkyl radicals (at its "upper" β-face) give hexa-coordinate organo-cob(III)alamins (such as AdoCbl) and occur with little restructuring of the corrin moiety: B12r thus fulfils the structural criteria of a very efficient "radical trap" and it reacts very fast (near diffusion control) with alkyl radicals (such as the 5′-deoxy-5′-adenosyl radical) (Endicott and Netzel 1979).

The second important type of organometallic reactivity of B12-derivatives represents a heterolytic mode of formation/cleavage of the Co–C bond and concerns

the inter-conversion between Co(I)-corrins and methyl-Co(III)-corrins, important in enzyme-catalysed methyl-transfer reactions (Kräutler et al. 1998; Banerjee 1999; Matthews 2001): Co(I)-corrins react as strong nucleophiles with methylating agents (the Co–CH3 bond is formed) and methyl Co(III)-corrins may be demethylated by nucleophiles (the Co–CH3 bond is cleaved).

Alkylation at the corrin-bound Co(I) centre of B12s normally proceeds via the "classical" bimolecular nucleophilic substitution (SN2) mechanism (Fig. 17.7), where the Co(I)-corrin acts as a "supernucleophile" (Schrauzer and Deutsch 1969). However, in certain cases alkylation of Co(I)-corrins occurs via an alternative two-step one-electron transfer path, where Co(I)-corrins act as strong one-electron reducing agents and the process proceeds via Co(II)-corrin intermediates (Kräutler and Caderas 1984; Kräutler 1998).

In Co(I)-corrins, like B12s, the nucleophilicity in alkylation reactions is virtually independent of the presence of the DMB-nucleotide. Co(I)-corrins, both "complete" and "incomplete", preferentially react at their β-face (Kräutler 1998). The immediate product of the β-alkylation is likely to be a penta-coordinate Coβ-alkyl-Co(III)-corrin (Fig. 17.7). Since most alkyl-cobalamins are hexa-coordinate in their more stable "base-on"-constitution, the methylation by the SN2-mode takes place in a two step mechanism. In aqueous solution and at room temperature methyl-cobalamin (MeCbl) is more stable in the "base-on"-form by about four kcal/mol than "base-off" Coα-aquo-Coβ-methyl-cobalamin (Kräutler 1987, 1998).

The reverse process, the nucleophile-induced dealkylations of methyl-Co(III)-corrins, is less studied. Intramolecular coordination of the nucleotide base stabilises MeCbl and slows the demethylation down: thiolates demethylate MeCbl to Co(I)corrinoids at approximately 1,000 times lower rate than methyl-corrinoids lacking a nucleotide base (Hogenkamp et al. 1985), reflecting the effect of the coordinated nucleotide (Kräutler 1987). Considerable axial base effects are thus expected for methyl-group transfer reactions involving enzyme-bound Co(I)- and methyl-Co(III)-corrins (Drennan et al. 1994; Kräutler 1998).

The two most relevant modes of formation and cleavage of the Co–C bond of the cobalt centre differ significantly in their structural requirements:

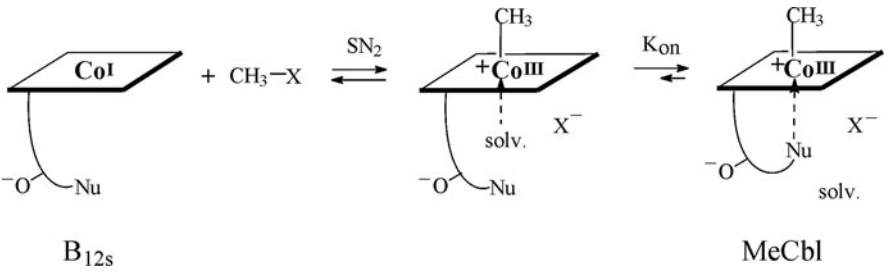

Fig. 17.7 The highly nucleophilic cob(I)alamin (B12s) reacts with methylating agents, to give methylcobalamin (MeCbl). For kinetic and thermodynamic reasons methylation of cob(I)alamin (B12s) occurs at the "upper" or β-face

– in the homolytic mode of cleavage, the complete cobalt-corrin part of organocobalamins (such as AdoCbl) hardly changes its structure (Fig. 17.6);
– in the heterolytic mode of cleavage and formation of the Co–C bond significant reorganization occurs at both faces of the corrin-bound cobalt centre. Cleavage of the Co–CH_3 bond is brought about by attack of a nucleophile at the readily accessible carbon of the cobalt-bound methyl group (Fig. 17.7).

Organocobalamins have long been know to be sensitive to visible light (Pailes and Hogenkamp 1968), which induces homolytic cleavage of the Co–C bond with a quantum yield of about 0.3 (Cole et al. 2002). Another mode of cleavage of the Co–C bond of organometallic B12-derivatives is the thermodynamically favourable radical-substitution at the cobalt-bound carbon centre, as observed in the demethylation of MeCbl by an organic radical (Kräutler et al. 1995; Mosimann and Kräutler 2000).

17.3 Occurrence of B12-Dependent Enzymes

B12-coenzymes are nowadays known to act as cofactors in four classes of distinct enzymes: B12-dependent methyl-transferases (Matthews 2001; Banerjee and Ragsdale 2003), B12-dependent isomerases and ribonucleotide reductases (both use AdoCbl) (Buckel and Golding 2006) and corrinoid dehalogenases (Siebert et al. 2002), some of which use a nor-vitamin B12-derivative (Kräutler et al. 2003). In addition, evidence for enzymatic catalysis of the biosynthetic incorporation of the organometallic groups of AdoCbl and of MeCbl (aside from such reactions in the methyl transferases and isomerases) is available (see below).

B12-dependent methyl transferases play an important role in amino acid metabolism in many organisms (including humans) as well as in one-carbon metabolism and CO_2 fixation in anaerobic microbes (Banerjee and Ragsdale 2003). The reactivity of the "supernucleophilic" Co(I)-corrins and of methyl-Co(III)-corrins make B12-derivatives ideal as cofactors in enzymatic methyl-group transfer reactions (Kräutler et al. 1998; Banerjee 1999; Kräutler and Ostermann 2003; Brown 2005). B12-dependent methionine synthase has been particularly well studied (see below and refs (Matthews 1999, 2001; Banerjee and Ragsdale 2003)) as have methyl transferases in methanogenesis (Sauer and Thauer 1999) and in aerobic acetogenesis (Ragsdale et al. 1998). In human metabolism, methionine synthase is crucial for the endogenous formation of methionine (Banerjee 1997).

About ten coenzyme B12-dependent enzymes are now known, which are distributed rather disproportionately in the living world (see refs (Kräutler et al. 1998; Banerjee 1999; Brown 2005)). Only one of these, the carbon skeleton mutase methylmalonyl-CoA mutase (hMMCM), is indispensable in human metabolism (Banerjee 1997). Three more microbial carbon skeleton mutases are known (besides MMCM (Banerjee and Chowdhury 1999)): glutamate mutase (Buckel and Golding 1996; Gruber and Kratky 2002), methylene glutarate mutase (Buckel and Golding

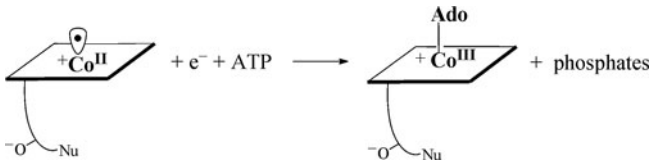

Fig. 17.8 Coenzyme B12 (AdoCbl, in its "base-off"-form) is biosynthesized (in humans) from "base-off" cob(II)alamin (B12r) by the mitochondrial enzyme adenosyltransferase

1996) and isobutyryl-CoA mutase (Zerbe-Burkhardt et al. 1999). In addition, five bacterial isomerases, diol and glycerol dehydratase, ethanolamine ammonia lyase and two amino mutases are B12-dependent, as is an anaerobic form of ribonucleotide reductase (Frey and Chang 1999; Stubbe 2000; Toraya 2003).

The labile and light sensitive coenzyme B12 (AdoCbl) is not typically taken up with the nutrition, but is enzymatically produced in the human mitochondrion by adenosyltransferase. A feature of this enzyme is its ability to bind cob(II)alamin (B12r) in a remarkable four coordinate "base-off"-constitution and, thus, to render the bound B12r more accessible to reduction by biological reducing agents (Stich et al. 2005). This biosynthetic enzyme catalyzes the reduction of B12r to cob(I)alamin (B12s) and adenosylation by ATP to give AdoCbl, which appears to be bound in a "base-off"-form also (Fig. 17.8) (Yamanishi et al. 2005). Direct delivery of AdoCbl in the "base-off"-constitution has been suggested from adenosyl-transferase to its target (apo-)enzyme, methylmalonyl-CoA mutase.

17.4 Methionine Synthase

Methionine synthase catalyzes the formation of methionine by methylation of homocysteine and demethylation of N5-methyl-tetrahydrofolate to tetrahydrofolate (Fig. 17.9). In B12-dependent methionine synthase, a methylcorrinoid, such as methyl-cobalamin (MeCbl) is the cofactor, whereas in cobalamin-independent methionine synthases, which have much slower turnover, homocysteine is merely activated by coordination to a zinc-centre (Matthews 2001).

17.4.1 B12-Dependent Methyl Group Transfer

In a catalytic cycle of B12-dependent methyl transferases (such as methionine synthase), the corrinoid cycles between enzyme-bound methyl-Co(III)- and Co(I)-forms (Matthews 2001). The transition between the hexa-coordinate methyl-Co(III)-form and the tetra-coordinate Co(I)-form must be accompanied by large structural changes (Figs. 17.5 and 17.7) and complementary changes in a protein environment.

This provides a means for controlling the organometallic reactivity of the bound cofactor at the same time (Kräutler 1998). The enzymatic methyl-group transfers

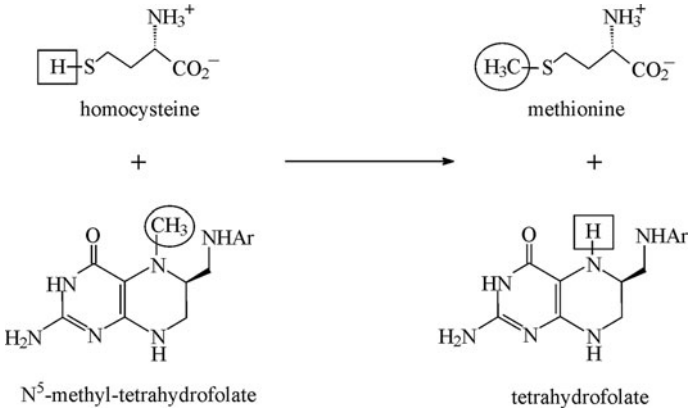

Fig. 17.9 Methionine synthase produces methionine and tetrahydrofolate from homocysteine and N5-methyl-tetrahydrofolate

(which formally involve nucleophile-induced heterolytic cleavage and formation of the organometallic Co–CH3 bond at the corrin bound cobalt centre) are thus expected to be subject to strict geometric control (mediated via the "regulatory" His-Asp-Ser-triad in methionine synthase (MetH) (Drennan et al. 1994; Matthews 2001), (Fig. 17.10 and below): The His-Asp-Ser-triad appears to have a dual role in MetH;

(i) it may participate in maintaining conformational control of the mutual placement of the corrinoid cofactors and the bound substrates (in their respective enzyme-modules) by a H^+-mediated conformational switch mechanism (Matthews 2001; Ludwig and Matthews 2002; Bandarian et al. 2003) and
(ii) it may exert a relevant thermodynamic effect on the strength of the Coβ–CH_3 bond by α-axial base-coordination (Drennan et al. 1994).

