

Relationship between paraoxonase and homocysteine: crossroads of oxidative diseases

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Abstract

Homocysteine (Hcy) is an accepted independent risk factor for several major pathologies including cardiovascular disease, birth defects, osteoporosis, Alzheimer's disease, and renal failure. Interestingly, many of the pathologies associated with homocysteine are also linked to oxidative stress. The enzyme paraoxonase (PON1) – so named because of its ability to hydrolyse the toxic metabolite of parathion, paraoxon – was also shown early after its identification to manifest arylesterase activity. Although the preferred endogenous substrate of PON1 remains unknown, lactones comprise one possible candidate class. Homocysteine-thiolactone can be disposed of by enzymatic hydrolysis by the serum Hcy-thiolactonase/paraoxonase carried on high-density lipoprotein (HDL). In this review, Hcy and the PON1 enzyme family were scrutinized from different points of view in the literature and the recent articles on these subjects were examined to determine whether these two molecular groups are related to each other like a coin with two different sides, so close and yet so different and so opposite.

Key words: homocysteine, paraoxonase, homocysteine thiolactonase, oxidative stress.

Homocysteine

In 1932 Burtz and du Vigneaud discovered a new amino acid by treating methionine with sulfuric acid at the University of Illinois [1]. Because this amino acid was similar in structure to cysteine and contained an extra carbon atom, they named it homocysteine. Du Vigneaud investigated the role of homocysteine in metabolism and the ability of homocysteine and choline to replace methionine as an essential nutrient for growth of animals. However, little else was known about the importance of homocysteine in medicine or vascular disease in the 1950s. Later, in monkeys homocysteine (Hcy) which suggested a relation between arteriosclerosis and sulfur amino acid metabolism [2].

Homocysteine has now become a rather famous amino acid. The name homocysteine (Hcy) is specific for thiol-containing amino acid. However, tissues and especially plasma contain related disulfides that are usually measured together with homocysteine. Common usage has given the sum of recorded and oxidized species the name total homocysteine (tHcy) or, more often, simply the imprecise, generic designation of homocysteine.

In 1962 children with mental retardation, dislocated ocular lenses, accelerated growth, osteoporosis, and a tendency to thrombosis of arteries and veins were discovered to excrete the amino acid homocystine in their urine. These children had a rare inherited enzymatic defect in homocysteine metabolism that was caused by deficiency of cystathionine synthase, an enzyme requiring pyridoxal phosphate (vitamin B₆) for normal activity [3-5]. According to current concepts, homocysteine damages cells and tissues of arteries by inciting the release of cytokines, cyclins, and other mediators of inflammation and cell division [6]. Although Hcy is a normal metabolite, its excess can be extremely toxic to human, animal, yeast, and bacterial cells [7]. The non-protein amino acid homocysteine (Hcy), owing to its structural similarity to the protein amino acids methionine, isoleucine, and leucine, enters the first steps of protein synthesis and is activated by methionyl-, isoleucyl-, and leucyl-tRNA synthetases *in vivo* [7]. Methionine contains sulfide sulfur, the general structure of which can be designated R-S-R'. Homocysteine and cysteine are sulfhydryl compounds (R-SH). Compounds that contain a free sulfhydryl group are known as thiols. Other low-molecular-weight biological *thiols* include glutathione, coenzyme A and dihydrolipoic acid.

A general chemical property of thiols is their ability to oxidize in the presence of an electron acceptor such as molecular oxygen to form disulfides. The homocysteine will autooxidize to form *homocystine*. Homocysteine can oxidize with other thiols such as cysteine and glutathione to form mixed disulfides, and these compounds are referred to as *homocysteine cysteine mixed disulfide* and *homocysteine-glutathione mixed disulfide*. Peptides and proteins may also contain free cysteine residues that can autooxidize or react with low-molecular-weight thiols to form stable disulfide bond complexes. Thus > 70% of the homocysteine in the circulation is bound to plasma protein cysteine residues through disulfide bonds.

There is also little doubt that excess Hcy is harmful to the human body. However, there is an ongoing debate as to why. Excess Hcy is harmful. Homocysteine is primarily found in its oxidized state (homocystine), homocysteine, homocysteine mixed disulfide and protein-bound homocysteine.

Why Hcy is toxic is not entirely clear and is a subject of intense studies. However, it is not clear whether homocysteine is a marker or a causative agent [8]. It is now well established that hyperhomocysteinaemia is an independent risk factor for coronary artery disease [9], cerebrovascular disease, and peripheral vascular occlusive disease, yet the question remains: Is homocysteine causal or merely an innocent marker? [10].

By affecting smooth muscle cells, homocysteine produces the connective tissue changes of arte-

riosclerotic plaques, causing fibrosis, calcification, proteoglycan deposition, and damage to elastic tissue layers. Homocysteine is a potent procoagulant that promotes the deposition of fibrin and mural thrombosis in artery walls. Homocysteine thiolactone is the reactive anhydride of homocysteine that interacts with low-density lipoprotein (LDL), causing aggregation, increased density, and uptake by vascular macrophages to form foam cells. Degradation of these aggregates leads to deposition of cholesterol and other fats in developing plaques [11-13].

In addition, reaction of Hcy-thiolactone with serum proteins leads to the production of new protein antigens and autoimmune antibodies, facilitating the inflammatory response. In addition, Hcy causes oxidant stress by effects on cellular respiration, leading to oxidation of LDL and other constituents of plaques. Hcy also antagonizes the vasodilator properties of nitric oxide by the formation of S-nitrosohomocysteine, leading to endothelial dysfunction, the earliest stage in atherogenesis [14, 15]. However, the underlying mechanisms of Hcy-induced vascular injury are still largely unknown in subjects with elevated plasma tHcy levels. Extensive clinical trials, spanning decades and tens of thousands of patients, have not yet established a definitive relationship between Hcy lowering using vitamin therapy and reduced cardiovascular disease risk.

Homocysteine and oxidative stress

Homocysteine is an accepted independent risk factor for several major pathologies including cardiovascular disease, birth defects, osteoporosis, Alzheimer's disease, and renal failure. Interestingly, many of the pathologies associated with homocysteine are also linked to oxidative stress. Reactive oxygen species (ROS) can in turn elevate tension of redox stress, and cause damage to cells. Oxidative stress is also linked to declines in pulmonary, brain, circulatory, and reproductive function [16].

It is now well established that when the production of ROS exceeds the capacity of antioxidant defences, oxidative stress might have a harmful effect on the functional and structural integrity of biological tissues. The best known ROS species generated from O₂ include the superoxide anion radical (dioxide, or O₂^{•-}), the hydroxyl radical (•OH), and the peroxynitrite anion (ONOO⁻). These can cause cellular dysfunction and sometimes cell death. Cells are equipped with excellent antioxidant defence mechanisms to detoxify the harmful effects of ROS. The antioxidant defences can be non-enzymatic (e.g. glutathione, vitamins C, A, E, and thioredoxin) or enzymatic (e.g. superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) [8].

