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Review: Chemical Pathology of Homocysteine. IV. Excitotoxicity, Oxidative Stress, Endothelial Dysfunction, and Inflammation

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Abstract. This review considers recent advances in the chemical pathology of homocysteine in atherogenesis, oxidative metabolism, and carcinogenesis. Homocysteine is a potent excitatory neurotransmitter that binds to the N-methyl-D-aspartate (NMDA) receptor and leads to oxidative stress, cytoplasmic calcium influx, cellular apoptosis, and endothelial dysfunction. According to the adsorption-induction theory, cytoplasmic calcium influx leads to depletion of cellular adenosine triphosphate (ATP) by reaction with cytoplasmic phosphate, leading to calcium apatite deposition. Oxidative stress is caused by failure of ATP synthesis and accumulation of reactive oxygen radicals, theoretically because of inhibition of thioretinaco ozonide function within mitochondria and endoplasmic reticulum. The toxicity of oxygen difluoride is theoretically explained by the displacement of ozone from thioretinaco ozonide, leading to inhibition of cellular respiration. Depletion of thioretinaco ozonide from cellular membranes is suggested to underlie the carcinogenic and atherogenic effects of fluoride and other electrophilic carcinogens. In atherogenesis the acute inflammatory response is related to cellular apoptosis and necrosis, autoantibodies to proteins containing peptide-bound homocysteine and oxidized low-density lipoprotein (Ox-LDL), and microbial products and antigens originating from homocysteinylated LDL aggregates trapped within vasa vasorum of developing atherosclerotic plaques. The trapping of lipoprotein aggregates and obstruction of the lumen of vasa vasorum are enhanced by high tissue pressure and by endothelial dysfunction because of narrowing of the lumen by swollen and hyperplastic endothelial cells, leading to the creation of vulnerable plaques.

Keywords: homocysteine, excitotoxicity, apoptosis, atherosclerosis, thioretinaco ozonide, carcinogenesis

Introduction

In the 15 years since publication of sections I, II, and III of this review [1-3], the field of homocysteine research has expanded. Many new developments and discoveries in the field were reported at the 6th World Congress on Hyperhomocysteinemia held in June 2007 [4]. Increased understanding of the key role of homocysteine in atherogenesis, carcinogenesis, and degenerative diseases of aging has led to new insights in the excitotoxicity of homocysteine, oxidative stress and endothelial dysfunction, and the acute inflammatory response. Section IV of this review describes how developments in these areas can be explained and interpreted by the theory of thioretinaco ozonide function in oxidative metabolism.

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Excitotoxicity

Homocysteine causes convulsions and death in rats following ip injection of high doses (LD_{50} 750 mg/kg) of DL-homocysteine or DL-homocysteine thiolactone [5]. No convulsions were observed when methionine, cysteine, or homocystine were injected, and protection against the convulsive

action of homocysteine was afforded by high doses of homoserine, serine, betaine, glycine, or glucose. The related oxidized derivatives of homocysteine and cysteine, homocysteic acid, cysteic acid, homocysteine sulfinic acid, and cysteine sulfinic acid, have long been known to be excitatory neurotransmitters, binding to receptors on brain cell membranes and causing excitation of neurons [6].

The excitotoxic properties of glutamate on retinal and brain cells were discovered by injection of the amino acid into newborn mice [7,8]. Glutamate causes acute neuronal necrosis in the arcuate nucleus of the hypothalamus, medial habenular nucleus, and rostral hippocampus, leading to massive obesity, decreased skeletal growth, and female sterility. In a systematic study of neurotoxicity of acidic and sulfur amino acids, N-methyl-D-aspartate (NMDA), homocysteate, and N-methyl glutamate were found to be the most potent excitatory neurotransmitters causing neuronal necrosis [9]. Glutamate, aspartate, cysteine, cysteate, and cysteine sulfinate were also shown to cause neuronal necrosis in infant mice. The concept of excitatory activation and necrosis of neurons is supported by extensive observations of the phenomenon of glutamate receptor function and the development of drugs that effectively antagonize glutamate in human and experimental in vivo and in vitro studies [10]. These studies have indicated that cytoplasmic influx of calcium ions plays a critical role in the phenomenon of neuronal excitotoxicity.

In a study of cultured neuronal and glial cells, homocysteine was demonstrated to act as an agonist at the glutamate binding site of the NMDA receptor, causing cytoplasmic calcium ion influx, decreased cellular viability, and increased reactive oxygen species within cells [11]. These experiments also showed that glycine potentiates the neurotoxic effect of homocysteine, similar to potentiation by glycine of the effect of glutamate on NMDA receptors [10]. Intracellular calcium ion concentrations are tightly regulated at about 10^{-7} M, while extracellular calcium ion concentrations are 10^{-3} M, creating a large electrochemical gradient. Receptor-mediated influx of cytoplasmic calcium ions occurs through the agonistic action of homocysteine and other neurotransmitters on ionotropic and metabotropic glutamate receptors, leading to cellular necrosis [12,13]. A variety of toxins, such as galactosamine, carbon tetrachloride, phylloidin, and silica particles, depend for their cytotoxic action upon influx of calcium ions into the cytoplasm of cultured liver cells [12,13].