Solution studies had shown a significant thermodynamic trans-effect of the DMB-coordination in methyl-cobalamin (MeCbl) and stabilization of the methyl-Co(III)-corrin MeCbl by about 4 kcal/mol against nucleophilic abstraction of the methyl group (Kräutler 1987, 1998) (a similar effect on heterolytic methyl group transfer reactions was deduced for imidazole-coordination in methyl-imidazoyl-cobamide (Me-ImCba) (Fasching et al. 2000).

The methyl group may originate from N5-methyl-tetrahydropterins (such as N5-methyl-tetrahydromethanopterin or N5-methyl-tetrahydrofolate) and a variety of other methylated substrates (Stupperich et al. 1998; Matthews 2001). Typically, thiols are the methyl group acceptors in methionine synthesis (homocysteine) (Matthews 2001; Bandarian et al. 2003) and methanogenesis (coenzyme M) (Sauer and Thauer 1999). In the anaerobic biosynthesis of acetyl-coenzyme-A from one-carbon precursors the methyl group acceptor is suggested to be a nickel centre (Drennan et al. 2004).

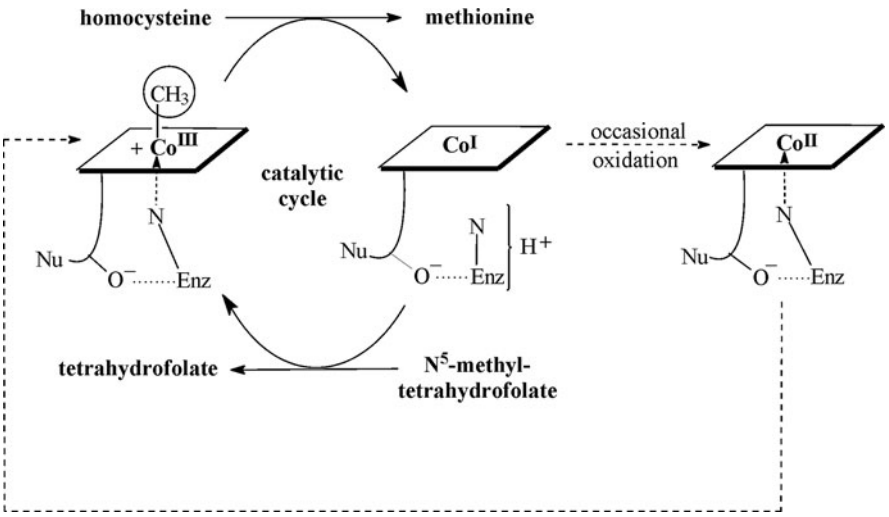

Fig. 17.10 Illustration of methionine formation catalyzed by MetH (Enz signifies the MetH-apoenzyme), where the bound corrinoid shuttles between MeCbl, in a "base-off/His-on" form, and cob(I)alamin (B12s) (Matthews 2001). In addition, reductive methylation with SAM and a reduced flavodoxin reactivates oxidized enzyme (with catalytically inactive cob(II)alamin)

17.4.2 Methionine Synthase from Escherichia coli

The methyl group transfer catalysed by methionine synthase from *E. coli* (MetH) (Matthews 2001; Ludwig and Matthews 2002) is indicated to proceed as a sequence of two nucleophilic displacement steps. This excludes free methyl cations or radicals as intermediates. The methyl group transfer relies on the inherent reactivities of enzyme-bound Co(I)corrins and methyl-Co(III)corrins (Matthews 2001). The great structural changes that must accompany the transitions from (tetra-coordinate) Co(I)corrins to (hexa-coordinate) methyl-Co(III)corrins (Kräutler 1998) also provide a means for control from the protein environment (on the methyl transfer, see above) (Ludwig and Matthews 2002).

MetH represents the most thoroughly studied B12-dependent methyl transferase (Matthews 1999, 2001; Bandarian et al. 2003). It is a modular enzyme containing four separate (and independently functioning) domains for binding (beginning at the N-terminus) homocysteine, N5-methyl-tetrahydrofolate, the B12-cofactor or S-adenosyl-methionine (SAM). The B12-binding domain interacts specifically with each of the other three domains (depending upon its three oxidation states): The Co(I) form with the N5-methyl-tetrahydrofolate domain, the Co(II) form with the SAM domain, and the CH3–Co(III) form with the homocysteine domain.

MetH catalyses the methylation of the bound and reduced cob(I)alamin cofactor by (N5-protonated) N5-methyltetrahydrofolate to give enzyme-bound MeCbl in a "base-off/His-on" form (see later) (Matthews 2001; Ludwig and Matthews 2002; Bandarian et al. 2003). The bound MeCbl is demethylated by homocysteine

(Fig. 17.10), whose sulfur is activated and deprotonated due to the coordination to a zinc ion (held by three cysteine residues) of the homocysteine domain. The two methyl-transfer reactions occur in a rapid sequential mechanism (kcat (MetH) = 27 s^{-1}) (Jarrett et al. 1996; Ludwig and Matthews 2002). The bound cob(I)alamin (B12s) may adventitiously be oxidized to inactive cob(II)alamin (B12r) and requires reactivation by reductive methylation with SAM and a reduced flavodoxin (Ludwig and Matthews 2002).

X-ray crystal analysis of the 28 kDa B12-binding domain of MetH provided the first insight into the three-dimensional structure of a B12-binding protein (Drennan et al. 1994, 1998; Ludwig and Matthews 2002). The cobalt-coordinating DMB-nucleotide tail of the protein-bound cofactor MeCbl was displaced by a histidine and extended into the core of the "Rossmann fold", two most astounding revelations of this work (Drennan et al. 1994). Consequently, in MetH the corrinoid cofactor is bound "base-off" and in a "base-off/His-on"-mode, due to histidine ligation to the metal centre. The crucial cobalt-ligating histidine residue is part of a Gly–X–X–His–X–Asp sequence, which was noticed to be a common sequence in some B12-binding proteins (Marsh and Holloway 1992). The B12-binding domain of MetH, therefore, provides both an anchoring site for the nucleotide tail and cobalt-ligation via the residues of the ("regulatory") His-Asp-Ser triad, holding the corrinoid cofactor with its "catalytic" β-side exposed at an inter-domain interface.

Further crystallographic studies provided more evidence for how the selection between the two ways of methylating the bound corrinoid (the "productive" methylation by N5-methyl-tetrahydrofolate or the "regenerative" methylation by enzyme bound SAM (Ludwig and Matthews 2002)) are achieved. This is controlled by a dynamic domain alternation (Bandarian et al. 2002). As shown by the crystal structure of the N-terminal (two domain) substrate-binding modules of MetH, the two substrates, homocysteine and N5-methyl-tetrahydrofolate, are bound and activated in orientations that position them for reaction with the bound corrinoid. However, the two active sites are separated by ≈ 50 Å and the B12-binding domain must shuttle back and forth between these distant active sites to complete the catalytic cycle (Evans et al. 2004).

The crystal structure and the finding of the "base-off/His-on" binding of the B12-cofactor in MetH explained ESR-spectroscopic evidence for histidine binding to the cobalt centre of p-cresolyl-cobamide (a "base-off" cobamide, with a non-coordinating cresolyl-"nucleotide") in the acetogen Sporomusa ovata (Stupperich et al. 1990).

17.4.3 Human Methionine Synthase

Human methionine synthase (hMS, E.C. 2.1.1.13) is a cytoplasmic enzyme with 4 domains, which shares 55% identity in deduced amino acid sequence with MetH (from *E. coli*). hMS shows extensive structural similarities with MetH and has all residues implicated in relevant B12-binding to MetH. hMS also catalyzes the methylation of homocysteine (with demethylation of N5-methyl-tetrahydrofolate)

in an apparently related mechanism to that of MetH (Banerjee 1997; Banerjee 1998). In both methionine synthases occasional oxidation (to an inactive cob(II)alamin carrying form) occurs (in the micro-aerophilic conditions of mammalian cells, hMS is oxidized about once every 2,000 turnovers). In contrast to MetH, which is reactivated via reduced ferredoxin (Matthews 2001), hMS is reactivated by a specific reductase, called hMS-reductase (hMSR) (Yamada et al. 2006). hMSR is a dual flavoprotein homologous to flavodoxin and appears to function as a chaperone in the assembly of holo-hMS from apo-hMS and B12a in the presence of NADPH (it also achieves the reduction of aquo-cobalamin (B12a) to B12r) (Yamada et al. 2006) and to reactivate oxidized hMS.

17.5 Methylmalonyl-CoA Mutase

The reversible isomerisation of methylmalonyl-CoA to succinyl-CoA is an enzyme catalyzed radical process (Fig. 17.11). Related isomerisations (malonates to succinates) have been achieved in model radical processes in solution (Halpern 1982; Buckel and Golding 2006).

17.5.1 Coenzyme B12-Dependent Enzymes

Coenzyme B12-dependent enzymes, such as methylmalonyl-CoA mutase (Fig. 17.11), thus performs "difficult" transformations. With the exception of the enzymatic ribonucleotide reduction (Stubbe et al. 1998), the results of coenzyme B12-catalysed enzymatic reactions correspond to isomerisations with vicinal exchange of a hydrogen atom and of a larger moiety (Buckel and Golding 1996). Homolytic cleavage of the Co–C bond of the protein-bound AdoCbl to a 5′-deoxy-5′-adenosyl radical and cob(II)alamin (B12r) (Fig. 17.6) was shown to be the entry to H-abstraction reactions induced by the 5′-deoxy-5′-adenosyl radical (Rétey 1999). Therefore, homolysis of the Co–C bond of AdoCbl is its biologically most significant reactivity (Halpern 1985; Finke 1998). The coenzyme B12-catalysed enzyme reactions occur with maximal rates of approximately 100 s^{-1} (Licht et al. 1999; Marsh and Drennan 2001). With MMCM, rapid formation of

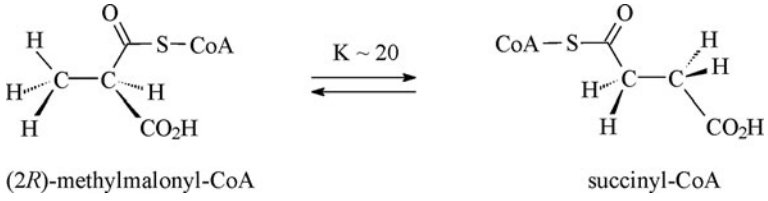

Fig. 17.11 (2R)-methylmalonyl-CoA is reversibly converted into succinyl-CoA (see below)

Co(II)corrins was observed when substrate was added to a solution of holoenzyme (or of apoenzymes and AdoCbl) (Rétey 1998).