It has been hypothesized that Hcy promotes oxidative stress via ROS generation upon disulfide

bond formation. Because homocysteine and other thiols have pro-oxidant activity, the oxidant stress hypothesis is frequently invoked to explain the damaging effects of homocysteine on vascular cells and tissues [17].

Homocysteine contains a reactive sulfhydryl group (–SH) and, like most thiols (RSH), can undergo oxidation to the disulfide (RSSR) at physiological pH in the presence of O₂:



The general reaction is catalysed by transition metals, and a variety of reactive oxygen species can be produced, including superoxide anion radical (O₂^{•−}) and hydrogen peroxide (H₂O₂) [8].

The daily flux of plasma homocysteine in a healthy 70-kg individual is estimated to be 1.3 mmol/day. This being the case, 0.65 mmol/day of hydrogen peroxide would be generated as a result of homocysteine oxidation, which is equivalent to 92 pmol/min × ml blood in a 70-kg individual (assuming an approximate blood volume of 70 ml/kg). Someone with mild hyperhomocysteinaemia would generate twice the level of hydrogen peroxide 184 pmol/min × ml under the same set of assumptions [8].

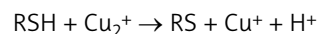
If homocysteine export fails to keep up with production, intracellular accumulation could become cytotoxic. One mechanism of cytotoxicity might involve the inactivation of the glutathione antioxidant defence system. In this scenario, the injury comes from within, not without, and may involve specific molecular targets [18]. Nishio and Watanabe have shown that treatment of rat aorta smooth muscle cells with D,L-homocysteine (0 to 500 mmol/l) decreased glutathione peroxidase activity but increased SOD activity in a dose-dependent manner. Homocysteine had no effect on catalase activity. They did observe that 5.0 mmol/l homocysteine (but not cysteine) decreased steady-state mRNA for glutathione peroxidase by 90% [18]. Using cultured porcine aortic endothelial cells, Lang *et al.* showed that 0.03 mmol/l to 1.0 mmol/l L-homocysteine but not L-cysteine or glutathione stimulated intracellular production of superoxide and induced higher levels of SOD activity [19]. These studies suggest that oxidative stress may be generated within vascular cells, but not necessarily as a result of thiol oxidation. Also, hyperhomocysteinaemia is accused of being responsible for elevating oxidative stress as a result of formation of Hcy-thiolactone or Hcy-thiyl radical, which may lead to impairment of cell signalling and cause pathology [20, 21].

In plasma, the concentration of homocysteine-free thiol is nanomolar, whereas albumin (thiol) is 400 µmol/l to 600 µmol/l, cysteine is 10 µmol/l to 20 µmol/l and glutathione is 3 µmol/l to 5 µmol/l.

Homocysteine-thiyl radical (•RS)

As a free amino acid it exists in either a reduced (homocysteine, a thiol RSH) or oxidized (homocysteine, a disulfide RSSR) form. Its redox chemistry is dominated by its thiol group (SH), which in contrast to most nucleophiles is readily oxidized.

Aliphatic thiols (RSH) are present in living organisms in high concentrations. Thiol compounds (RSH) are frequently oxidized in the presence of iron or copper ions:



The thiyl radical (•RS) generated by the one-electron oxidation of a thiol reacts rapidly with another thiol to yield the radical disulfide anion (RSSR^{•−}).

Thiols such as homocysteine and glutathione have a dark side that makes them potentially deleterious to cells. In the presence of metal ions and oxygen, they can autooxidize, generating highly reactive partially reduced oxygen species.

As noted previously, thiol oxidation can generate thiyl radicals. These species abstract hydrogen atoms from polyunsaturated fatty acids at a constant rate.

The Hcy-thiyl radical can undergo a kinetically favoured hydrogen atom transfer (HAT) reaction to afford an α-amino carbon-centred radical. α-Amino carbon radical formation in Hcy occurs under the same conditions that promote thiyl radical mediated disulfide formation. This is due to the thiyl radical's participation in the HAT mechanism [23].

Importantly, HAT has displayed enhanced carbonyl formation at physiological temperature without added oxidants, simply upon homocysteamide enrichment. Studies of the properties of Hcy-N-protein showed that homocystamides render proteins more prone to oxidation, as evidenced by enhanced disulfide formation and alteration of the redox status of cytochrome C. The kinetically favoured formation of α-carbon radicals renders Hcy-N-protein capable of initiating oxidative damage under the natural conditions that induce thiyl radical formation. Elucidation of the altered redox status of hyperhomocysteinaemic patients is needed as a step toward targeted therapy [23].

Homocysteine-thiolactone

Homocysteine thiolactone was first prepared from methionine in 1934. Homocysteine thiolactone is an intramolecular thioester of homocysteine. The thiolactone is prepared by boiling either homocysteine for ten minutes with HCl or methionine for 4 h with hydriodic acid.

Although Hcy-thiolactone was obtained by chemical synthesis in 1934, the first indication of its biological significance came almost 50 years later with

the discovery of the enzymatic conversion of Hcy to Hcy-thiolactone in error editing reactions of some aminoacyl-tRNA synthetases. Homocysteine thiolactone, the product of amino acyl tRNA synthetase editing reactions, is the five-membered condensed ring form of homocysteine. Thiolactone formation is unique to homocysteine owing to the extra carbon atom within the side chain (cysteine has only a single carbon within its side chain, whereas homocysteine has two) (Figure 1).

Homocysteine thiolactone, which is formed from an intramolecular condensation reaction between thiol and carboxylic acid, may also occur in plasma in submicromolar concentrations. Many researchers, and especially Jakubowski, suggest that metabolic conversion of Hcy to Hcy-thiolactone followed by subsequent spontaneous protein N-homocysteinylation by Hcy-thiolactone may contribute to Hcy toxicity in humans [24].

This hypothesis suggests that the conversion to Hcy-thiolactone contributes to Hcy toxicity and is linked to atherosclerosis in humans. The formation of Hcy-thiolactone can be detrimental for two reasons. First, its reaction requires ATP and thus causes non-productive consumption of cellular energy [25]. Second, Hcy-thiolactone is a reactive intermediate that causes protein N-homocysteinylation through the formation of amide bonds with ϵ -amino groups of protein lysine residues. Resulting protein damage necessitates the removal of N-homocysteinylation by proteolytic degradation, which would further deplete cellular energy and limit cell growth. Eventually Hcy-thiolactone appears to be more toxic to human cells than Hcy. Homocysteine-containing proteins are also toxic and induce an autoimmune response, which is associated with atherosclerosis in humans [26, 27].

Excess homocysteine and homocysteine-thiolactone

The only known source of Hcy in the human body is dietary protein methionine. After meal ingestion, methionine is liberated from dietary protein in the digestive system. Free methionine is transported in the blood to body organs and taken up by cells [28].