Neurotoxicity mediated by activation of the NMDA receptor is dependent upon compromise of cellular energy production [14]. Cultured neurons are refractory to the excitotoxic effects of glutamate in the presence of glucose and magnesium ions, which block the entry of calcium ions into cytoplasm. Inhibition of cellular oxidative metabolism by glucose deprivation, potassium cyanide, oxygen deprivation, or ouabain, a sodium/potassium ATPase inhibitor, leads to neurotoxicity by glutamate [14]. Notably, potassium cyanide without glutamate is not toxic to cultured neurons, but potassium cyanide facilitates glutamate toxicity in the presence of glucose and magnesium ions. The conclusion from these experiments is that glutamate is toxic in glucose-starved cells because of decreased ATP synthesis, leading to reduction in the block of the NMDA calcium channel, which is known to be voltage-dependent [14]. Homocysteine is a potent inhibitor of cellular sodium/potassium ATPase, whereas cysteine has no inhibitory effect [15]. Studies with cultured cerebellar granular cells concluded that activation of the NMDA receptor by homocysteine leads to neurotoxicity from free radical formation [16]. The ability of glucose to prevent the convulsive effect of homocysteine [5] may be attributed to increased ATP synthesis within cerebral neurons.

Many studies of glutamate and homocysteine activation of the NMDA glutamate receptor utilized cultured neurons [10]. However, NMDA glutamate receptors are also expressed by cultured rat cerebral endothelial cells [17] and by human neuroepithelial cells [18]. Moreover, NMDA glutamate receptors are present in cultured rat aortic endothelial cells, showing that these receptors are widely distributed in the peripheral vasculature [19]. Activation of the NMDA glutamate receptor of cultured endothelial cells by homocysteine produced cellular proliferation, suggesting a role of these receptors in the intimal hyperplasia produced by hyperhomocysteinemia [19]. NMDA glutamate

receptors were also demonstrated within cultured rat smooth muscle cells, and activation of these receptors by homocysteine produces cellular proliferation, increased expression of matrix metalloproteinase-9 and interleukin-1beta, reduced vascular elaboration of nitric oxide, and increased endogenous elaboration of the nitric oxide synthase inhibitor, asymmetric dimethylarginine [20]. These results implicate homocysteine in endothelial dysfunction through activation of the NMDA glutamate receptors.

Studies with cultured human cerebral endothelial cells demonstrate a loss of endothelial barrier integrity through activation of the NMDA receptor by glutamate and NMDA [21]. Moreover, human cerebral endothelial cells respond to glutamate by generating intracellular oxidant stress by NMDA receptor activation [22]. Studies employing several glutamate receptor agonists, antagonists, and second message blockers showed that endothelial dysfunction depends upon intracellular calcium ions and is mediated by oxidants derived from reduced nicotinamide adenine dinucleotide oxidase, cytochrome P-450, and mitochondria [22].

In a review of the literature concerning excitotoxins, Blaylock [23] points out that dietary glutamate causes higher blood levels of glutamate in human subjects than in monkeys, mice, or rats. He also notes that developing human brain, human brain injury, and aging increase potential susceptibility to neurotoxicity of dietary excitotoxins because of reduced integrity of the blood brain barrier. Blaylock further suggests that dietary exposure to glutamate, aspartame, and cysteine may exacerbate important human neurodegenerative diseases, including Alzheimer's dementia, amyotrophic lateral sclerosis, Parkinson's disease, and Huntington's chorea. Dietary intake of glutamate, aspartate, and cysteine from hydrolyzed protein extracts, flavorings, and seasonings in processed foods is unknown because of a lack of labeling requirements by the Food and Drug Administration. Blood homocysteine levels are elevated in several chronic neuropsychiatric disorders such as Alzheimer's dementia [24], schizophrenia [25], cognitive dysfunction [26], and multiple sclerosis [27]. Activation of NMDA receptors by hyperhomocysteinemia resulting from developmental, dietary, and aging factors may affect the progression and exacerbation of these disorders.

The normal concentration of total homocysteine in plasma of adults is approximately 10 μM [28]. Over 90% of total plasma homocysteine is bound to plasma proteins, and only traces of free homocysteine, approximately 0.1 μM, are present in plasma [29]. Only free homocysteine in the reduced form activates NMDA receptors, and protein-bound homocysteine and the disulfide form, homocystine, in plasma are inactive. The homocysteine levels in normal cerebrospinal fluid are 0.007-0.020 μM [30]. In a report of cerebrospinal fluid homocysteine levels in patients with fibromyalgia and chronic fatigue syndrome, the controls averaged 0.19 μM, and the patients averaged 0.61 μM [31].

Concentrations of the excitatory neurotransmitters, glutamate, aspartate, and cysteine, are much lower in cerebrospinal fluid, compared to plasma, as indicated in Table 1. These data show that the concentrations of the neurotransmitters, cysteine and homocysteine, are rigorously controlled in cerebrospinal fluid, creating a plasma/cerebrospinal fluid gradient of 245 to 500. Injection of a high dose of cysteine (3 mg/kg) into newborn mice causes necrosis of hypothalamic neurons and uniform fatality; a lower dose (1 mg/kg) causes no fatalities but results in widespread, delayed neuronal necrosis affecting the thalamus, hippocampus, amygdala, and cerebral cortex, impairing learning capacity as adults in rats [33]. These observations show that increased cysteine and homocysteine

Table 1. Concentrations of excitatory amino acid neurotransmitters in plasma and cerebrospinal fluid (CSF).* Data are given in μmol/L.