Homolysis of the Co–C bond of protein-bound AdoCbl needs to be accelerated by a factor of $>10^{12}$ to relate the observed rates of catalysis by the coenzyme B12-dependent enzymes to the rate of homolysis of AdoCbl in aqueous solution (Halpern 1985; Finke 1998). This dramatic labilisation of the bound cofactor towards homolysis of the Co–C bond is an intriguing feature of coenzyme B12-dependent enzymes (Halpern 1985; Finke 1998). The mechanism of the (enzyme- and substrate-) induced labilisation of AdoCbl towards cleavage of its Co–C bond is a much discussed problem. A "conformational distortion" of the cobalt-corrin part of AdoCbl was suggested as its basis (Halpern 1985). However, the crystal structures of several coenzyme B12-dependent enzymes (Mancia et al. 1996; Reitzer et al. 1999; Shibata et al. 1999) and of cob(II)alamin (B12r) (Kräutler et al. 1989) have not supported this mode of action. Labilisation of the Co–C bond appears to mostly result from protein- and substrate-induced strain on the organometallic group, separation of the largely non-strained homolysis fragments and strong binding of the separated pair, 5′-deoxy-5′-adenosyl radical and B12r (in "base-off/His-on" or "base-on" form) (Kräutler et al. 1989; Toraya 2003; Gschösser et al. 2005). Fixed placement of the corrin moiety at the interface of the B12-binding and substrate-binding/activating domains is given a high significance and movements of the corrin moiety are not required. The nature of the axial trans ligand (histidine or DMB) has a lesser effect on the homolytic Co–C bond dissociation (Fasching et al. 2000). The "regulatory triad" appears not to be involved in proton-transfer steps and may conserve its structure largely during enzymatic turnover (Ludwig and Evans 1999).

The 5′-deoxy-5′-adenosyl radical is the established reactive partner in the actual coenzyme B12-dependent enzymatic reactions, so that AdoCbl functions as a "pre-catalyst" (or catalyst precursor) (Kräutler 1998). The further enzyme reactions all involve bound organic radicals, which are formed (directly or indirectly) by a H-atom abstraction by the 5′-deoxy-5′-adenosyl radical (that originates from AdoCbl). The rearrangement steps of B12-dependent enzymatic rearrangements are assumed to be accomplished by tightly protein-bound radicals that are controlled in their reaction space by the protein (Toraya 2003; Buckel and Golding 2006). The role of the Co(II)-corrin fragment of the coenzyme (as a "spectator" or a "conductor") is a matter of ongoing discussions (Buckel et al. 2006). The major functions of the enzyme concern not only the catalysis of its proper reactions but also the reversible generation of the radical intermediates and the effective protection from non-specific radical chemistry, called "negative catalysis" (Rétey 1990).

Four of the known coenzyme B12-dependent enzymatic reactions concern carbon skeleton rearrangements, in which two vicinal groups (a hydrogen atom and an organic substituent) exchange their positions in a (pseudo-) intra-molecular fashion (Marsh and Drennan 2001; Buckel and Golding 2006). The B12-cofactor is bound "base-off/His-on" at an interface between two modules, the B12-binding and substrate activating domains (or subunits) as revealed by the analysis of the crystal structures of methylmalonyl-CoA mutase (MMCM) from *Propionibacterium shermanii* (Hodgkin et al. 1956) and of glutamate mutase from Clostridium cochlearium

(Reitzer et al. 1999; Gruber and Kratky 2002). The B12-binding motif (Gly–X–X–His–X–Asp) (Marsh and Holloway 1992) is common to the carbon skeleton mutases (Marsh and Drennan 2001). The B12-binding domain of MMCM exhibits considerable sequence homology, even with the B12-binding domain of MetH (Ludwig and Evans 1999). Such homology can be rationalized by the related way of "base-off/His-on" binding the cobalamin cofactor and does not extend to the substrate binding domains (subunits) of the carbon skeleton mutases (Marsh 2000).

17.5.2 Methylmalonyl-CoA Mutase from Propionibacterium shermanii

Methylmalonyl-CoA mutase has been most thoroughly studied as the *P. shermanii* mutase (MMCM), which interconverts (2R)-methylmalonyl-CoA and succinyl-CoA (Banerjee and Chowdhury 1999) (Fig. 17.12). The main function of MMCM in *P. shermanii* concerns the formation of (2R)-methylmalonyl-CoA from succinyl-CoA. Binding of the substrate to holo-MMCM triggers a very fast homolysis of the Co–C bond of the bound AdoCbl. The overall turnover for the radical rearrangement occurs with a rate of about 60 s^{-1} at 25°C. The 5′-deoxy-5′-adenosyl (5′-Ado) radical induces the rearrangement reaction by abstracting an H-atom from the methyl group of enzyme-bound methylmalonyl-CoA. A large deuterium isotope effect of the abstracted H-atoms on the rate of homolysis of the Co–C bond was observed (Chowdhury and Banerjee 2000a). Labilisation of the Co–C bond towards homolysis by about 16 kcal/mol is due largely to a decrease of the activation enthalpy (Chowdhury and Banerjee 2000b). Recent studies (Brooks et al. 2004) indicated the stabilisation of the separated homolysis fragments, 5′-Ado radical and cob(II)alamin (B12r) to be a dominant contribution to the activation (for homolysis) of the Co–C bond of AdoCbl, consistent with an earlier suggestion, based on the crystal structure of B12r (Kräutler et al. 1989).

H-atom abstraction gives the 2-methylmalon-2′-yl-CoA radical which rearranges rapidly to the succin-3-yl-CoA radical (Golding et al. 1998; Banerjee and Chowdhury 1999). Both, fragmentation/ recombination and intramolecular addition/elimination, via a cyclopropyloxyl radical, pathways have been considered for this rearrangement (Buckel and Golding 2006). However, computational studies indicate that the energetic barrier for the addition/elimination pathway is lower than the dissociative pathway (Smith et al. 1999). Experimental evidence has also been used to support this theory, in which (i) the succin-3-yl-CoA radical arises from an intramolecular radical rearrangement and (ii) occurs without noticeable participation of the bound cob(II)alamin (B12r) (Golding et al. 1998). The succin-3-yl-CoA radical (resulting from the rearrangement) then re-abstracts an H-atom from 5′-deoxyadenosine to give succinyl-CoA and the 5′-deoxy-5′-adenosyl radical (which recombines with B12r to give enzyme-bound AdoCbl).

X-ray analysis of the 150 kDa heterodimeric MMCM from *P. shermanii* was the first accurate structure of a coenzyme B12-dependent enzyme. It showed the B12-cofactor to be bound "base-off/His-on". The cobalt centre was coordinated to

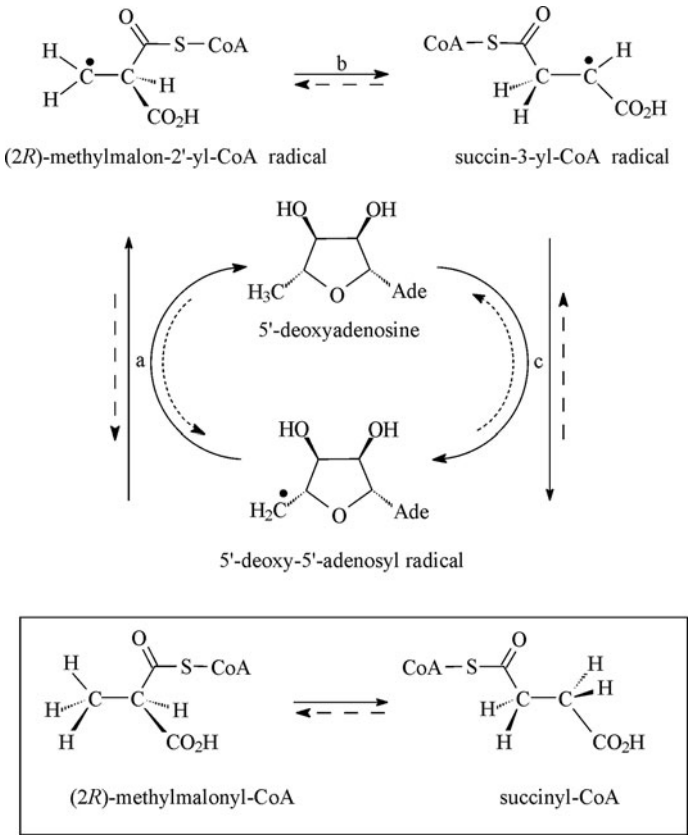

Fig. 17.12 Methylmalonyl-CoA mutase (MMCM) interconverts (2R)-methylmalonyl-CoA and succinyl-CoA. Proposed reaction mechanism, involving H-atom abstraction (**step a**), radical rearrangement (**step b**) and back transfer of H-atom (**step c**). The 5′-deoxy-5′-adenosyl radical (and cob(II)alamin (B12r)) originate(s) from homolysis of protein bound AdoCbl (Fig. 17.6) (Banerjee and Chowdhury 1999; Buckel and Golding 2006)

the histidine of the "regulatory triad" His-Asp-Lys. As in MetH the nucleotide tail of the corrinoid was tightly inserted into the protein and the corrinoid was bound at an interface between two domains (Evans and Mancia 1998; Ludwig and Evans 1999).

The question of how enzymes recognize and bind their corrinoid cofactor in a "base-off"-form is intriguing (in aqueous solution, AdoCbl and MeCbl prefer to be "base-on"). The solution structure of the cofactor-free B12-binding subunit of glutamate mutase from Cl. tetanomorphum (Tollinger et al. 1998, 2001), derived by NMR-spectroscopy, showed the B12-binding subunit to be largely pre-organized for B12-binding. However, the apo-protein was seen to include a flexible loop and a "nascent" helix, which were both suggested to structure upon binding of the B12-cofactor. A model for the events in binding of the "base-off/His-on" corrinoid by the

B12-binding subunit was derived, in which the "base-off"-form of the B12-cofactor was trapped by its nucleotide tail and the bound nucleotide moiety, in turn, stabilized the protein (Tollinger et al. 2001).