At the nutritional level, there is little doubt that excess Hcy is a direct consequence of excess intake of protein methionine. Since Hcy is formed from an essential dietary amino acid, methionine, high intakes of methionine increase the plasma tHcy concentrations. In clinical practice the term tHcy refers to the sum of free, oxidized and protein-linked Hcy. Elevation of plasma tHcy occurs, for example, in the oral methionine loading test, in which a large dose of methionine (0.1 g/kg body weight of L-methionine) is ingested to diagnose hyperhomocysteinaemia. It could be speculated

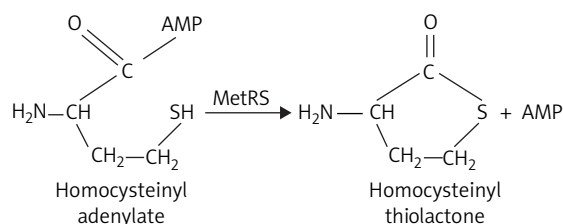


Figure 1. Production of homocysteine thiolactone

that a long-term high methionine intake from the diet could lead to modest but chronic plasma tHcy concentrations [29].

Inside cells, methionine is used for synthesis of new proteins and S-adenosylmethionine (AdoMet), a universal methyl donor. As a result of biological methylation reactions, AdoMet is converted to adenosylhomocysteine (AdoHcy), which is subsequently hydrolysed to adenosine and Hcy. Under normal circumstances, most, but not all, of the Hcy formed in transmethylation reactions is remethylated back to methionine or converted into cysteine in transsulfuration reactions. This happens because the capacity of the transsulfuration pathway is exceeded and Hcy is excreted from cells [30].

The condition of excess Hcy can be exacerbated by inadequate intake of folic acid, vitamin B₁₂, and vitamin B₆ as well as by the allelic variation in genes encoding enzymes participating in Hcy metabolism [31, 32].

Homocysteine is also metabolized to the cyclic thioester Hcy-thiolactone. The synthesis of thiolactone occurs in all human cell types investigated. The Hcy-thiolactone pathway becomes predominant when remethylation or transsulfuration reactions are impaired by genetic alterations of enzymes involved in Hcy metabolism, such as cystathionine β -synthase (CBS), methionine synthase (MS), or methylenetetrahydrofolate reductase (MTHFR) or by inadequate supply of folate, vitamin B₁₂, or vitamin B₆. Homocysteine-thiolactone is formed by methionyl-tRNA synthetase (MetRS) in an error-editing reaction in protein biosynthesis when Hcy becomes mistakenly selected in place of methionine [30].

In all organisms, Hcy enters the first step of protein biosynthesis and forms an Hcy-AMP intermediate in a reaction catalysed by methionyl-tRNA synthetase (MetRS). However, the misactivated Hcy is then edited, with the formation of the thioester Hcy-thiolactone [30] (Figure 2).

Homocysteine thiolactone is synthesized by MetRS in all cell types tested thus far, including human vascular endothelial cells. The energy of the anhydride bond of Hcy~AMP is conserved in an intramolecular thioester bond of Hcy thiolactone.

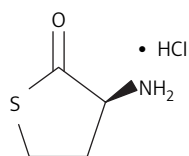


Figure 2. Chemical formula of homocysteine thiolactone

Consequently, Hcy-thiolactone is a reactive intermediate that causes protein N-homocysteinylation through the formation of amide bonds with ϵ -amino groups of protein lysine residues [30].

Homocysteine thiolactone is harmful to cells

Homocysteine-thiolactone is cytotoxic [24]. Early evidence suggesting that Hcy-thiolactone is cytotoxic to the cardiovascular system was obtained in the 1970s, well before metabolism of Hcy-thiolactone in humans was deciphered and its physiological significance established [24, 33]. Infusions with Hcy-thiolactone have been used as an early model of clinical homocystinuria. Tissue culture studies show that Hcy-thiolactone induces apoptotic death in human vascular endothelial cells, promyeloid HL-60 cells, and placental trophoblasts, and inhibits insulin signalling in HTC rat hepatoma cells transformed with insulin receptor [34, 35]. Apoptosis is also induced by Hcy, but at much higher concentrations than Hcy-thiolactone. Homocysteine-thiolactone has also been demonstrated to be more effective than Hcy in inducing endoplasmic reticulum (ER) stress in a retinal pigmented epithelial cell line [34].

In particular, homocysteinylation of LDLs increases their susceptibility to oxidation and facilitates their uptake by macrophages [36]. Additionally, homocysteinylation of LDLs elicit an autoimmune response and increase vascular inflammation, which are known modulators of atherosclerosis. Clots formed from homocysteinylation of fibrinogen present a higher resistance to lysis, contributing to a higher risk of thrombosis and vascular disease [37, 38].

Disposal mechanisms of homocysteine thiolactone

To minimize Hcy-thiolactone toxicity, cells had to evolve the mechanism of its disposal [39]. Indeed, in all organisms, the bulk of Hcy-thiolactone is eliminated by excretion from cells into the extracellular media. However, Hcy-thiolactone is not a reliable marker of plasma tHcy. In healthy volunteers, it contributes only 0.14-0.28% of tHcy, has a half-life of 1 h, and is below the detection limit in approximately one half of volunteers [39].

In humans, Hcy-thiolactone is cleared out from the circulation by urinary excretion in the kidney. Urinary

concentrations of Hcy-thiolactone are ~100-fold higher than those found in plasma. Normal urinary Hcy-thiolactone levels vary from 11 nmol/l to 485 nmol/l. Urinary Hcy-thiolactone accounts for 2.5-28% of urinary tHcy and thus contributes significantly to urinary tHcy pools [40]. Whereas relative renal clearance of tHcy is only about 0.001-0.003, the clearance of Hcy-thiolactone is 0.2-7.0 of creatinine clearance [41]. This suggests that in some individuals Hcy-thiolactone is not only filtered in the glomeruli but also secreted into the tubular lumen. Although it contributes very little to the daily flux of tHcy in a healthy individual, renal excretion removes a large fraction of Hcy-thiolactone, which would otherwise cause protein N-homocysteinylation [39].

The metabolic conversion of Hcy to Hcy-thiolactone has been reported as the major cause of Hcy pathogenesis. It was hypothesized that inhibition of the thiol group of Hcy by S-nitrosation will prevent its metabolic conversion to Hcy-thiolactone. The kinetics, reaction dynamics, and mechanism of reaction of Hcy and nitrous acid to produce S-nitrosohomocysteine (Hcy-NO) were studied in mildly to highly acidic pHs. Transnitrosation of this non-protein-forming amino acid by S-nitrosoglutathione (GS-NO) was also studied at physiological pH 7.4 in phosphate buffer.

Copper ions were found to play dual roles, catalysing the rate of formation of Hcy-NO as well as its rate of decomposition. Transnitrosation of Hcy by GS-NO produced Hcy-NO and other products including glutathione (reduced and oxidized) and homocysteine-glutathione mixed disulfide. This study has shown that it is possible to modulate homocysteine toxicity by preventing its conversion to more toxic Hcy-thiolactone by S-nitrosation [24].

Excess homocysteine thiolactone is toxic to proteins

As previously mentioned, Hcy-thiolactone is a reactive intermediate that causes protein N-homocysteinylation through the formation of amide bonds with ϵ -amino groups of protein lysine residues. In the event homocysteinylation of proteins may lose their biological activity [24].