Amino acid	Plasma	CSF	plasma/CSF
Aspartate	16	0.9	18
Glutamate	58	7.0	8
Cysteine	49	0.2	245
Homocysteine	10	0.02	500

*Adapted from Scriver & Rosenberg [32], Refsum et al [29], and Blom et al [30].

concentrations in cerebrospinal fluid have the potential for causing focal or widespread neuronal necrosis, depending upon the age of the animal or subject and the increased concentrations achieved within cerebrospinal fluid and neurons.

Apoptosis, or programmed cell death, is a feature of advanced human atheromas and experimental vascular injury [34,35]. Adenosine alone or in combination with homocysteine causes apoptosis in pulmonary endothelial cell cultures because of increased intracellular synthesis of adenosyl homocysteine [36]. Homocysteine increases apoptosis in cultured human leukemic cells exposed to 3-deazaadenosine [37]. The apoptotic damage to DNA caused by homocysteine thiolactone in cultured human leukemic cells is mediated by increased hydrogen peroxide production and caspase activation [38]. Homocysteine elicits apoptosis, mitochondrial dysfunction, oxidative stress, and caspase activation in cultured rat hippocampal neurons [39]. This study showed that homocysteine produces delayed mitochondrial oxidative stress and membrane depolarization that sensitizes neurons to the excitotoxic effects of glutamate. In addition, homocysteine potentiates hippocampal neuronal necrosis induced by the potent excitotoxin, kainate, in mice [39]. Hyperhomocysteinemia induced by dietary deficiency of vitamins B_{12}, B_2, folate, and choline produces apoptosis and deficient neurobehavioral development in rat pups of mothers fed the deficient diet [40]. This immunohistochemical study demonstrated homocysteine-containing cells and apoptotic cells in the hippocampus of 21-day-old rats. Taken together, these experimental studies relate homocysteine exposure to excitotoxicity, apoptosis, and mitochondrial oxidative stress. These studies support an important role of excitotoxicity by homocysteine in the apoptosis observed in atherogenesis [34,35] and neuronal necrosis in neurodegenerative diseases [24-27]. These findings also help to explain the mental retardation observed in some individuals with homocystinuria caused by cystathionine synthase deficiency [41] and the degeneration of brain parenchyma, both grey and white matter, observed in methionine synthase deficiency [42].

Oxidative Stress and Endothelial Dysfunction

Electron microscopic studies of hepatocytes of patients with homocystinuria caused by deficiency of cystathionine beta synthase [43] or methylenetetrahydrofolate reductase [44] demonstrate cytoplasmic fat droplets, proliferative smooth endoplasmic reticulum, and abnormal mitochondria with bizarre shapes, breaks in outer membranes, and enlarged dense granules. Some mitochondria have finger-like projections and giant shapes. Similar mitochondrial abnormalities occur in normal or hypertensive rats given high doses of methionine or cholestane triol in the diet [45]. An example of bizarre, polymerized mitochondria formed from trunk-like projections is illustrated in an aortic smooth muscle cell from one of these rats (Fig. 1). These studies show that intracellular

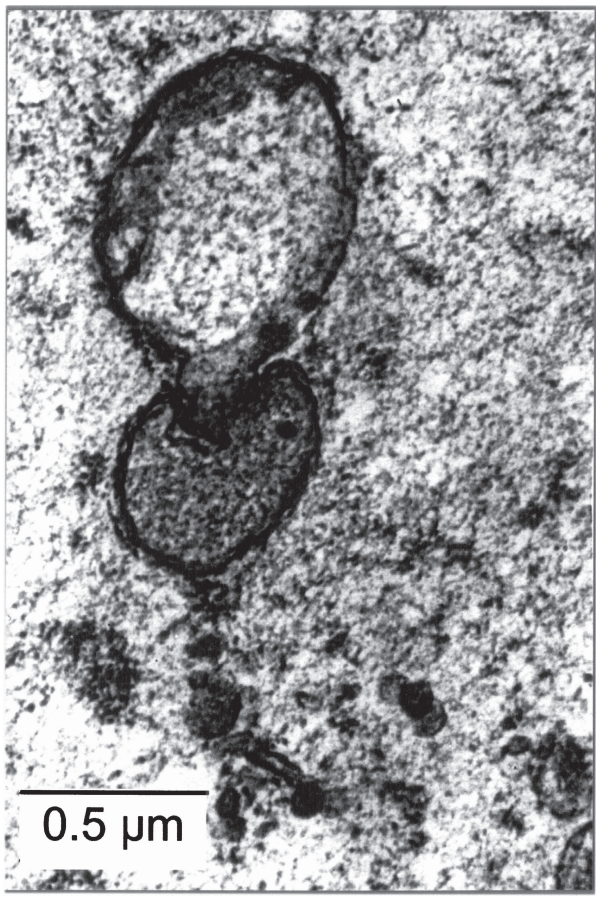

Fig. 1. Deep invagination of mitochondrial processes into adjacent degenerative mitochondrion is observed in a smooth muscle cell of a spontaneously hypertensive rat with hyperhomocysteinemia from oral methionine, 200 mg/day [45].

accumulation of homocysteine produces abnormalities in the structure of mitochondria, which are the site of oxidative phosphorylation and ATP synthesis within cells.

The current view of the origin of oxidative stress in cells exposed to increased levels of homocysteine is that auto-oxidation of thiol groups generates hydrogen peroxide and the reactive radical oxygen species, superoxide and hydroxyl radical [46], leading to oxidant stress [47]. Previous studies implicated homocysteine in the production of superoxide and oxidation of low-density lipoprotein by cultured arterial smooth muscle cells [48]. A requirement for transition metal cations, including ferric and cupric ions, is implicated in the oxidative modification of low-density lipoprotein by homocysteine [49].