In crystallographic work, substrate free MMCM was investigated, as well as with bound pseudo-substrate: both structures indicated the organometallic group to be strained or detached from the cobalt centre (Mancia and Evans 1998; Mancia et al. 1999). Substrate binding appears to squeeze the adenosyl group off from the cobalt-corrin and to assist Co–C bond homolysis (Ludwig and Evans 1999).

17.5.3 Human Methylmalonyl-CoA Mutase

Human methylmalonyl-CoA mutase (hMMCM, EC 5.4.99.2) is a mitochondrial enzyme and catalyzes the isomerisation of (2R)-methylmalonyl-CoA to succinyl-CoA (as final part of the catabolic pathway of uneven numbered and branched chain fatty acids) (Banerjee and Chowdhury 1999). Impairment of hMMCM leads to methyl-malonic aciduria, a metabolic error that leads to developmental retardation and mortality in infants. hMMCM binds two AdoCbl per (homodimeric) enzyme (predicted mass: about 159 kDa) and is specific for the 2R-epimer of methylmalonyl-CoA. The (monomer-)chain of hMMCM and the B12-binding α-chain of MMCM are homologous; evidence for a related mechanism in both mutases has been provided (Buckel and Golding 2006). Apo-hMMCM has been suggested to be loaded with its cofactor, AdoCbl, in a direct and "targeted" interaction with adenosyl-transferase. In the presence of the latter enzyme, hMMCM exhibits a striking resistance towards deactivation (Yamanishi et al. 2005).

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Chapter 18
Physiological and Molecular Aspects of Cobalamin Transport

Sergey N. Fedosov

Abstract Minute doses of a complex cofactor cobalamin (Cbl, vitamin B12) are essential for metabolism. The nutritional chain for humans includes: (1) production of Cbl by bacteria in the intestinal tract of herbivores; (2) accumulation of the absorbed Cbl in animal tissues; (3) consumption of food of animal origin. Most biological sources contain both Cbl and its analogues, i.e. Cbl-resembling compounds physiologically inactive in animal cells. Selective assimilation of the true vitamin requires an interplay between three transporting proteins – haptocorrin (HC), intrinsic factor (IF), transcobalamin (TC) – and several receptors. HC is present in many biological fluids, including gastric juice, where it assists in disposal of analogues. Gastric IF selectively binds dietary Cbl and enters the intestinal cells via receptor-mediated endocytosis. Absorbed Cbl is transmitted to TC and delivered to the tissues with blood flow. The complex transport system guarantees a very efficient uptake of the vitamin, but failure at any link causes Cbl-deficiency. Early detection of a negative B12 balance is highly desirable to prevent irreversible neurological damages, anaemia and death in aggravated cases. The review focuses on the molecular mechanisms of cobalamin transport with emphasis on interaction of corrinoids with the specific proteins and protein-receptor recognition. The last section briefly describes practical aspects of recent basic research concerning early detection of B12-related disorders, medical application of Cbl-conjugates, and purification of corrinoids from biological samples.

Keywords Adsorbent · Affinity binding · Analogues · B12 · Cobalamin · Conjugates · Haptocorrin · Intrinsic factor · Receptor · Transcobalamin

18.1 Introduction

Cobalamin (Cbl, vitamin B12) is the most complex cofactor existing in the nature (Pratt 1972; Kräutler and Ostermann 2003), Fig. 18.1. Its molecule consists of two invariable moieties: (i) the corrin ring with cobalt ion; and (ii) the nucleotide loop

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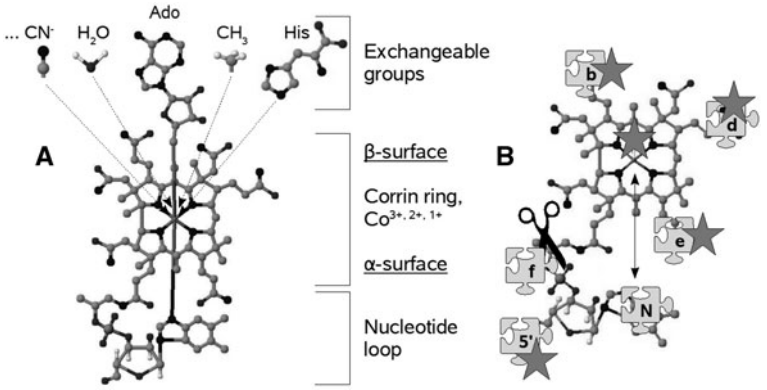

Fig. 18.1 Schematic structures of (**a**) cobalamins; and (**b**) cobalamin analogues containing substitutes (*puzzle symbols*). *Stars* indicate potential sites of chemical conjugation

coordinated to cobalt at the lower α-surface. The third component, the active group coordinated to cobalt at the upper β-surface, is exchangeable. A few typical β-groups are shown in Fig. 18.1, but only two of them, 5′-deoxy-adenosyl (Ado) and methyl (Me), form a physiologically active cofactor. The three major cobalamins in biological objects are AdoCbl (≈60%), MeCbl (≈20%) and H2OCbl (≈20%), (Mervyn 1971; Linnell et al. 1974; Quadros et al. 1979).

The most abundant natural cobalamin is AdoCbl. In animal cells it is involved in catabolism of fatty acids with a branched structure or an odd number of carbons (Banerjee and Ragsdale 2003). The process takes place inside mitochondria, where an AdoCbl-dependent enzyme converts a metabolite methylmalonic acid (MMA) to succinate. Inadequate Cbl concentration results in high level of MMA in blood plasma which is considered as a characteristic indication of Cbl-deficiency (Carmel et al. 2003).

The second cofactor, MeCbl, acts in cytoplasm and mediates transfer of Me-group from the inactive form of folate (methyl tetrahydrofolate, MeTHF) to homocysteine (Hcy), (Carmel et al. 2003; Toohey 2006). This process reactivates THF and produces methionine, precursor of a methylating agent S-adenosyl methionine (SAM). Thus, MeCbl controls most methylation reactions including THF-dependent synthesis of DNA and SAM-dependent methylation of lipids and creatine precursor guanidinoacetate. Low activity of MeCbl-dependent enzyme causes insufficient production of erythrocytes and components of nerve membranes. This metabolic disorder is exposed by elevated Hcy in blood plasma (Carmel et al. 2003).

The two cofactors are occasionally converted to H2OCbl because of abnormal termination of their catalytic cycles (Kräutler and Ostermann 2003; Banerjee and Ragsdale 2003). This makes H2OCbl the third ubiquitous cobalamin available from the natural sources. Cobalamins with the upper group differing from Ado and Me (e.g. water, cyanide, histidine, glutathione) cannot perform catalysis but are easily

transformed to the cofactors inside the animal cell. Although all the above forms serve as vitamins, the notation "B12" corresponds, strictly speaking, to CNCbl, the main industrial form of the vitamin (Martens et al. 2002).

Beside true cobalamins, there is a large group of Cbl-like molecules of natural or synthetic origin called analogues (Pratt 1972; Kräutler and Ostermann 2003). The differences between these molecules and vitamin B12 are related to other structural elements than the upper group (Fig. 18.1b). The most typical natural analogues have either substituted or absent nucleotide (Fig. 18.1b, positions N, f). Structural changes in the nucleotide often reduce its affinity for cobalt ion (Kräutler and Ostermann 2003). The analogues are inactive inside the animal cells, but many of them can catalyse Cbl-dependent reactions in bacteria (Pratt 1972; Kräutler and Ostermann 2003). Both cobalamins and analogues belong to a common group called "corrinoids" (Cor).

18.2 Nutritional and Physiological Facets of Cobalamin Intake

18.2.1 Dietary Cbl and Its Gastrointestinal Absorption

The process of Cbl assimilation is rather complex. Firstly, this cofactor is produced exclusively by bacteria (Pratt 1972; Kräutler and Ostermann 2003; Martens et al. 2002), and secondly, the amount of Cbl available from natural sources is extremely low (Mervyn 1971; Fedosov et al. 1996; Ortigues-Marty et al. 2005). Yet, vertebrates manage to provide themselves with sufficient amount of Cbl from either internal or external sources. Thus, herbivores (Fig. 18.2a) obtain the vitamin from bacteria inhabiting their digestive tract (Pratt 1972; Ortigues-Marty et al. 2005). These microorganisms convert indigestible cellulose to monosaccharides and synthesize corrinoids, sharing the excessive products with their host. Cbl is accumulated in animal tissues, where it can be stored for years or utilized. It is known that low amount of cobalt in the soil causes malproduction of bacterial Cbl with accompanying undergrowth of microorganisms in the fore-stomach of a grassing cow (Pratt 1972; Ortigues-Marty et al. 2005). The consequence is a low velocity of grass fermentation and starvation of the animals.

Humans are ultimately dependent on animal sources of vitamin B12: meat, milk, eggs, etc. (Mervyn 1971; Fedosov et al. 1996; Ortigues-Marty et al. 2005), Fig. 18.2b. The highest concentration of Cbl ever reported (5,000 nmol/kg) was found in a Japanese salted and fermented salmon kidney "Mefun" (Adachi et al. 2005). The normal daily dose of vitamin B12 for an adult human corresponds to 1–2 nmol of Cbl (1 nmol = 1.58 μg of AdoCbl or 1.35 μg of CNCbl). This is an extremely low amount when compared to other vitamins, but limited availability of Cbl is compensated by an efficient and complex capturing mechanism. Three transporting proteins – intrinsic factor (IF), transcobalamin (TC) and haptocorrin (HC) – as well as a number of receptors, are the key players in this process (Allen 1975; Nexø 1998; Moestrup 2006). Gastrointestinal uptake of B12 includes several