The levels of N-linked Hcy present in individual human blood proteins are roughly proportional to the proteins' abundance. Thus, most of N-linked Hcy is carried on haemoglobin (75%), albumin (22%), and g-globulin (2%) in the human blood. All other blood proteins contain about 1% of N-linked Hcy. In human plasma, most of N-linked Hcy, 90%, is carried on albumin. The concentration of N-linked protein Hcy carried on albumin in plasma is about 2.8 mmol/l, which is 25% relative to plasma tHcy. The substitution of the ϵ -amino group of a protein lysine residue with an Hcy residue containing a free

thiol group is expected to affect protein structure and function [42, 43].

The immediate result of protein N-homocysteinylation is a decrease of the net positive charge on a protein because the highly basic ϵ -amino group of a protein lysine residue (pKa 10.5) is replaced by a less basic α -amino group of N-linked Hcy (estimated pKa 7) [43].

In addition to these modifications, protein N-homocysteinylation leads to secondary structural changes. For example, after incorporation of just one N-linked Hcy/mol protein, N-Hcy-cytochrome C becomes prone to aggregation as a result of intermolecular disulfide bond formation. Trace amounts of aggregates of a variety of proteins might occur spontaneously or as a result of protein modification, particularly during aging, and could account for subtle impairments of cellular function even in the absence of an evident amyloid phenotype. Thus, the observation that N-Hcy-proteins occur in the human body and tend to form aggregates *in vitro* raises an interesting question of whether such aggregates can be cytotoxic [44].

A possible mechanism contributing to vascular injury by Hcy involves ER stress and activation of the unfolded protein response. Exactly how Homocysteine causes protein unfolding is not clear. One possibility is that Hcy is metabolized to Hcy-thiolactone, which then causes protein N-homocysteinylation in the ER, leading to damage to secretory proteins. Homocysteine itself could participate in disulfide exchange reactions with ER proteins. Endoplasmic reticulum stress is manifested by dysregulation of lipid metabolism, activation of inflammatory pathways, impaired insulin signalling, and possibly cell death. Essentially, Hcy-thiolactone and N-Hcy-proteins are known to be formed in human cells, and the magnitude of their synthesis depends on the concentration of Hcy [30, 44, 45].

Another example that can be given is structural distortions. Clots formed from N-Hcy-fibrinogen are more resistant to lysis than control clots from native fibrinogen. The presence of small amounts of N-linked Hcy in native human fibrinogen suggests that it is a target for the modification by Hcy-thiolactone in the human body. Clots formed from Hcy-thiolactone-treated normal human plasma lyse more slowly than clots from untreated control plasma, and the magnitude of this effect depends on the concentrations of Hcy-thiolactone used [46, 47].

These results suggest that N-homocysteinylation of fibrinogen can lead to abnormal resistance of fibrin clots to lysis and contribute to increased risk of cardiovascular disease in hyperhomocysteinaemia. Also, fibrinogen represents a major step in platelet aggregation while homocysteine impairs nitric oxide production and also contributes to the generation of oxidized species [46].

Incorporation of multiple N-linked Hcy residues has been shown to be detrimental to the function

of other proteins. Complete loss of enzymatic activity occurs after N-homocysteinylation of 8 lysine residues in MetRS (33% of total lysine residues) or 11 lysine residues in trypsin (88% of total lysine residues) [30].

Homocysteine-thiolactone also inactivates enzymes by other mechanisms. For example, lysine oxidase, an important enzyme responsible for post-translational collagen modification essential for the biogenesis of connective tissue matrices, is inactivated by Hcy-thiolactone, which derivatizes the active site tyrosine quinone cofactor with a half-life of 4 min. Homocysteine-thiolactone has also been shown to decrease enzymatic activity of lysine oxidase in cultured porcine aortic endothelial cells. These observations can account for the reduced number of collagen cross-links observed in patients with homocystinuria [24, 48].

N-Homocysteinylation may also be detrimental to the normal function of LDL. For example, N-homocysteinylation of LDL, in which 10% or 25% of lysine residues have been modified (i.e., containing 36 mol and 89 mol N-linked Hcy/mol LDL), is taken up and degraded by human monocyte-derived macrophages significantly faster than native LDL. The mechanism underlying N-Hcy-LDL toxicity may involve a decrease in endothelial Na^+, K^+ -ATPase activity, leading to an overload with sodium and, subsequently, with calcium. This in turn causes reduced production of nitric oxide and generation of peroxynitrate, a highly reactive nitrogen metabolite. Taken together, these observations suggest that protein N-homocysteinylation may contribute to endothelial dysfunction, a key event initiating the development of atherosclerotic plaque. It is known that in a healthy endothelium NO rapidly reacts with Hcy to form S-nitrosohomocysteine [25, 30, 50-52].

More recently, Jakubowski *et al.* provided direct evidence showing that free N ϵ -Hcy-Lys arises by proteolytic degradation of N-Hcy-protein in mouse liver extracts. Free N ϵ -Hcy-Lys can also arise by a reaction of free lysine with Hcy-thiolactone. Thus, N-Hcy-protein is the major source of free N ϵ -Hcy-Lys while the contribution of the reaction of free lysine with Hcy-thiolactone to the pool of free N ϵ -Hcy-Lys is expected to be very small [43]. Herewith, *in vitro*, Hcy thiolactone can modify essentially all plasma proteins, as well as other proteins that have been examined, at rates proportional to the protein's concentration and lysine content.

Pathophysiological responses to protein N-homocysteinylation

Jakubowski *et al.* have found that male stroke patients have significantly higher serum levels of anti-N ϵ -Hcy-Lys-protein IgG than healthy male controls. Higher levels of anti-N ϵ -Hcy-Lys-protein

autoantibodies most likely reflect higher levels of tHcy observed in these patients. In addition, they also found that male patients with angiographically documented coronary artery disease have higher serum levels of anti-N^ε-Hcy-Lys-protein IgG and tHcy than healthy male controls [13]. Induction of an autoimmune response may explain why the relatively small amounts of N-Hcy protein occurring in the human body can be detrimental.

Hyperhomocysteinaemia causes connective tissue pathology. Toohey *et al.* have explained the toxicity of Hcy-thiolactone on connective tissue by the conversion of Hcy-thiolactone to mercaptoaldehyde. The reaction may occur *in vivo* and may be pathogenic to connective tissue in four ways: (1) the reaction may deplete ascorbic acid that is required for collagen synthesis, (2) the mercaptoaldehyde product may interfere with collagen synthesis, (3) the mercaptoaldehyde may cause abnormal cross-linking of collagen molecules, and (4) the mercaptoaldehyde may attach to collagen molecules, rendering them antigenic and triggering an autoimmune response [31]. According to Hermmann *et al.*, tissue specific accumulation of Hcy in bone may be a promising mechanism explaining adverse effects of hyperhomocysteinaemia on bone. A reduced methylation capacity of bone cells might be another relevant pathomechanism [48].