The important mediator of endothelial, platelet, and smooth muscle function, nitric oxide, reacts with homocysteine to form S-nitroso-homocysteine, counteracting the adverse vascular effects of homocysteine, including endothelial dysfunction, vasoconstriction, and platelet aggregation [50]. Hyperhomocysteinemia produced by dietary deficiency of folate and increased methionine in monkeys induces endothelial dysfunction, as shown by impaired vascular relaxation response to acetyl choline or adenosine diphosphate, decreased thrombomodulin anticoagulant activity, and increased platelet-mediated vasoconstriction to collagen infusion [51]. Further evidence for the interaction of homocysteine with nitric oxide in endothelial dysfunction is the stimulation of endothelial nitric oxide production in aortic endothelial cells by increased endothelial nitric oxide synthase by homocysteine [52]. In addition, homocysteine decreases nitric oxide production by decreasing transcription of the mRNA for glutathione peroxidase in aortic endothelial cells [53]. These experiments suggest that increased oxidative stress induced by homocysteine promotes endothelial dysfunction by decreased availability and activity of nitric oxide. Human studies show that hyperhomocysteinemia is associated with impaired endothelium-dependent vasodilation, presumably by decreased bioavailability of nitric oxide [54]. Additional evidence for endothelial dysfunction in hyperhomocysteinemia is the observation of endothelial hyperplasia, swelling and vacuolization of endothelial cells, and fibrin deposition in cerebral arterioles in patients with homocystinuria [55]. Recent reviews have summarized the extensive literature on vascular oxidant stress in hyperhomocysteinemia [56] and the key role of glutathione peroxidase in modifying endothelial dysfunction from oxidant stress [57].

According to the adsorption/induction hypothesis, adenosine triphosphate (ATP) is required for maintenance of the high-(negative)-energy, low-entropy state that characterizes living cells [58]. This concept theorizes that adsorption of ATP to cellular proteins, for example myoglobin in muscle cells, results in electron withdrawal and maintenance of the living state. The theoretical function of thioretinaco ozonide, composed of thioretinamide, cobalamin, and ozone, is to form the active site for oxidative phosphorylation of adenosine diphosphate and phosphate to ATP [2]. During this process, oxygen is bound to the ozone of thioretinaco ozonide, and electrons from the electron transport enzymes of cells form oxygen radicals, releasing ATP from binding to the active site, and simultaneously reducing oxygen to water through multiple reduction steps.

Calcium ion influx caused by excitotoxic activation of NMDA receptors or the cytotoxic effect of cellular toxins [12,13] presumably inhibits the oxidative phosphorylation process by formation of the insoluble salt, calcium hydroxyl apatite. Depletion of phosphate by this process, therefore, leads to failure of ATP synthesis and accumulation of oxygen radicals, creating cellular oxidant stress. Thus oxygen molecules delivered to cells by transport from hemoglobin produce a continuous supply of oxidative potential that overcomes the ability of thioretinaco ozonide to convert oxygen radicals to water and generate ATP from phosphate and adenosine diphosphate. The result of this oxidative stress produces oxidative modification of cellular proteins and lipids [59]. Oxidative stress within cell cytoplasm, arising from inhibition of ATP synthesis by thioretinaco ozonide by calcium ions, is a more likely source of intracellular radical oxygen species than auto-oxidation of thiols in the extracellular fluid and plasma [60].

Table 2. Toxicity of selected gases containing sulfur, nitrogen, fluorine, and carbon.*

Toxic gas	LC_{50} (ppm)
Hydrogen cyanide (HCN)	544 (rats) 169 (mice) 300 (dogs)
Hydrogen fluoride (HF)	1278 (rats) 500 (mice) 1780 (monkeys)
Oxygen difluoride (OF_2)	2.6 (rats) 1.5 (mice)
Sulfur dioxide (SO_2)	irritating
Nitrogen dioxide (NO_2)	100-200 (rats)
Hydrogen sulfide (H_2S)	713 (rats) 673 (mice)
Fluorine (F_2)	185 (rats) 150 (mice) 170 (guinea pigs)
Ethylene (CH_2CH_2)	narcosis 950,000 (mice)

*Data are taken from the Merck Index, 11th Ed, 1989. Lethal concentration for 50% of animals (LC_{50}).

In reviewing the toxicity of a selected series of gases formed from sulfur, nitrogen, fluorine, and carbon, pronounced differences are observed in the LC_{50} in experimental animals, as summarized in Table 2. The striking toxicity of oxygen difluoride, OF_2, is in contrast to the other toxic gases in this group. This gas is toxic at concentrations two to three orders of magnitude less than all of the other examples in Table 2, with LC_{50} values of 2.6 ppm in rats and 1.5 ppm in mice. Nitrogen dioxide, NO_2, and elemental fluorine, F_2, have LC_{50} values of 100 to 185 ppm. Hydrogen sulfide, H_2S, and hydrogen cyanide, HCN, have LC_{50} values of 713 and 544 ppm, respectively, in rats. The least toxic gas in this group is hydrogen fluoride, HF, with an LC_{50} of 1278 ppm in rats. For comparison, ethylene, CH_2CH_2, induces narcosis and has an LC_{50} of 950,000 ppm in mice.