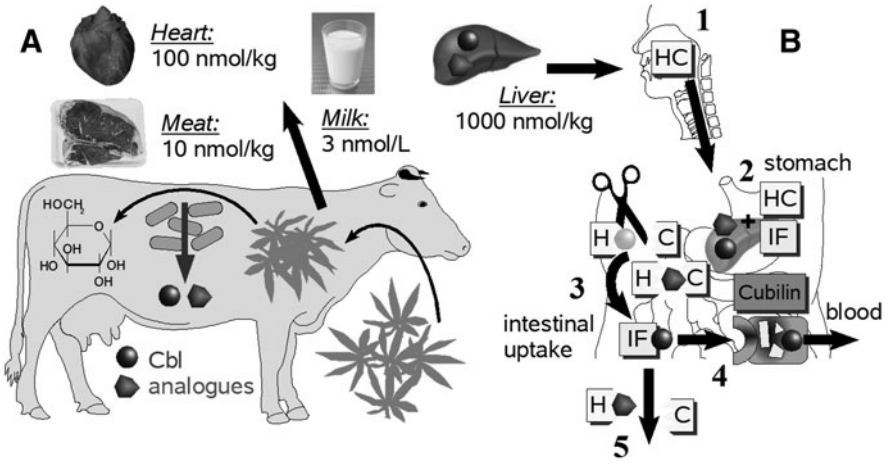

Fig. 18.2 Assimilation of cobalamin by (**a**) ruminant animals; and (**b**) humans

steps as depicted in Fig. 18.2b. First step might occur in the mouth, where the corrin-binding glycoprotein HC (called previously R-binder) is secreted to saliva at concentration of ≈50 nM (Nexø et al. 1988), Fig. 18.2b, step 1. Specificity of this carrier is low, and it binds both vitamin B12 and Cbl-analogues. Nevertheless, it hardly does so in the mouth, since the ligands are not yet released from the food components. HC of salivary origin proceeds to the stomach, where some amount of gastric HC is also produced. Total concentration of HC in gastric juice can be estimated as ≈10 nM (Allen et al. 1978). Simultaneous effect of low pH and proteolytic activity of pepsin causes liberation of 60–70% of corrinoids encapsulated in food (Del Corral and Carmel 1990; Kittang and Schjonsby 1987). They interact primarily with HC (Fig. 18.2b, step 2) despite high excess of another Cbl-binding glycoprotein, gastric IF ≈ 50 nM (Del Corral and Carmel 1990; Allen et al. 1978b; Kittang and Schjonsby 1987). Interaction of IF with Cbl is pH sensitive; therefore this carrier binds the vitamin only in the intestine after the digest is neutralized (Allen et al. 1978a, b; Gueant et al. 1986). Pancreatic enzymes accomplish liberation of the dietary Cbl; in addition, they cleave HC in two fragments (Allen et al. 1978a; Gueant et al. 1986). Nicked HC partially loses its affinity for Cbl, and the major part of the vitamin (≈80%) transfers to IF (Allen et al. 1978a, Gueant et al. 1986), Fig. 18.2b, step 3. The latter carrier has a preference for the true vitamin and serves as the first filter sorting out Cbl-analogues (Allen et al. 1978b; Gueant et al. 1984). The produced IF·Cbl complex is recognized by the receptor cubilin (Moestrup 2006) and internalized in the distal ileum, Fig. 18.2b, step 4. The unsaturated IF does not bind to cubilin and is gradually digested (Gueant et al. 1984; Gordon et al. 1991). The specific intestinal assimilation of Cbl is exceptionally efficient and guarantees uptake of the minute vitamin doses, passive diffusion accounts for only ≈1% of absorption (Mervyn 1971).

In contrast to a widespread citation, HC is not completely proteolysed and HC-fragments with the remaining Cbl-binding activity were found even in faeces (Gueant et al. 1984). They seem to retain the bound Cbl-analogues, since faecal excretions are enriched by corrinoids differing from Cbl (Gueant et al. 1984), Fig. 18.2b, step 5.

18.2.2 Transport of Cbl in Blood

Two carriers of Cbl, protein TC ≈ 0.8 nM and glycoprotein HC ≈ 0.4 nM, are present in blood due to internal secretion (Allen 1975; Nexø 1998; Moestrup 2006). Thus, practically all tissues synthesize TC, though the patterns vary among different species. The highest production is typically associated with kidney, lymphatic nodes and liver (Li et al. 1994; Fedosov et al. 1999). TC circulates mostly as the unsaturated apo-form (Allen 1975; Nexø 1998), which is fairly fast-metabolising with $t_{1/2}$ ≈ 1 h (Allen 1975). The Cbl-saturated form holoTC ≈ 0.06 nM accounts for ≈10% of the TC-pool under steady-state (Hvas et al. 2006; Miller et al. 2006, Nexø et al. 2002; Ulleland et al. 2002). Clearance of TC·Cbl from blood plasma is biphasic, when monitoring radioactive Cbl associated with TC (Hom and Olesen 1969). The half-life times were estimated as ≈1 (Allen 1975; Hom and Olesen 1969) and ≈15 h (Carkeet et al. 2006; Hom and Olesen 1969).

The second plasma transporter, HC, exists in two variants, which are probably secreted by liver (Boisson et al. 1999) and granulocytes (Allen 1975). The essential difference between the two forms is composition of the attached carbohydrates (Allen 1975). Thus, high content of sialic acids in HC of the assumed liver origin (called also form I) slows down the protein metabolism to $t_{1/2}$ ≈ 10 days (Allen 1975; Hom and Olesen 1969). On the contrary, HC from granulocytes (form III) has low amount of sialic acids and is cleared very rapidly by liver ($t_{1/2}$ ≈ 5 min) without discrimination between HC·Cbl and HC (Allen 1975). Blood plasma under steady state conditions contains almost exclusively the slow metabolising form saturated by 80% with Cbl, which corresponds to holoHC ≈ 0.3 nM (Allen 1975; Mørkbak et al. 2006).

The scheme of Cbl transport cycle is depicted in Fig. 18.3. At the first step, intestinal IF·Cbl degrades in lysosomes of enterocytes, and the vitamin is released into blood (Fig. 18.3, step 1). Most of Cbl becomes attached to TC, and 7 h after ingestion of B12 the plasma level of TC·Cbl (holoTC) increases by 50% (Carkeet et al. 2006; Bor et al. 2004). Prevalence of TC·Cbl complex can be explained by either (i) high excess of the unsaturated TC in plasma (Allen 1975), or (ii) binding of Cbl to TC already inside the intestinal cells (Quadros et al. 1999). The main targets for the newly produced plasma holoTC are liver (Allen 1975) and kidney (Alsenz et al. 2000).

Hepatocytes bind TC·Cbl (Fig. 18.3, step 2) and to some extent TC via a ubiquitous TC-receptor (Moestrup 2006; Quadros et al. 2005, Seetharam and Li 2000; Seligman and Allen 1978; Nexø and Hollenberg 1980), which identity is still debated (Quadros et al. 2005, Seetharam and Li 2000). Absorbed holoTC degrades

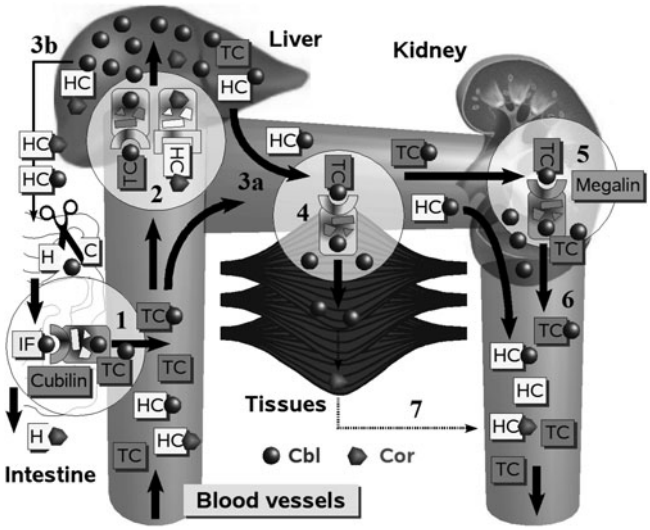

Fig. 18.3 Transportation of Cbl in blood

inside liver cells, and the liberated Cbl is partially stored, partially secreted back to blood bound to HC or TC of liver origin (Allen 1975; Hom and Olesen 1969; Bor et al. 2004), Fig. 18.3, step 3a. Primary absorption of TC·Cbl complex by the liver and its following resynthesis and recirculation may explain the biphasic clearance of TC·Cbl (Hom and Olesen 1969). Liver seems to verify the physiological usefulness of absorbed corrinoids, whereupon the TC-bound vitamin is distributed among other tissues and transferred to the enzymes, Fig. 18.3, step 4.

Cbl turnover in kidney stands apart from that in other organs. Thus, both TC and TC·Cbl are prone to glomerular filtration (≈10% sieving) because of their relatively small size, M ≈ 46 kDa, r ≈ 2.5 nm (Moestrup 2006; Nexø 1998). The filtered TC·Cbl complex is reabsorbed from primary urine by the specific receptor megalin, Fig. 18.3 step 5, (Moestrup 2006; Birn 2006). The endocytosed protein degrades, but the vitamin returns to blood bound to freshly synthesized TC, Fig. 18.3 step 6. Therefore, kidney both produces significant quantities of TC (Li et al. 1994) and temporarily stores Cbl, which concentration reaches 500 nmol/kg (Mervyn 1971).

The role of the other Cbl-transporting protein, plasma HC, is not clarified. Thus, HC seems to be absorbed only by liver (Allen 1975). It is not subjected to glomerular filtration due to heavy glycosylation, which increases its apparent radius to ≈ 4.5 nm (Nexø et al. 1988). HC appears to be a scavenger occasionally capturing products of Cbl degradation, Fig. 18.3, step 7. These Cbl-analogues account for 10–20% of all corrinoids present in the tissues and plasma (Gueant et al. 1984; Djalali et al. 1990; Kanazawa and Herbert 1983; Kondo et al. 1980). Fast and slow metabolising forms of HC are expected to deliver all bound corrinoids to liver interacting with a galactose-specific receptor expressed on the surface of hepatocytes (Allen 1975; Nexø 1998). Absorbed HC generally degrades, and the corrinoids are stored

or secreted to blood as TC·Cbl (Allen 1975) or HC·Cbl (Hom and Olesen 1969), Fig. 18.3, step 3a. Approximately 10% of the endocytosed HC-corrinoid complexes are excreted to bile without degradation, 2–4 nmol/day (Allen 1975; Fedosov et al. 2005b), Fig. 18.3, step 3b. The latter process seems to be associated with disposal of Cbl-analogues.

18.3 Molecular Aspects of Cbl Binding and Receptor Recognition

18.3.1 Structure of the Cbl-Transporting Proteins

Structural investigations of Cbl-transporting proteins have made an essential progress in recent years. Successful DNA cloning of HC (Allen 1975), IF (Hewitt et al. 1991) and TC (Platica et al. 1991) supplied information about their primary structures and pointed to homology between these proteins, Fig. 18.4. The Cbl-binders have similar molecular masses of 45.5 kDa (TC), 43.4 kDa (IF), 45.6 kDa (HC) and demonstrate significant pairwise identity of 23–30%. Expression of TC (Quadros et al. 1993; Fedosov et al. 1999, 2000) and IF (Gordon et al. 1991; Fedosov et al. 2003) in different recombinant organisms provided material for various protein-consuming experiments including crystallisation studies (Garau et al. 2001; Wuerges et al. 2006). The three-dimensional structures of bovine and human TCs in complex with H2OCbl are now established at atomic resolution (Wuerges et al. 2006), Fig. 18.5.