Cell energy is wasted for producing homocysteine-thiolactone

Excess Hcy is known to inhibit growth of microbial cells, such as the bacterium *Escherichia coli* and yeast. The conversion of Hcy to Hcy-thiolactone requires ATP and thus causes nonproductive dissipation of cellular energy which could inhibit growth, as demonstrated in yeast. Supplementation with Hcy greatly increases Hcy-thiolactone accumulation and causes growth inhibition in *E. coli*. Growth inhibition caused by supplementation of *E. coli* cultures with Hcy is accompanied by greatly increased synthesis of Hcy-thiolactone, which requires increased unproductive ATP dissipation [53].

Strategic enzymes: paraoxonase family

The presence of an organophosphate-hydrolysing enzyme was first demonstrated in mammalian tissue, an observation that ultimately led to the identification of a human paraoxonase in serum in 1953 [54, 55]. Paraoxonase – so named because of its ability to hydrolyse the toxic metabolite of parathion, paraoxon – was also shown early after its identification to manifest arylesterase activity. Although the preferred endogenous substrate of paraoxonase remains unknown, lactones (especially Hcy-thiolactone) comprise one possible candidate class. Paraoxonase 1 (PON1) has also been shown

to metabolize a number of drugs and pro-drugs via its lactonase activity [56].

The three PON genes (PON1, PON2, PON3) share 65% similarity at the amino acid level and are located adjacent to each other on chromosome 7 in humans. The cloning of the gene in 1993 resulted in the identification of over 200 single-nucleotide polymorphisms (SNPs) in PON1 in different regions of the gene [57, 58]. Further studies were conducted to identify the impact of these polymorphisms on the activity and protein level of the enzyme. Researchers' attention has focused on SNPs of the coding region at positions 192, 55, and the –108 promoter region. Glutamine (Q)/arginine (R) substitution at codon 192 results in different hydrolytic activity of the alleles towards various substrates [59, 60].

Two other members of the PON gene family, termed PON2 and PON3, have been identified. Although the expression of PON1 and PON3 is restricted primarily to the liver, PON2 is not detectable in serum, although PON2 is more widely expressed and is found in a number of tissues, including brain, liver, kidney, and testis [61]. Paraoxonase 2 (PON2) enzyme resides in many tissues and is not released from the cells. It is located in the cell membrane with its active side exposed to the outer side of the cell. Paraoxonase 1 is similarly orientated in the cell membrane before it is excreted to the serum and bound to HDL [61].

Phylogenetic analysis reveals PON2 to be the oldest member of the family. The exact function of PON2 in humans is not known. Dihydrocoumarin is the only substrate reported to date for PON2. The results of Devarajan *et al.* illustrate that the anti-atherogenic effects of PON2 are, in part, mediated by the role of PON2 in mitochondrial function [62].

Like PON1, PON3 is also found in HDL, but PON2 and PON3 lack paraoxonase or arylesterase activities but are similar to PON1 in that both hydrolyse aromatic and long-chain aliphatic lactones. Paraoxonase 3 in particular hydrolyses widely used drugs such as the statin lactones lovastatin and simvastatin and the diuretic spironolactone [63].

The high similarity observed in the amino acid sequence between the PON proteins suggests that PON2 may possess a biochemical function similar to that of PON1 and PON3. Like PON1, both human PON2 and PON3 have been shown to prevent cell-mediated oxidative modification of LDL.

However, only PON1 hydrolyses organophosphates, and thus, reference to the other 2 isoforms as paraoxonases is a misnomer [61, 64]. So, paraoxonase nomenclature is a good example of the power of language to mislead [61]. Really, there is no judicious reason for mammals to have evolved an enzyme that can only hydrolyse synthetic organophosphates or drugs. This focus on one aspect of the enzyme's function, overemphasized

by its denotation, delayed an appreciation of other potential – and perhaps more relevant – roles [65]. However, the exact endogenous substrates and mechanism of the PONs' protective activities remain to be elucidated.

Natural substrates of paraoxonase

Extensive research has focused on serum arylesterase/paraoxonase, an organophosphate-detoxifying enzyme whose natural substrate and function remain unknown. Also remaining unknown is the relationship between the catalytic efficiencies of the enzyme variants toward the natural substrate and the 'artificial substrate' (organophosphate or arylester). In the absence of an acknowledged biological substrate for PON1, its activity is measured through its degrading function towards the artificial substrates paraoxon and phenyl acetate. Recent studies indicate that arylesterase activity best reflects the antioxidant activity of PON1, although it is not directly responsible for it [67, 68].

Although the notion that PON1 has an antioxidant function is assumed, we know of no biochemical basis for that exact function. It seems unlikely that the paraoxonase or arylesterase activities measured are related to the suggested antioxidant function of PON1; paraoxon and phenylacetate are artificial substrates, convenient for monitoring hydrolytic activity of PON1, which is not a putative redox activity [69].

The lactonase activity of paraoxonase may be an important consideration here, especially if one endogenous substrate is the naturally occurring Hcy-thiolactone. It seems that PON1 has a wide range of biological substrate specificity. However, the variety of lipids postulated as substrates for PON1 may also stem from the technical difficulties in identifying and analysing specific lipid oxidation products, as they are often unstable and depend on the type and length of the oxidation process. The enzyme also reduces lipid hydroperoxides to hydroxides and presents a peroxidase-like activity, as PON1 was shown to degrade hydrogen peroxide (H_2O_2), a major ROS produced under oxidative stress [70].

Why is the naturally endogenous substrate Hcy-thiolactone not appropriate for measurement of PON1 enzyme activity? Actually there is not a very easy answer to this question. Since the serum PON1 is present extracellularly, it was unknown whether Hcy-thiolactone can be detoxified intracellularly. Homocysteine-thiolactone can also be disposed of by enzymatic hydrolysis by the serum Hcy-thiolactonase/paraoxonase (PON1) carried on high-density lipoprotein. Homocysteine-thiolactonase (HTLase) activity of the PON1 protein detoxifies Hcy-thiolactone in human blood and could thus confer a vascular protective role. Billecke *et al.* showed that

human serum PON1 is able to hydrolyse lactones and cyclic carbonate esters [71].

Also, Carey *et al.* found that PON2 (lactonase) is constitutively expressed in both primary and immortalized human endothelial cells and human aortic smooth muscle cells. Furthermore, the same researchers show that PON2 has antioxidant properties. Paraoxonase 2 overexpression lowers the intracellular oxidative state of cells that have been treated with either hydrogen peroxide or oxidized- α -1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (Ox-PAPC) [72].

These data suggest that PON2 may play an anti-atherogenic role by reducing the oxidation of LDL and/or by reducing the production of intracellular hydroperoxides [72]. There are many studies showing the harmful effects of intracellular Hcy, etc. Huang *et al.* demonstrated that Hcy exerted its genotoxic effects on HL-60 cells through an apoptotic pathway, which is mediated by the activation of caspase 3 activity induced by an increase in intracellular hydrogen peroxide [73].

Whether Hcy-thiolactone is detoxified inside cells was unknown. Zimny *et al.* made a suggestion in this regard and in a study of theirs showed that Hcy-thiolactone is hydrolysed by an intracellular enzyme, which they purified to homogeneity from human placenta and identified by proteomic analyses as human bleomycin hydrolase (hBLH). They have also purified an Hcy-thiolactonase from the yeast *Saccharomyces cerevisiae* and identified it as yeast bleomycin hydrolase [74].