Because of the extreme toxicity of oxygen difluoride, one of the most toxic gases known, this compound was investigated in World War I as a chemical weapon. The effectiveness of oxygen difluoride as a weapon is limited, however, by the decomposition of the compound in the presence of traces of water vapor or humidity in the atmosphere. This compound is suspected of causing injuries and deaths in the Manhattan Project in World War II because of its production from elemental fluorine and atmospheric oxygen in the synthesis of uranium hexafluoride [61].

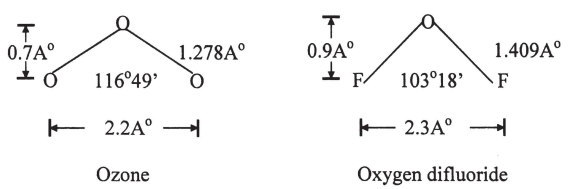

Fig. 2. The molecular structure of ozone is compared to the molecular structure of oxygen difluoride [62].

As shown in Fig. 2, the molecular structure of oxygen difluoride is similar to that of ozone [62]. Although the bond angle of oxygen difluoride, 103°18', is more acute than that of ozone, 116°49', the spacing of the two fluorine atoms, approximately 2.3 Å, is similar to the spacing of the two oxygen atoms about the central oxygen atom of ozone, approximately 2.2 Å. The reason is that the oxygen fluorine bonds of oxygen difluoride are slightly longer, 1.409 Å, than the oxygen oxygen bonds of ozone, 1.278 Å. The result of this bond configuration is that the central oxygen atom of oxygen difluoride is displaced from the plane of the fluoride atoms, approximately 0.9 Å, compared to the corresponding displacement of the central oxygen atom of ozone from the plane of the attached oxygen atoms, approximately 0.7 Å. The oxidizing power of ozone is exceeded only by that of fluorine, atomic oxygen, and OH radicals, and similar species [62].

The function of ozone in oxidative phosphorylation is considered to produce an active site with oxygen to form an oxygen ozone complex bound to the sulfonium centers of thioretinaco ozonide [2]. This complex is suggested to bind ATP synthesized by the F1 complex of ATPase of mitochondrial membranes from adenosine diphosphate and phosphate, as shown in Fig. 3. Because of its molecular structure and chemical reactivity, the extreme toxicity of oxygen difluoride is attributable to displacement of ozone from thioretinaco to form an inactive oxygen difluoride thioretinaco complex. Toxicity presumably results

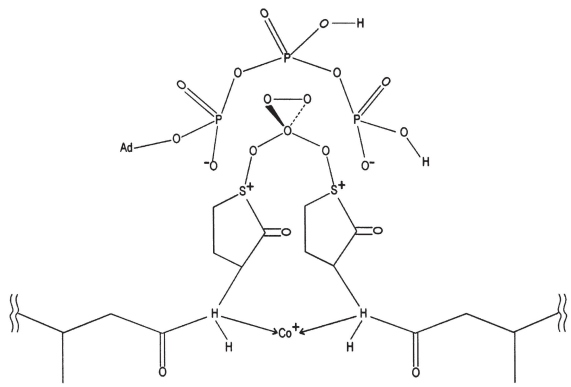

Fig. 3. Stereochemistry of disulfonium active site of thioretinaco ozonide accommodates binding of the alpha and gamma phosphate anions of ATP [2].

from inability of the oxygen difluoride complex to bind molecular oxygen or oxygen radicals, thereby preventing ATP synthesis. Thus the stereochemistry of the oxygen difluoride thioretinaco complex prevents binding of the alpha and gamma phosphates of ATP, inhibiting oxidative phosphorylation. Loss of ATP synthesis leads to cellular disintegration and decreased cellular viability because of loss of the high energy low entropy state of living cells [58].

The quantity of cobalamin in human liver is approximately 1.5 mg and the total body content is 2-5 mg [63]. These quantities correspond to 1 ppm in liver and 0.07 ppm in total body stores. Adenosyl cobalamin and methyl cobalamin are the principal forms of vitamin B_{12}, comprising 50-70% of tissue cobalamins [63]. The quantity of thioretinaco ozonide in tissue is unknown, as no method for determination of this complex has yet been reported. The LC_{50} of oxygen difluoride gas is 1.5 to 2.6 ppm (Table 2), corresponding to 1.5 to 2.6 times the concentration of total cobalamin in liver and 20 to 35 times the total body concentration of cobalamin. These comparisons suggest that oxygen difluoride is capable of displacing ozone from thioretinaco ozonide, explaining the extreme toxicity of this gas.

Fluoride is a toxic anion that stimulates oxygen consumption [64] and increases superoxide production in resting polymorphonuclear leukocytes [65]. Intracellular calcium ions are required for superoxide production by neutrophils during phagocytosis [66]. The effect of fluoride on superoxide production in neutrophils is reversible, causing superoxide production in the presence of fluoride, decreasing superoxide production when fluoride is removed, and restoring superoxide production when fluoride is again added [67]. Cellular viability is maintained during superoxide production. These experiments show that superoxide radical production by leukocytes is modulated by fluoride, providing a means for producing intracellular oxidative stress. In leukocytes, oxidative stress is an essential process by which phagocytosed microbes are inactivated and killed.