TC consists of two domains connected by a short flexible link. The ligand H2OCbl is sandwiched between the two domains (Fig. 18.5). The larger N-terminal unit of 300 amino acid residues contains two circular layers of α-helices, where the odd and even segments belong to the external and internal bundles, respectively (Wuerges et al. 2006), Figs. 18.4 and 18.5. The N-domain is stitched by three disulfide bridges (Fedosov et al. 1999), which are conservative in all known sequences. Disruption of bridges C98(99)–C291(303) and C147(151)–C187(193) negatively affects Cbl binding, while C3(4)–C249(261) does not seem to play any essential role (Kalra et al. 2003). The C-terminal domain of 100 residues is built mostly of β-strands and has no disulfide bridges. The two domains have 17+17 bonds with Cbl, where double contacts are found for the residues N267(279) and Y137(141) (Wuerges et al. 2006). The ligand is practically buried inside TC with only 7% of Cbl surface open to solvent around 5′OH-group of ribosyl.

One particular bond connects the central cobalt ion of Cbl with H173(179) of human TC (or H175 of bovine TC). This His-residue attacks a weakly associated water molecule at the upper coordination position of the TC-bound H2OCbl. The substitution occurs as a well-defined conformational transition $\left(k + 2 = 0.02\ s^{-1}\right)$ visualized via changes in the Cbl absorbance spectrum (Fedosov et al. 2000). His-coordination may be advantageous because of a vulnerable nature of H_2OCbl,

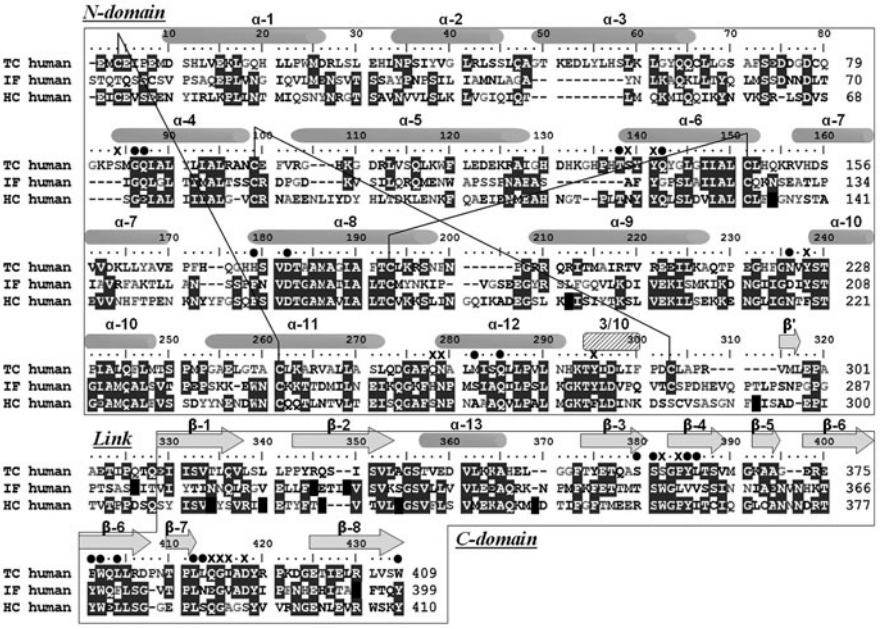

Fig. 18.4 Alignment of human TC, IF and HC (mature proteins), where N-/C-domains are *boxed*. The following notation is used: (α)-helices; (β)-strands; (3/10)-helix; (•) residues bound to Cbl directly and (x) via a solvent molecule; (■) Asn where carbohydrates can be attached. Links depict S–S-bonds. Numbering of residues in the main text (e.g. C148(151) of TC) refers to both individual protein sequences (C148 of TC) and the total alignment (C(151))

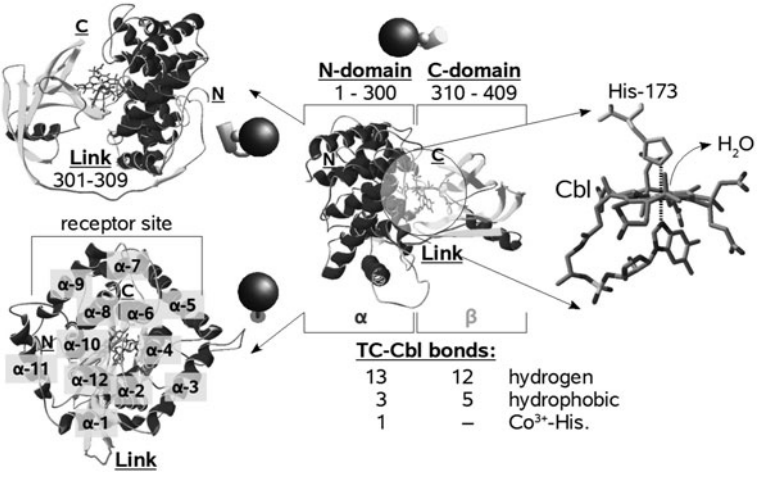

Fig. 18.5 Structure of human TC in complex with H_2OCbl

which readily reacts with external nucleofiles and reducing agents (Kräutler and Ostermann 2003; Pratt 1972). The His–Co^{3+} contact is absent in TC–CNCbl and TC–AdoCbl complexes, where the original upper group of Cbl is firmly attached to cobalt ion (Fedosov et al. 1999, 2002). Yet, the abnormal spectrum of AdoCbl bound to TC may point to essential elongation of the Ado–Cbl bond (Fedosov et al. 2002). Two other transporting proteins do not substitute water in H2OCbl (Fedosov et al. 2000, 2002), since H(179) is absent in IF and HC.

A two-domain organisation was demonstrated for TC (Fig. 18.5), and the same architecture can be conjectured for all Cbl-binding proteins. For instance, recombinant IF from plants is partially cleaved into two stable fragments – a 30 kDa peptide IF30 and a 20 kDa glycopeptide IF20 (Fedosov et al. 2004) – analogous to N- and C-terminal domains of TC. The split IF-fragments can be efficiently assembled by Cbl into a firm complex IF30·Cbl·IF20 (Fedosov et al. 2004), apparently with a "sandwich"-like structure, resembling TC·Cbl. This arcitecture was recently corroborated by the crystallographic analysis of the split IF in complex with Cbl (Mathews et al. 2007).

While TC is a true protein, IF and HC are glycoproteins supplemented with ≈10 kDa and ≈40 kDa of carbohydrates, respectively (Allen 1975; Nexø 1998). The potential N-glycosylation sites (Fig. 18.4, black bars) seem to be remote from the expected Cbl-protein contacts. All carbohydrates are placed on the C-terminal fragment of IF increasing its mass from 13 to 20 kDa, as established for the recombinant IF from plants (Fedosov et al. 2004). Carbohydrates of HC can be conjugated at three positions in the N-terminal domain and five positions in the C-terminal unit. The highest density of glycosylation sites is observed in the segment 310–350 of the total alignment.

Protruding sugar residues increase the apparent sizes of IF and HC, and they behave during gel-filtration as proteins of MwIF ≈ 60 kDa and MwHC ≈140 kDa (Allen 1975). Since oligosaccharides are not involved in the protein–Cbl and IF–receptor interactions (Gordon et al. 1991; Mørkbak et al. 2006), their role can be confined to (i) partial protection of HC and IF from proteolysis in the intestine (Gordon et al. 1991; Gueant et al. 1984); (ii) prevention of glomerular filtration of HC; and (iii) recognition of HC by a hepatic galactose-specific receptor (Allen 1975).

IF is the only Cbl-transporter prone to oligomerization. Thus, the full-length protein associates to dimers (IF·Cbl)2 after saturation with the ligand (Fedosov et al. 2004). Cleaved form IF30·Cbl·IF20 does not dimerize (Fedosov et al. 2004), suggesting involvement of the native inter-domain segment in the association of IF·Cbl monomers. Since the dimers have a dissociation constant of about 1 μM, they are hardly present under physiological concentrations (IF ≈ 50 nM), unless an external stabilising factor exists. If proven, the ability of IF to dimerize would have doubled the velocity of Cbl uptake from the intestine.

18.3.2 Cbl Binding and Ligand Specificity

It has long been known, that all Cbl-transporting proteins have a very high affinity for the true substrate B12. However, the reported dissociation constants KCbl = 10^{-9} – 10^{-15} M show exceptional dispersion (Allen 1975; Nexø 1998; Fedosov et al. 2002; Gordon et al. 1991; Brada et al. 2001). The sources of ambiguity were analysed in a number of publications (Fedosov et al. 2005a; Fedosov et al. 2006, 2007b), but the insufficient incubation time (10–20 min) during the equilibrium binding deserves to be mentioned specifically. The pseudo-saturation curve, produced after a non-equilibrium experiment, noticeably deviates from the correct shape, especially at the equivalent concentrations of the biding species. This leads to essential overestimation of Kd (Fedosov et al. 2005a). Examination of this effect led to the conclusion that the equilibrium methods cannot be used for analysis of Kd in a nearly irreversible binding reaction (Fedosov et al. 2005a). The alternative way is determination of the rate constants k+Cbl and k–Cbl in two separate reactions. The performed kinetic measurements pointed to extremely low dissociation constants (KCbl ≈ 5 fM) for all Cbl-transporting proteins (Fedosov et al. 2005a, 2006, 2007b). Note, that IF's affinity for Cbl heavily depended on the method of preparation of the apo-form (Fedosov et al. 2006).

Significant progress in elucidation of the mechanisms of Cbl – protein interaction was achieved during work with the fragments: IF30 (N-domain of IF), IF20 (C-domain of IF) (Fedosov et al. 2004, 2005a, 2006) and TC12 (C-domain of TC) (Fedosov et al. 2005b). The results are summarized in Fig. 18.6. It was found that the small C-terminal fragments IF20 or TC12 bound Cbl thousandfold stronger than the large N-terminal peptide IF30 (Fedosov et al. 2005a, b, 2006). These results agree with the atomic structures of TC·Cbl (Wuerges et al. 2006) and IF30·Cbl·IF20 (Mathews et al. 2007) as well as the epitope mapping of TC (Fedosov et al. 2005b), where high density of the ligand binding residues was found on a short C-terminal stretch, Fig. 18.4, residues 380–420 in the alignment. Preferential attachment of Cbl to the C-terminal fragment may be considered as the first binding step in the mixture IF30+IF20+Cbl. It is followed by assembly of the two protein units, as shown in the binding reaction IF20·Cbl + IF30 (Fedosov et al. 2004, 2005a, 2006), Fig. 18.6a. Stability of the produced complex IF30·Cbl·IF20 is impressive. Thus, the dissociation of IF30 from the assembled structure is characterized by Kd = 10–100 pM, depending on the refolding technique used for preparation of the unsaturated fragments (Fedosov et al. 2005a, 2006).

Interaction of Cbl with the full-length binding proteins seems to follow a two-step pattern. Thus, initial attachment of a ligand to the C-terminal domain is accompanied by assembly of N- and C-units subdivided into multiple transitions (Fedosov et al. 2005a, 2006, 2007b), Fig. 18.6b. Convergence of the two domains is facilitated by their attachment to each other via a flexible link.