Paraoxonase and atherosclerosis

Traditional risk factors of coronary artery disease (CAD) include age, sex, hypercholesterolaemia, arterial hypertension, diabetes, and smoking. Other risk factors, such as homocysteine, and PON 1/HTLase activities, were recently added to the list [75]. Atherosclerosis is the underlying cause of 50% of mortality in the Western world, and organophosphates (OPs) use presents an environmental risk and a terrorist threat in today's society. Concentration and activity of PON1 are highly variable in human populations. There is still uncertainty as to the exact biological substrate for paraoxonase. It seems that the enzyme serves several roles and may have a number of substrates [76].

Paraoxonase 1 has been presented as a potential therapeutic agent against both OP poisoning and atherosclerosis development and as such has been the focus of intensive research. Paraoxonase 1^{-/-} mice are also more susceptible to poisoning by the OP toxins chlorpyrifos-oxon and diazoxon, although interestingly not paraoxon. Injection of purified PON1 into the mice restores OP resistance to normal levels [76].

There are two polymorphisms in the PON1 coding region at positions Gln192→Arg (Q192R) and Leu55→Met (L55M). Paraoxon is hydrolysed six times faster by PON1192R than by PON1192Q, whereas the Q form is more active towards sarin, soman and diazoxon. The Q192R polymorphism also alters the enzyme's ability to protect LDL from oxidation *in vitro* with the Q form being the most protective. For some substrates, there is no difference in hydrolytic rates, e.g. phenylacetate and dihydrocoumarin [77].

Two mechanisms are currently proposed in which PON1 is thought to participate in atherosclerosis prevention: antioxidant protection and hydrolysis of homocysteine thiolactone [76].

There is evidence in some studies that PON1's antioxidant function begins at the level of lipoprotein (LDL and HDL) protection against oxidative modification by ROS. The enzyme also reduces lipid hydroperoxides to hydroxides and presents a peroxidase-like activity, as PON1 was shown to degrade hydrogen peroxide (H₂O₂), a major reactive oxygen species produced under oxidative stress. Most evidence that PON1 inhibits oxidized lipid formation comes from general measurements of lipid oxidation, such as thiobarbituric acid-reactive substances (TBARS), lipoperoxides, and conjugated diene formation. The increased production of mitochondrial ROS can be induced, for example, by oxidized LDL (OxLDL). So mitochondrial dysfunction leads to decreases in aerobic capacity, which is a strong predictor of mortality. Increased production of mitochondrial ROS also causes endothelial dysfunction/apoptosis and vascular smooth muscle cell (VSMC) proliferation/apoptosis, which finally leads to the development of atherosclerosis [78-81].

Relationship between paraoxonase and homocysteine

Hepatic expression of the PON1 gene is down-regulated in hyperhomocysteinaemic mice; it is plausible, therefore, that the proatherogenic effects of Hcy may involve diminished serum PON1 activity, leading to impaired antioxidant function and decreased capacity to degrade Hcy-thiolactone [30, 38].

Normal human plasma levels of Hcy-thiolactone vary from 0 to 34.8 nmol/l and account for 0.002-0.3% of plasma tHcy. There was no linear correlation between plasma Hcy-thiolactone and plasma tHcy. Although somewhat surprising, this finding suggests that Hcy may not be a major determinant of plasma Hcy-thiolactone in humans [39, 82].

Two major constituents of this protective mechanism are proposed to be: 1) Hcy-thiolactonase, a component of HDL, which hydrolyses and therefore detoxifies Hcy thiolactone; and 2) serum albumin, which by binding Hcy to form albumin-S-S-

Hcy would prevent its cellular uptake and conversion to Hcy thiolactone. It is worth noting that protein-S-S-Hcy and free Hcy comprise 82% and 2%, respectively, of tHcy in human serum [17].

Other possible determinants of plasma Hcy-thiolactone, such as folic acid, methionine, MetRS (or other aminoacyl-tRNA synthetases), or renal function may also be important [39].

Paraoxonase is a crossroad of homocysteine and activities of thiolactonase on HDL

Evidence indicates that hyperhomocysteinaemia, which occurs in 5-7% of the general population, is a risk factor for CVD, but how homocysteine mediates its potential proatherogenic effects is still debated [83].

Whereas epidemiological data indicate that elevation of plasma homocysteine is not associated with a significant change in plasma total cholesterol, some studies have reported a negative correlation with HDL concentrations [84-86].

The mechanistic relationship between low plasma HDL and atherosclerosis has not been fully elucidated but involves the ability of HDL to promote reverse cholesterol transport from peripheral tissue to the liver as well as its anti-inflammatory properties. Moreover, Mikael *et al.* recently suggested that the decrease in plasma HDL in humans and animals with hyperhomocysteinaemia is caused in part by decreased hepatic expression of apoA-1. There are several reports on alterations in HDL composition during inflammation leading to attenuated anti-inflammatory activities or even inflammatory properties of HDL [87].

Because low plasma HDL concentration sometimes is associated with increased risk of CVD, whereas other conditions with low plasma HDL concentration are associated with improved prognosis, it seems that it is not only the concentration per se but also the function of the HDL particles that is important for its anti-atherogenic effects [87, 89].

HDL particles are susceptible to structural modifications mediated by various mechanisms, including oxidation, glycation, or enzymatic degradation, affecting their functional properties. Moreover, *in vitro* studies have shown that homocysteinylated HDL may reduce the activity of the enzyme PON, which is associated with human HDL, thus rendering the HDL particle more susceptible to oxidative damage. Formation of inflammatory HDL has been suggested to correlate with decreases in the activities of various HDL-associated enzymes, such as PON, a multifunctional enzyme with antioxidant capacity, and the ability to detoxify the homocysteine metabolite homocysteine thiolactone [89, 90]. Indeed, Holven *et al.* found that hyperhomocysteinaemic subjects, and particularly those with extreme hyperhomocysteinaemia, were characterized by significantly less PON activity than healthy controls [91].

Thiolactonase activity: cysteine residue 284

Homocysteine thiolactonase was shown to have an identical amino acid sequence as PON1. Paraoxonase 1's free cysteine (residue 284) is important for lactonase activity. The thiolactonase and arylesterase/paraoxonase activities co-migrate at all steps of purification, suggesting that the three activities represent that same enzyme [89, 92]. These data suggest that thiolactonase/paraoxonase protects proteins against homocysteinylation by detoxifying Hcy-thiolactone. The finding that Hcy thiolactonase is tightly associated with HDL also suggests a link between metabolism of Hcy and lipoproteins [92].