Fluoride has been known as a metabolic inhibitor for many years, causing inhibition of oxidative metabolism and decreased ATP synthesis [68]. Fluoride is believed to inhibit the activity of many enzymes by disrupting the molecular structure of proteins by interfering with normal hydrogen bonding. In addition the effects of fluoride on chromosomal sturucture, genetic damage, and carcinogenesis may be attributable to inhibition of DNA repair enzyme structure [68]. Fluoride forms unexpectedly strong hydrogen bonds in amide-fluoride systems, as determined by ab initio calculations and spectroscopic studies [69]. The effect of fluoride inhibition of hydrogen bonding was determined by analysis of the three-dimensional crystal structure of yeast cytochrome c peroxidase [70]. These findings support the view that fluoride inhibits enzyme function by altering the conformation of the polypeptide backbone of proteins through interaction of peptidyl amide groups with fluoride [68].

The effect of fluoride in the induction of oxidant stress is attributable to interaction of fluoride ions with the amide groups of thioretinaco ozonide (Fig. 3). Conformational change in the binding of thioretinamide groups to the cobalt of thioretinaco by hydrogen bonding with fluoride would be expected to inhibit the binding of superoxide and other oxygen radicals to the active site of oxidative phosphorylation, resulting in inhibition of ATP synthesis and accumulation of oxygen radical species within cells.

Homocysteine enhances superoxide anion release and NADPH oxidase assembly by human

neutrophils [71]. In addition, homocysteine enhances production of hydrogen peroxide and stimulates migration of neutrophils, either in suspension or adherent to fibrin [71]. The possible effect of homocysteine on thioretinaco ozonide to enhance oxygen radical production needs further investigation. One possibility is that homocysteine leads to increased formation of homocysteine thiolactone, which displaces thioretinamide from binding to cobalamin, forming thioco, a complex that inhibits oxidative phosphorylation and leads to accumulation of oxygen radicals and oxidative stress [2].

Inflammation

The presence of inflammatory cells in atherosclerotic plaques has been known for over 150 years, and Virchow gave the name "endoarteritis chronica deformans nodosa" to the arterial abnormalities that he demonstrated in arteriosclerosis [72]. These abnormalities include infiltration of inflammatory cells, fatty deposition in the intima, mucoid degeneration of arterial wall, fibrosis, calcification, and atheroma with crystal deposition. Virchow suggested that endothelial damage and increased permeability of arterial intima led to increased filtration of plasma and deposition of plasma fats, associated with the degenerative changes of arterial wall. In 1914 Aschoff described deposition of cholesterol crystals in aortic atheromas [73]. The contemporary version of the inflammatory origin of atherosclerosis is the "response to injury hypothesis," advocated by Ross [74]. According to this hypothesis, the factor leading to inflammation in atherosclerosis is considered to be endothelial dysfunction and intimal damage from low-density lipoprotein (LDL), oxidized LDL, hyperhomocysteinemia, hypertension and elevated angiotensin II, and infectious organisms such as *Herpesvirus* and *Chlamydia pneumoniae*.

A number of observations appear to contradict the "response to injury hypothesis." The concept that LDL cholesterol causes endothelial dysfunction and intimal damage is contradicted by the observation that there is no association between LDL levels in blood and the degree of endothelial dysfunction [75]. The concept that intimal damage causes influx of LDL cholesterol is contradicted by the observation that atherosclerotic plaques in hyperhomocysteinemia caused by inborn errors of methionine metabolism in children contain no lipid deposition despite pronounced intimal damage [55]. No study of unselected subjects has demonstrated an association between blood cholesterol concentration and the degree of atherosclerosis at autopsy [76]. A study of 194 consecutive autopsies showed that two-thirds of individuals with severe atherosclerosis had no elevation of blood cholesterol concentration and no evidence of renal failure, hypertension, or diabetes [77]. Hypercholesterolemia is not a risk factor in women of any age or in men over 50 years old, even though most cardiovascular deaths occur in subjects over 65 [78]. Among subjects with familial hypercholesterolemia, there is no association between LDL-cholesterol and the prevalence or progression of cardiovascular disease [79]. These studies cast doubt on the ability of hypercholesterolemia to cause intimal damage and trigger the inflammatory reaction in human atherosclerosis.

In the late 19th and early 20th centuries, many investigators suspected micro-organisms or toxic factors related to infection as the cause of atherosclerotic plaques. However, most contemporary experimental models failed to support this view. Instead, investigators focused on dietary protein [80] or dietary cholesterol [81] as pathogenic factors, leading to the lipid/cholesterol hypothesis and the homocysteine theory of arteriosclerosis [82]. Nevertheless, in the 20th century evidence accumulated to support the view that infection by viruses, bacteria, and protozoan organisms is important in the formation of atherosclerotic plaques [74,78]. Thus remnants of organisms such as *Herpesvirus*, *Cytomegalovirus*, periodontal bacteria, and *Chlamydia pneumoniae* have been demonstrated within atherosclerotic plaques by immunohistochemistry, polymerase chain reaction for DNA, and electron microscopy. For example, remnants of more than 50 different organisms have been demonstrated by polymerase chain reaction for DNA within human atherosclerotic plaques, and no evidence for these organisms was found in normal arteries [83]. In addition, lipoproteins participate in a nonspecific immune defense system

that inactivates micro-organisms by formation of aggregated lipoprotein complexes [78].