The described above two-domain organization of Cbl-binding proteins may provide a structural basis for different ligand specificities observed for IF, HC and TC. Kinetic analysis of the interactions between a number of corrinoids and the three binding proteins revealed essential details of ligand recognition (Fedosov et al.

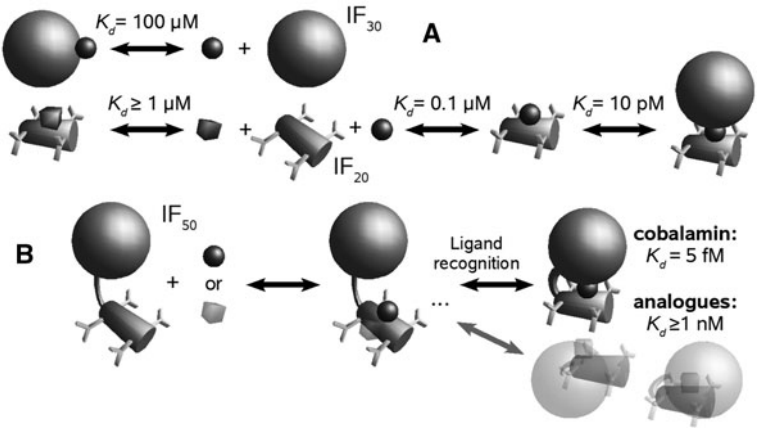

Fig. 18.6 Binding of Cbl (*small sphere*) or an analogue (*small cube*) to (**a**) IF-fragments; and (**b**) full-length IF

2006, 2007b). It was found that the rate constants of attachment k + Cor ≈ 70 $\mu M^{-1}s^{-1}$ were similar in all cases. Nevertheless, the following adjustments and fastening of the bound ligand obeyed different mechanisms for HC, TC and IF. A firm attachment to HC was observed for all corrinoids immediately after initial contact with the protein, though a slow conformational transition without any visible effect on affinity was found in some cases. On the other hand, several slow transitions were observed during fastening of Cbl-analogues bound to TC and IF. The number of conformational steps directly correlated with Cbl-specificity, i.e. the most complex kinetic patterns were observed for IF – Cor interactions (Fedosov et al. 2006, 2007b). The transitions were ascribed to the mutual re-arrangements of the protein domains, thus, the ligand selectivity was related to the internal flexibility of the protein molecule. It was concluded that recognition of the true substrate by, for example, IF is tightly associated with assembly of the two domains and following adjustments at the domain-domain interface (Fedosov et al. 2006, 2007b), Fig. 18.6b. This statement is corroborated by the fact that many Cbl-analogues are not able to induce association of the cleaved fragments IF30+IF20 despite successful binding to IF20 (Fedosov et al. 2007b). It seems that the sequence of bond formation (e.g. 1, 2, 3, 4. . .) is a crucial factor during ligand recognition. The correct order leads to firm binding of Cbl, while binding of the distorted corrinoids follows the ramified schemes giving multiple protein-ligand complexes of different dissociation stability (Fedosov et al. 2007b), Fig. 18.6b.

18.3.3 Receptor Recognition

It was already mentioned, that the efficient uptake of vitamin B12 by the organism is mediated by several receptors (Allen 1975; Moestrup 2006; Birn 2006; Nexø 1998;

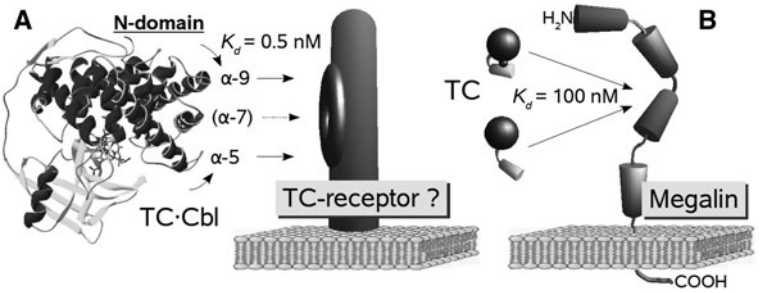

Fig. 18.7 Interaction of TC with (**a**) ubiquitous receptor; and (**b**) renal receptor megalin

Seetharam and Li 2000). The simplest mechanism describes absorption of HC from plasma by the liver cells. HC, with or without attached Cbl, uniformly interacts with a receptor, which binds in the presence of Ca^{2+} all glycoproteins containing the terminal galactose residues (Allen 1975).

TC, the main distributor of Cbl among all the tissues, binds in the presence of Ca^{2+} to a membrane-associated glycoprotein of still debated identity (Seligman and Allen 1978; Nexø and Hollenberg 1980; Seetharam and Li 2000; Quadros et al. 2005). The receptor-binding site on the surface of TC involves α-5, α-9 and possibly α-7 helices in the N-terminal domain (Figs. 18.5 and 18.7a) according to mapping with the monoclonal anti-TC antibodies (Fedosov et al. 2005b). The above external segments seem to converge after the neighbouring helices α-4, α-6 and α-8 in the inner bundle have attached Cbl. This causes formation of an assembled surface α-5, 7, 9 with better compatibility and a 2- to 30-fold higher affinity for the receptor (Seligman and Allen 1978; Nexø and Hollenberg 1980; Quadros et al., 2005; Moestrup 2006), Fig. 18.7a. Difference between conformations of apo- and holoTC was confirmed by existence of a monoclonal antibody characterized by low affinity for apoTC and high affinity for holoTC (Örning et al. 2006).

Another TC-related receptor is a well-described glycoprotein megalin present in a number of tissues (Moestrup 2006; Seetharam and Li 2000; Fedosov et al. 2002; Moestrup et al. 1996). This is a complex molecule of 600 kDa (Fig. 18.7b) which consists of four extracellular clusters enriched by cysteines and similar to those of low density lipoprotein (LDL) receptors. Megalin is associated with Cbl homoeostasis in kidney, where it binds filtered apo/holoTC and mediates return of the vitamin to the blood circulation (Fig. 18.3, step 5). Apart from TC, this receptor binds variety of other ligands (Birn 2006; Moestrup et al. 1996; Seetharam and Li 2000).

The most complex mechanism of recognition describes interaction between IF·Cbl and the intestinal receptor cubilin (Fig. 18.2, step 4). The latter is a 460 kDa glycoprotein constructed of 8 epidermal growth factor (EGF) repeats and 27 CUB domains with a characteristic β-folding (Birn et al. 1997; Birn 2006; Kristiansen et al. 1999; Moestrup 2006), Fig. 18.8. This receptor has no transmembrane segment of its own and is anchored to the cell surface via a protein amnionless (AMN)

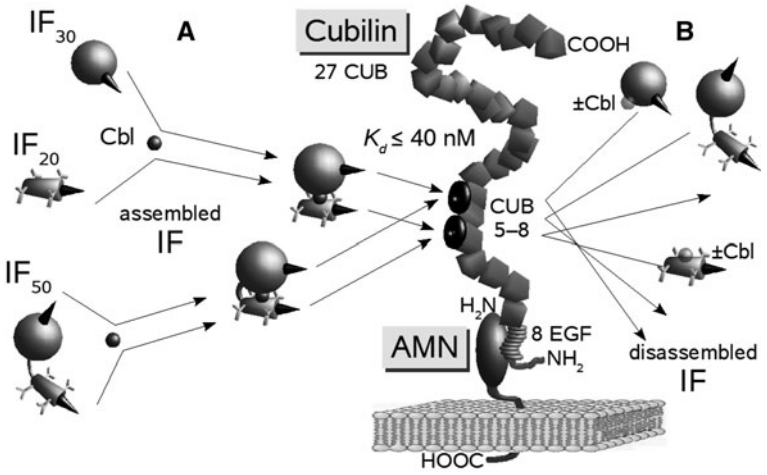

Fig. 18.8 Binding of IF to cubilin while IF-domains are (**a**) assembled; or (**b**) disassembled

(Fyfe et al. 2004). In the absence of AMN, cubilin is detached from the membranes, whereupon the cell loses the ability to absorb IF·Cbl (Fyfe et al. 2004). Apart from intestine, cubilin is also found in a number of tissues, where it mediates absorption of ligands other than IF·Cbl (Moestrup 2006). The domains CUB 5–8 of the receptor bind IF·Cbl with high affinity (Kristiansen et al. 1999), where estimations by different methods give Kd = 2 nM or 40 nM (Birn et al. 1997; Kristiansen et al. 1999). Apo-form of the full-length IF or its isolated fragments (with or without Cbl) do not interact with the receptor (Fedosov et al. 2005a), Fig. 18.8b.

Exclusive recognition of the complex IF·Cbl by cubilin is based on the essential difference in the structures of apo- and holo-forms of full-length IF. Receptor recognition site of IF seems to have a composite organisation, where the function of ligand is to induce association of the two domains, thereby assembling the scattered elements into a cubilin-compatible interface (Fedosov et al. 2005a).

All described receptors mediate endocytosis of the Cbl-transporting proteins, whereupon the receptors return to the cell surface, the Cbl-carriers are cleaved in lysosomes, and the vitamin is released to the cytoplasm or on the opposite side of the cell.

18.4 Practical Implementation of Recent Basic Research

18.4.1 Novel Methods for Estimation of Cbl-Status

Efficient uptake of dietary B12 is ensured by the complex transport mechanism. Yet, complexity has its drawbacks, and breakdown of the transportation chain at any link results in Cbl-malabsorption. Determination of the current Cbl-status is,

nevertheless, not as easy as it might appear (Carmel et al. 2003). Thus, the normal level of total plasma B12 often coexists with the signs of deficiency, as well as a low total B12 does not necessarily cause metabolic or clinical manifestation of the disease (Carmel et al. 2003; Miller et al. 2006; Hvas and Nexø 2005; Herbert et al. 1990). The inconsistency between total B12 and clinical signs can be explained by inert character of the prevailing form of B12 in plasma – HC·Cbl. Other detection methods are based on the elevated levels of metabolites MMA and Hcy in plasma. They aim to reveal decreased catalytic activities of the Cbl-dependent enzymes due to low B12 balance. Yet, folate deficiency and renal dysfunction may provoke similar symptoms (Carmel et al. 2003; Hvas and Nexø 2005). Additionally, complexity of the measurements of MMA and Hcy hinders their widespread application. Eventually, low concentration of the Cbl-saturated TC (holoTC) was suggested as a sensitive marker for developing Cbl-deficiency (Herbert et al. 1990), though no accurate method of measurement existed at that time.

Recent advances in characterization of the anti-TC antibodies (Fedosov et al. 2005b) allowed a quantitative determination of holoTC. The available techniques (Fedosov et al. 2006; Nexø et al. 2002) are based on sequestration of the total TC-pool using an immobilized antibody (Fig. 18.9a, step 1) or a Cbl-matrix followed by an antibody-matrix (Fig. 18.9b, steps 1 and 2). Afterwards, Cbl is either released from TC and measured by a standard procedure (Fig. 18.9a, steps 2 and 3) or the adsorbed complex TC·Cbl is detected by a second antibody (Fig. 18.9b, step 3). The most recent method uses a holoTC-specific antibody as a capturing substance, whereupon the bound TC is measured by a labelled antibody (Örning et al. 2006), Fig. 18.9c. This setup seems to be very convenient, and we are looking forward to its practical implementation.