Kinetic properties of human serum thiolactonase

Jakubowski, of those working on this issue, is doing the most extensive studies. Human serum thiolactonase, a 45-kDa protein component of high density lipoprotein, requires calcium for activity and stability. Human serum thiolactonase could not be purified in the absence of CaCl_2 because the enzyme quickly lost its activity. The inactivated enzyme could not be re-activated by addition of any divalent metal. The enzyme is inhibited noncompetitively by isoleucine ($K_i = 2 \text{ mmol/l}$) and penicillamine ($K_i = 0.2 \text{ mmol/l}$). The substrate specificity studies indicate that homocysteine thiolactonase exhibits a certain degree of selectivity toward L-Hcy thiolactone. The amount of human serum thiolactonase present in human serum is sufficient to hydrolyse Hcy-thiolactone within a few hours. Substrate specificity studies suggest that homocysteine thiolactone is a likely natural substrate of this enzyme ($K_m = 23 \text{ mM}$). However, thiolactonase also hydrolyses non-natural substrates, such as phenyl acetate ($K_m = 0.6 \text{ mM}$), *p*-nitrophenyl acetate ($K_m = 2.5 \text{ mM}$), and the organophosphate paraoxon ($K_m = 0.9 \text{ mM}$) [25].

Although K_m for Hcy thiolactone is relatively high (23 mM), other enzymes also have high K_m values for physiologically important substrates [25]. This suggests that Hcy thiolactone is a likely natural substrate of human serum thiolactonase.

Taken together, these data indicate that Hcy thiolactone, phenyl acetate, and paraoxon are hydrolysed by the same enzyme, but probably at different sites. It is possible that human serum thiolactonase possesses multiple binding sites for phenyl acetate or paraoxon.

The N-terminal amino acid sequence of pure thiolactonase is identical to that of human paraoxonase. Purified human serum thiolactonase yielded the following N-terminal amino acid sequence: AKLIALTLLGMG. A search of data banks revealed a 100% match of this sequence with the N terminus of human serum paraoxonase. Human serum thiolactonase activity, like paraoxonase activity, was

present in HDL and absent in the LDL fraction. In the absence of detergents, the thiolactonase copurifies with apolipoprotein AI [93].

By detoxifying homocysteine thiolactone, thiolactonase/paraoxonase would protect proteins against homocysteinylation, a potential contributing factor to atherosclerosis. Recently, Perla-Kaján *et al.* found that plasma N-Hcy-protein was negatively correlated with serum Hcy-thiolactonase activity. They also found that enzymatic activities of the PON1 protein measured with artificial substrates correlated less strongly. These findings provide evidence that the Hcy-thiolactonase activity of PON1 is a determinant of plasma N-Hcy-protein levels and that Hcy-thiolactonase/PON1 protects proteins against N-homocysteinylation *in vivo*, a novel mechanism likely to contribute to atheroprotective roles of HDL in humans [92].

We now know that Hcy-thiolactone is hydrolysed to Hcy by serum Hcy-thiolactonase/paraoxonase carried on HDL and that rabbits have about 10 times higher Hcy-thiolactonase activity than an average human being does, which could explain the greater resistance of rabbits than primates to Hcy-thiolactone [30]. In fact, Hcy-thiolactone is unlikely to be efficiently hydrolysed to Hcy in the chicken because of the lack of Hcy-thiolactonase [30].

Homocysteine-thiolactonase, which hydrolyses Hcy-thiolactone to Hcy, could be in fact paraoxonase. If it is so, paraoxonase can hydrolyse Hcy-thiolactone back to Hcy and Hcy may be then converted either back to methionine (by a reaction which needs folate and vitamin B_{12} as co-factors), or condensed with serine to form cystathionine in a reaction that is dependent on vitamin B_6 [94]. It is possible that folic acid supplementation or high folate intake decreases plasma tHcy (and plasma Hcy-thiolactone levels) and affects serum PON activity by this mechanism [95]. Weijun *et al.* reported that short-term oral folic acid (5 mg/day) supplementation with or without methylcobalamin appeared to be an effective approach to decrease Hcy levels and increase Hcy-thiolactonase/PON activity in patients with type 2 diabetes [96]. These findings suggest that the anti-atherogenic function of PON1 is more complex and is not only restricted to inhibition of LDL oxidation. In humans, the homocysteine thiolactonase activity of PON1 inversely correlates with homocysteine concentration and predicts cardiovascular disease. Thus, perhaps the most important anti-atherogenic function of PON1 is attributable to its ability to hydrolyse Hcy-thiolactone, a considered risk factor of cardiovascular disease [68].

Other factors affecting the activity of homocysteine-thiolactonase activity

According to the results of Ferretti *et al.*, modifications of apoprotein conformation and physico-

chemical properties in Hcy-HDL and the decrease of PON1 activity could affect the protective effect of HDL against oxidative damage and/or homocysteinylolation [36]. Because it contains tightly bound Hcy-thiolactonase as one of its components, HDL should inhibit accumulation of Hcy-thiolactone.

Indeed, supplementation of human umbilical vein endothelial cell (HUVEC) cultures with HDL led to 80% inhibition of thiolactone synthesis. However, a small amount of N-homocysteinylated HDL (N-Hcy-HDL) is present in human plasma, suggesting that homocysteinylolation of plasma lipoproteins occurs *in vivo* [70]. A significant decrease of paraoxonase and lactonase activity of HDL-bound PON1 has also been observed in Hcy-HDL [97]. In addition to this finding, Gong *et al.* have demonstrated the positive effects of quercetin on activity of Hcy-thiolactonase and PON1 [98].

Clinical studies showing the relationship between paraoxonase and homocysteine

Vascular diseases

Paraoxonase 1 is thought to influence serum homocysteine concentrations, at least in part, due to its homocysteine thiolactonase activity and to play a role in atherosclerosis [99]. Homocysteine-thiolactonase activity is influenced by both PON1 and MTHFR genotypes and there is a direct relation between Hcy and Hcy-thiolactone levels. In relation to this matter, Jakubowski *et al.* hypothesized that high thiolactonase associated PON1 R and L alleles should confer significant cardiovascular protection in subjects with high Hcy levels [100].

Similarly, Koubba *et al.* showed that total plasma Hcy levels were negatively correlated with Hcy-thiolactonase activities in patients with coronary syndrome in whom the TT (MTHFR C677T) genotype was significantly more frequent [101]. Aydın *et al.* found that genetic variants of PON1 55/192 and MTHFR were associated with CAD. Since it has been shown that Hcy or its metabolites are associated with endothelial dysfunction and cardiovascular disease, hyperhomocysteinaemia was suggested to have a role in promoting endothelial dysfunction in cardiovascular diseases [102]. Man *et al.* found that the genotype distributions of PON1 Q192R and MTHFR A222V, which affect lipid and homocysteine metabolism, differed significantly between patients with stroke and healthy controls. The presence of at least one R allele in PON1 Q192R and a TT allele was associated with concurrent stenoses [103].