Homocysteine thiolactone, the reactive cyclic anhydride of homocysteine, reacts with free amino groups of proteins to form peptide-bound homocysteine groups, a process called thiolation because of the introduction of a free sulfhydryl group [1]. When increased concentrations of homocysteine thiolactone react with human LDL, the resulting homocysteinylated LDL becomes aggregated and susceptible to precipitation in vitro [84]. The homocysteinylated LDL aggregates are phagocytosed by cultured human macrophages, forming foam cells with greatly increased cytoplasmic cholesterol and cholesterol esters [84]. Foam cells are generally considered to be an important initiating factor in formation of atherosclerotic plaques, and rupture of vulnerable plaques leads to hemorrhage and occlusive thrombosis [85].

Thiolation of LDL produces an altered potentially antigenic apoB protein, and antibodies to thiolated LDL have been produced in rabbits [86]. Autoantibodies to N-thiolated albumin are present in human coronary heart disease [87,88], and although thiolated LDL is present in human serum at low concentrations (0.04-0.10%), autoantibodies to thiolated LDL have not been reported [89]. The possibility that autoantibodies to thiolated LDL may participate in atherogenesis is suggested by the finding of hyperhomocysteinemia and susceptibility to vascular disease in autoimmune diseases such as lupus erythematosus, rheumatoid arthritis, Behcet's disese, inflammatory bowel disease, and myelodysplastic syndrome [90]. These diseases are characterized by activation of the immune response and inflammation. Homocysteine activates cytokines and pro-inflammatory molecules, such as IL-1beta, IL-6, Il-12, IL-18, IL-1 receptor antagonist, C-reactive protein, adhesion molecules (P-selectin, E-selectin, ICAM-1), and metalloproteinases (MMP-9). In addition, homocysteine up-regulates reactive oxygen species, leading to activation of NF-kappaB, the pro-inflammatory nuclear regulatory molecule [91].

Oxidatively modified LDL (Ox-LDL) has long been considered to be a factor in initiation and progression of atherosclerotic plaques. However, the titer of autoantibodies to Ox-LDL does not reflect or predict vascular disease [92,93]. A population study of carotid atherosclerosis demonstrated a correlation between Ox-LDL and titers of antibodies to *Escherichia coli*, *Chlamydia pneumoniae*, mycobacterial heat shock protein, *Helicobacter pylori,* and *Cytomegalovirus* [94].

In order to explain the association of atherogenesis with infections, autoantibodies to thiolated LDL and Ox-LDL, hyperhomocysteinemia, and lipoprotein complexes with microbes, a new hypothesis describes how these factors conspire to produce vulnerable plaques [95]. Lipoproteins provide a nonspecific immune system that inactivates a wide variety of bacteria, viruses, and protozoans by formation of complexes with the lipopolysaccharides and lipoteichoic acids of microbial cell wall and with the membrane envelopes of viruses and protozoans. According to this hypothesis, these aggregated complexes become trapped in the vasa vasorum of artery walls, obstructing the lumen to cause ischemia, intramural cell death, rupture of capillaries, hemorrhage, and release of microbes into intima, where macrophages phagocytose these complexes to become foam cells. Thus this process leads to creation of a vulnerable plaque containing lipid deposits, hemorrhage, inflammatory cells, microbial remnants, and foam cells with some characteristics of a micro-abscess. This suggested chain of events explains the presence of micro-organisms in atherosclerotic plaques, the association of infections with acute coronary syndromes, and why cholesterol accumulates in plaques. It also explains the origin of the chronic inflammatory state associated with atherogenesis because of activation of the inflammatory response by micro-organisms and their toxins. Because of increased extra-capillary tissue pressure in vasa vasorum, it also explains why arteriosclerotic plaques are localized to areas of elevated pressure in arteries and why atherogenesis is promoted by systemic hypertension.

Endothelial dysfunction is generally considered to be the earliest manifestation of vascular disease [96]. Many factors lead to endothelial dysfunction, including oxidant stress, hyperhomocysteinemia, and antagonism of the vascular actions of nitric oxide, as outlined in this review. Some other factors