Measurements of holoTC in plasma were carried out and correlated with total plasma B12, MMA and Hcy, as well as with clinical manifestations of Cbl-deficiency, see recent references (Goringe et al. 2006; Hin et al. 2006; Hvas and Nexø 2005; Hvas et al. 2006; Miller et al. 2006). It seems, that none of the mentioned parameters taken alone is an absolutely reliable indicator of Cbl deficiency (Goringe et al. 2006; Hvas and Nexø 2005; Miller et al. 2006). Yet, there was a

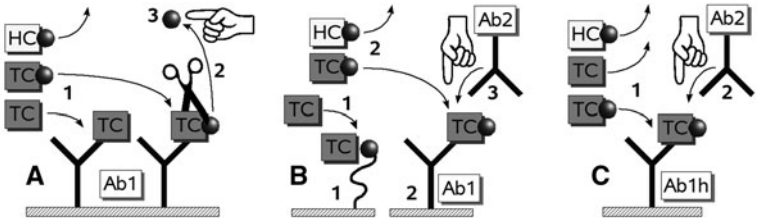

Fig. 18.9 Determination of holoTC (TC·Cbl) in blood plasma using (**a**) adsorption of total TC on anti-TC antibody Ab1 followed by liberation and measurement of TC-associated Cbl (Fedosov et al. 2006); (**b**) removal of apoTC by a Cbl-matrix followed by adsorption of the remaining holoTC on Ab1 and measurement of the sequestered TC by Ab2 (Nexø et al. 2002; (**c**) adsorption of holoTC on the holoTC-specific antibody Ab1h and measurement of the sequestered TC by Ab2 (Örning et al. 2006)

correlation between the elevated concentrations of both metabolites (MMA > 0.35 μM, Hcy > 13 μM) and low concentrations of Cbl in both Cbl-pools (B12 < 150 pM and holoTC < 35 pM) (Miller et al. 2006). In this regard, application of the combined coefficients $(B12 \times holoTC)^{1/2} < 78$ pM and $(MMA \times Hcy)^{1/2} > 2.3$ μM as indicators of Cbl-deficiency seems to be convenient and mathematically justified (Fedosov 2006b).

Measurements of plasma holoTC also characterize the intestinal B12 uptake. Thus, a pronounced spike in plasma holoTC (+50%) was detected 1 day after ingestion of three sequential 9 μg (6.7 nmol) doses of B12 (Bor et al. 2004; Hvas et al. 2006). It seems to reflect the normal absorption of the vitamin. Examination of patients by this method might be a convenient alternative to the Schilling test.

18.4.2 Cbl-Conjugates and Their Medical Application

All animal cells require Cbl to maintain DNA synthesis. Hence, accumulation of Cbl is a characteristic feature of the fast growing tissues, and it may serve as a good marker of cancer cells (Russell-Jones 1998). Unfortunately, the radioactive vitamin [57Co]Cbl is not applicable for imaging in vivo because the half-life time of 57Co is too long (272 days). Cbl-conjugates with the attached imaging or therapeutic groups can be used instead of [57Co]Cbl to visualize and treat cancer cells (Russell-Jones 1998; Collins et al. 2000; McGreevy et al. 2003; Bagnato et al. 2004).

Chemical modification of Cbl molecule must not affect its affinity for IF or/and TC, since it would hinder targeted delivery of the synthesized conjugate to the tissues. A popular strategy of Cbl modification includes acidic hydrolysis of amides b, d or e (Fig. 18.1b), separation of monocarboxylic derivatives, and attachment of an active substance via amine-containing spacer (Russell-Jones 1998; Collins et al. 2000; Hogenkamp et al. 2000). Yet, the above method does not seem to be optimal in the frame of recent structural analysis of TC·Cbl, where each of the derivatized amides is involved in bonding with TC residues (Wuerges et al. 2006). At the same time, the 3D-structure shows that 5′OH-group of ribosyl (Fig. 18.1b, position 5′) is open to solvent (Wuerges et al. 2006) and can be used for conjugation of different probes without disturbing the Cbl-binding site of TC (Horton et al. 2003; Fedosov et al. 2006). This approach was examined after synthesis of a fluorescent 5′OH-conjugate CBC-244 (Fedosov et al. 2006). High affinities were established when binding this derivative to IF and TC, as well as IF·CBC complex to cubilin (Fedosov et al. 2006). Increase in the quantum yield of CBC-244 by 50–300%, induced by its binding to the Cbl-carriers, makes it a sensitive probe for investigations of protein-corrinoid interactions (Fedosov et al. 2006, 2007b).

Another way to attach a supplementary group to Cbl without loosing affinity is carbon–cobalt bonding at the upper position of corrin ring (Bagnato et al. 2004; Smeltzer et al. 2001), Fig. 18.1. The produced cobalamins are, however, light sensitive (Kräutler and Ostermann 2003; Pratt 1972; Smeltzer et al. 2001).

Synthesis of the Cbl-conjugated drugs, their targeted delivery and application in cancer treatment is another developing field. A toxic compound attached to

Cbl is expected to be physiologically neutral until it is liberated. Therefore, a Cbl-conjugate can be safely delivered by TC to cancer cells, where the active toxic component is released by influence of light (Smeltzer et al. 2001; Bagnato et al. 2004), low lysosomal pH (Bagnato et al. 2004) or mild radiation (Hogenkamp et al. 2000). In such case, the poisonous effect would be essentially confined to the target tissue with a relatively low negative impact on the rest of the organism.

A recently suggested strategy of the targeted delivery of therapeutic Cbl-conjugates to cancer cells is based on the binding to HC but not TC (Waibel et al. 2008). It was demonstrated that many tumours produce and accumulate high amounts of HC resulting in a significant uptake of the HC-specific conjugates by the malignant tissues in mouse.

18.5 Affinity Purification of Cbl

Purification of Cbl from biological sources or isolation of Cbl-derivatives after organic synthesis is a multi-step procedure. Thus, purification from a fermentation extract (Martens et al. 2002) typically requires adsorption on several unspecific materials, e.g. charcoal, Amberlite XAD/IRC(H^+), Dowex(Cl^-) (Adachi et al. 2005; Barchielli et al. 1960; Lamm et al. 1980; Martens et al. 2002; Vogelmann and Wagner 1974). Application of an affinity adsorbent would essentially simplify this process. Guidelines for the development of such material are, however, not so evident.

Analysis of the environment in the Cbl-binding site of TC provides several useful observations (Figs. 18.4 and 18.5). First of all, most of TC–Cbl contacts belong to the well-known hydrogen and hydrophobic bonds, which are also responsible for retention of Cbl inside the unspecific microporous resins. Much higher affinity of Cbl for TC is caused exclusively by spacial organization of TC-residues around the ligand and better compatibility between the interacting species. Clearly, the TC-like scaffolding of residues cannot be reproduced by organic synthesis. Yet, one bond between TC and H2OCbl has a completely different nature, and this is the coordination contact of His-173(179) with cobalt ion (Fig. 18.5). Coordination binding has never been exploited for Cbl purification, and this approach required a thorough examination.

It was found that H_2OCbl binds to heterocyclic tetrazole (TZ) derivatives with Kd $\approx$ 4 μM (Fedosov et al. 2007a), i.e. 60-fold better than to His (Fedosov et al. 2003, 2007a). Treatment of the CNBr-activated Sepharose with a neutral solution of 50–100 mM azide easily initiates tetrazole groups in a solid material. After 2–10 h of incubation the matrix acquires the H_2OCbl-binding properties manifested at pH 3–8 (Fig. 18.10). Concentration of the active groups in the packed material corresponds to $\approx$10 mM (Fedosov et al. 2007a). Interaction of H_2OCbl with Sepharose-TZ occurs in two steps: (1) fast coordination of one TZ-group at the upper surface of Cbl (K1 $\leq$ 10 μM); and (2) slow substitution for the nucleotide base N at the low surface rendered by the second TZ-residue (K1·K2 $\leq$ 1 μM).

AdoCbl and MeCbl, prevailing in the biological extracts, both have the protected cobalt ion. Yet, illumination with visible light efficiently converts them to H2OCbl

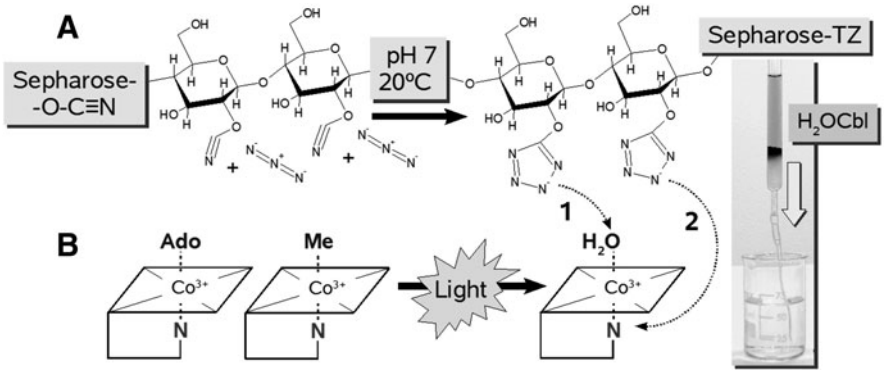

Fig. 18.10 Affinity adsorption of aquo-corrinoids. (**a**) Preparation of tetrazole-(TZ)-containing Sepharose; (**b**) conversion of Ado/MeCbl to H_2OCbl, and binding of the latter to Sepharose-TZ

when working in an oxygenated aqueous solution (Pratt 1972; Kräutler and Ostermann 2003), Fig. 18.10b. Some biological compounds (e.g. His-containing peptides, reduced glutathione) can substitute for water of H_2OCbl (Pratt 1972; Fedosov et al. 2007a) and hinder adsorption on TZ-material. In such case, the medium should be acidified (pH 3–4) to protonate the competing compounds. The developed affinity purification of Cbl included: (i) hot acidic extraction of biological cobalamins in the absence of cyanide; (ii) illumination of the solution followed by the binding to a TZ-column; (iii) washing and elution of Cbl with 20 mM KCN at alkaline pH (Fedosov et al. 2007a). Optional desalting on charcoal or XAD can be performed.

The tetrazole-containing matrix is also applicable for adsorption of other aquo-corrinoids (Co^{3+} and Co^{2+}), e.g. diaquo-cobinamide, cyano-aquo-cobinamide, aquo-forms of pseudo B12 and factor A (Fedosov et al. 2007a).

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