Recently, Lakshman *et al.* not only found a 47% decrease in PON1 activity, but also a 30% decrease in Hcy-thiolactonase activity in diabetic subjects with prior coronary artery bypass surgery. Moreover, the PON1 activity was significantly inversely correlated with the extent of intracoronary lesions

determined at catheterization [104]. Marcucci *et al.* reported that patients with non-valvular atrial fibrillation had increased plasma Hcy levels, but did not probe the relationship between plasma Hcy levels and atrial dimensions [105]. Shimano *et al.* presented a study in which they evaluated plasma levels of Hcy in a series of patients scheduled to undergo catheter ablation of atrial fibrillation [106]. Their results confirm those of other researchers documenting a significant increase in plasma Hcy in patients with persistent (but not paroxysmal) atrial fibrillation. The links between hyperhomocysteinaemia and endothelial dysfunction are clear and suggest that elevated Hcy may contribute to the increased stroke risk in patients with persistent atrial fibrillation [107, 108]. Kerkeni *et al.* showed that hyperhomocysteinaemia and low PON1 concentration are associated with chronic renal disease and markedly associated in patients with cardiovascular complications [110].

Diabetes and metabolic diseases

In addition, Hcy-thiolactonase activity varies in some metabolic disorders. Jakubowski *et al.* found that serum Hcy-thiolactonase was lower in hyperleptinaemic than in control animals. A recent study showed the relationship between diabetes and Hcy-thiolactonase. This study showed that increased lysophosphatidylcholine (lyso-PC) levels in LDL were associated with suppressed Hcy-thiolactonase activity, and Hcy-thiolactonase activities may be related to lyso-PC in type 2 diabetic patients [110].

In a cohort of healthy overweight/obese women, Kotani *et al.* showed for the first time reduced PON1 lactonase activity during a low caloric diet intervention on weight loss, with a significant correlation with the reduction of LDL-C. A tendency for a negative association between homocysteine thiolactonase activity and the thickness of the carotid intima media was observed in patients with type 2 diabetes mellitus [112].

Baráthi *et al.* reported that the PON1 activity was significantly lower in the uraemic hypertensive group than in the controls [115], whereas the plasma homocysteine level was significantly higher in the uraemic hypertensive patients as compared with the controls. Isbilen *et al.* found that tHcy level in the plasma of pre-eclamptic women was increased in comparison with healthy pregnant [114].

Other diseases

A significant increase in Hcy-thiolactone and PON-Hcy-thiolactonase activity was observed in proliferative diabetic retinopathy [114]. The *in vitro* studies on bovine retinal capillary endothelial cells showed a dose and time dependent increase in the PON-Hcy-thiolactonase activity and mRNA expres-

sion of PON2 on exposure to Hcy-thiolactone and Hcy [115].

Ateş *et al.* reported that serum PON1 activity was significantly lower in patients with age-related macular degeneration (AMD) than that in the controls [116]. In contrast, the serum levels of homocysteine were significantly higher in the patients than those in the controls. In AMD patients, a significant negative correlation was found between PON1 activity and homocysteine level [117, 118]. Angayarkanni *et al.* reported that central retinal venous occlusion patients showed significantly lowered serum PON1-ARE activity along with a significant increase in the levels of plasma Hcy when compared to the control subjects. There was a negative correlation between serum PON1-ARE and plasma Hcy levels as well as between PON1-ARE levels in the central retinal venous occlusion patients [119].

Mungan *et al.* found a significant negative correlation between serum PON1 and MDA levels, and serum PON1 activity was also negatively correlated with homocysteine levels in Behcet disease patients [120].

Many researchers have reported that paraoxonase activity was decreased in Alzheimer disease and in mixed dementia as compared with the control group. The same forms of dementia homocysteine levels were increased. Alzheimer disease paraoxonase activity was negatively correlated with homocysteine levels [121-123].

The study by Paşca *et al.* showed that in children with autism there are higher levels of tHcy, which is negatively correlated with GPx activity, low PON1 arylesterase activity and suboptimal levels of vitamin B₁₂ [124, 125].

Nutrition and drugs

Noll *et al.* found that red wine polyphenolic extract supplementation at low dose significantly reduced plasma Hcy levels and restored the hepatic and plasma-decreased PON1 activity induced by chronic hyperhomocysteinaemia. Immediately following the month of white wine consumption there was a significant increase in HDL cholesterol PON1 levels. However, there was also a clear increase in homocysteine. The beneficial influence of light alcohol intake on PON1 activity is probably also caused by its ability to increase the concentration of HDL in serum [126].

Moderate alcohol intake causes an increase in PON1 activity, while the serum of alcoholics presents a decrease in PON1 activity compared with non-alcoholics. Light alcohol intake stimulates paraoxonase by upregulating liver mRNA in rats and humans. Heavy alcohol intake inhibits gene expression and PON1 activity, independently of the PON1 polymorphism. It has been demonstrated that polyphenolic compounds could modulate the expression level of the PON1 gene *in vitro* [127-129].

Hamelet *et al.* reported that a high-methionine diet significantly increased serum homocysteine levels, decreased hepatic CBS activity, and down-regulated PON1 mRNA and its activity [130].

The effect of the type of fat taken in the diet on the activity of serum PON and Hcy-thiolactonase was investigated by Varatharajalu *et al.* According to their results, high omega-3 polyunsaturated fatty acid (omega-3 PUFA) consumption decreased liver PON1 mRNA expression, serum PON1, and Hcy-thiolactonase activity compared to the low ω -3 PUFA group [127].

However, chronic administration of catechin but not quercetin significantly reduced plasma homocysteine levels, attenuated the reduction of the hepatic CBS activity, and restored the decreased paraoxonase-1 gene expression and activity induced by chronic hyperhomocysteinaemia [131].

Statins are the drugs used most frequently in cardiovascular disease prevention. Gouedard *et al.* reported that in HuH7 human hepatoma cells, the PON1 secreted enzymatic activity and mRNA levels were increased by fenofibric acid and decreased by several statins [132]. The opposite effects of fenofibrate and statins could be consistent with clinical data on homocysteine levels after hypolipidaemic drug treatment. Because Hcy-thiolactone can be converted into its parent form Hcy by PON1, an increase in PON1 activity could raise the level of homocysteine and detoxify Hcy-thiolactone. The regulation of the PON1 gene reported here is consistent with clinical data showing the different effects of cardiovascular disease preventing drugs on plasma homocysteine levels. Indeed, fenofibrate and bezafibrate were shown to increase Hcy levels, whereas gemfibrozil had no effect and high-dose statins led to a decrease. The well-established clinical benefit of statins is likely to involve PON1-independent mechanisms. Together with the hydrolysis of toxic endogenous compounds (oxidized phospholipids and homocysteine thiolactone), probably accounting for its anti-atherogenic capacity, PON1 is also a xenobiotic metabolizing enzyme that detoxifies organophosphates [132].

Also, atorvastatin therapy is beneficial in decreasing oxidative stress related to hypercholesterolaemia, mainly affecting lipid profile and PON1 activity. Bolayirli *et al.* found that 4 weeks of atorvastatin therapy significantly increased HDL-C and PON1 activity, and reduced LDL-C, TBARS and lipid peroxide concentrations [133].

Furthermore, the PON1 3D structure will be a tremendously useful frame for building and refining a model of the catalytic mechanism for the hydrolysis of different substrates. It will particularly help to determine the structural basis of the substrate specificity in the PON family. This should lead

to PON1 engineering, that is the design of mutants with improved functional properties, mainly organophosphatase and antioxidative activities. The main therapeutic uses of these mutants are expected to be for the prevention of atherogenesis [134, 135].

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