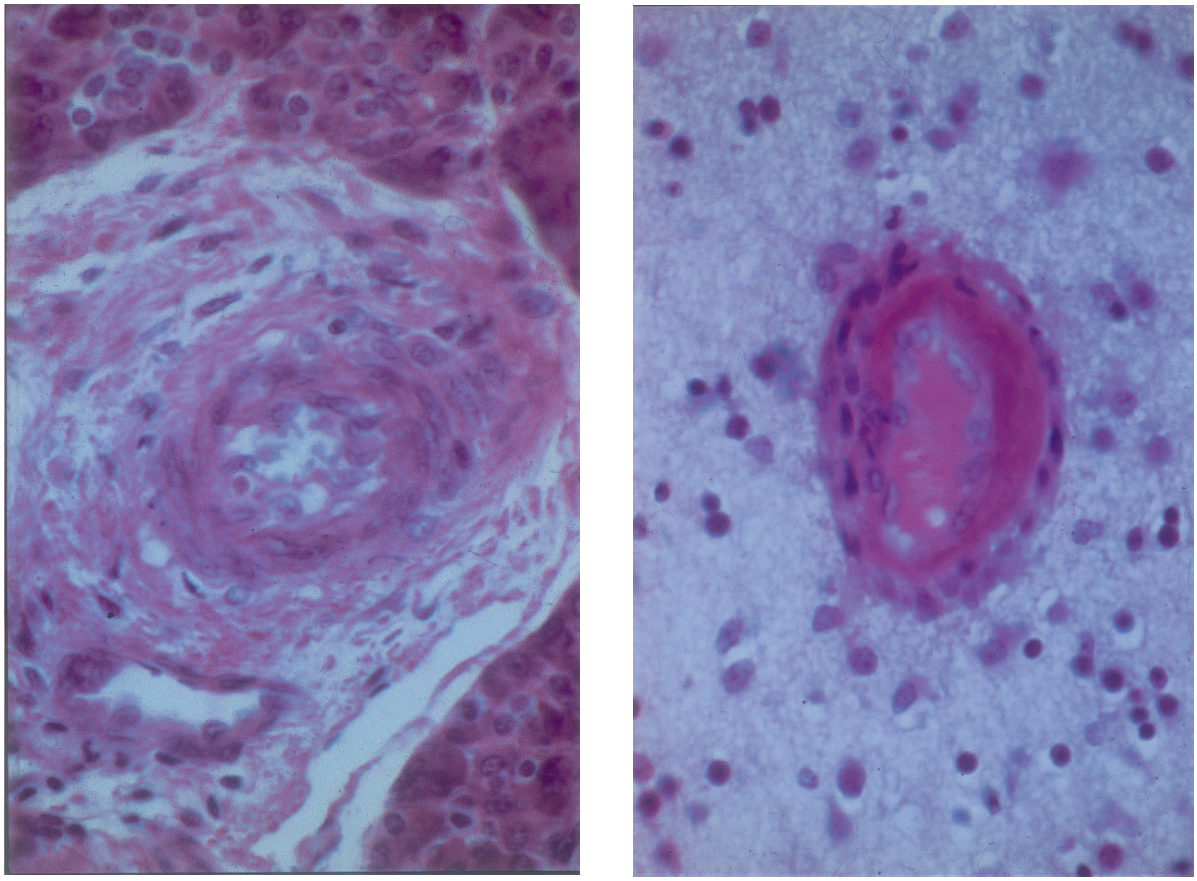

Fig. 4. Left panel: Pancreatic arteriole lumen is constricted by swollen, vacuolated, hyperplastic, endothelial cells in a patient with hyperhomocysteinemia from methionine synthase deficiency [55]; H&E x530. Right panel: Cerebral arteriole lumen is constricted by swollen, vacuolated, hyperplastic, endothelial cells and deposition of fibrin in a patient with hyperhomocysteinemia from methionine synthase deficiency [55]; H&E x530.

leading to endothelial dysfunction are renal failure, smoking, hypertension, and diabetes, all of which promote atherogenesis. Another factor leading to endothelial dysfunction is ascorbate deficiency, which leads to intimal lipid deposition along elastica interna in aortas of scorbutic guinea pigs [97]. Oxidation of homocysteine thiolactone is inhibited in scorbutic guinea pigs, leading to accumulation of free homocysteine in liver [98]. Endothelial dysfunction in human subjects with atherosclerosis is reversed by iv ascorbate, potentially ameliorating the early stages of atherogenesis [99].

Morphologically, endothelial dysfunction is characterized by vacuolization, cytoplasmic swelling, and hyperplasia of endothelial cells, all of which are observed in children with homocystinuria [55], as shown in Fig. 4. Constriction of the lumen of vasa vasorum by these changes in endothelial cell morphology would be expected to enhance trapping of lipoprotein complexes with micro-organisms, with homocysteinylated lipoprotein aggregates, and with autoantibodies to thiolated LDL and to Ox-LDL, causing obstruction of the lumen. Thus atherogenesis and the creation of vulnerable plaques are dependent upon numerous pathophysiological factors leading to endothelial dysfunction, hyperhomocysteinemia, infection, and inflammation.

Evidence to support this formulation is provided by a recent study demonstrating evidence of hypoxia in cells and tissues of vulnerable plaques in human atherosclerosis [100]. In this study of 7 patients with carotid atherosclerosis, infusion of pimonidazole prior to surgery demonstrated

hypoxia-inducible factor (HIF) by immunohistochemistry, by messenger ribonucleic acid (mRNA), and by HIF-responsive genes, especially within the macrophage-rich center of the carotid plaques removed at surgery. Hypoxia correlated with the presence of a thrombus, angiogenesis, and the expression of the macrophage marker, CD68, with HIF and with vascular endothelial growth factor (VEGF). The hypoxia of cells and tissues of vulnerable plaques demonstrated in this study is attributable to ischemia caused by trapping of LDL aggregates and obstruction of the lumen in vasa vasorum narrowed by endothelial dysfunction.

This review summarizes the current state of knowledge of the chemical pathology of homocysteine concerning the molecular, cellular, and tissue processes involved in excitotoxicity, oxidative stress, endothelial dysfunction, and inflammation. The creation of vulnerable plaques in advanced atherosclerosis is suggested to be related to formation of aggregates of homocysteinylated lipoprotein complexed with microbes, leading to trapping in vasa vasorum narrowed by endothelial dysfunction. The resulting obstruction of vasa vasorum causes ischemia of arterial wall, cell death, foam cell formation, rupture of capillaries with hemorrhage, and deposition of lipid in the intima, all of which are constituents of vulnerable plaques. This chain of events explains how risk factors for hyperhomocysteinemia, atherosclerosis, and endothelial dysfunction contribute to the pathogenesis of atherosclerotic plaques.

Acknowledgements

Dr. Curt-H. Becker, Max Delbrűck Center for Molecular Medicine, Berlin, provided the electron micrograph of mitochondria within smooth muscle cells of hyperhomocysteinemic rats. Charles F. Foltz, Medical Media Service, VA Medical Center, West Roxbury MA, helped to prepare the figures.

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