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Homocysteine and oxidative stress

Review Article

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Summary. Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (ischemic disease, such as stroke and myocardial infarction, and arterial and venous thrombotic events) in the general population. We can assume that the association is causal, based on the example of homocystinuria, and on the evidence put forward by several basic science and epidemiological studies; however, the results of large intervention trials, which will grant further support to this hypothesis, are not yet available. In addition, the mechanisms underlying this relationship, and also explaining the several toxic effects of homocysteine, related or not to cardiovascular disease, are unclear. Oxidation is one of the most favored postulated mechanisms; others are nitrosylation, acylation, and hypomethylation. Regarding the relative importance of these mechanisms, each of these hold pros and cons, and these are weighed in order to propose a balance of evidence.

Keywords: Homocysteine – Homocystinuria – Cardiovascular risk – Mechanisms of toxicity – Uremia – Chronic renal failure

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (ischemic disease, such as stroke and myocardial infarction, and arterial and venous thrombotic events) in the general population. Cardiovascular disease remains the major cause of morbidity and mortality, at least in the developed world. Well-studied risk factors, such as hypercholesterolemia, hypertension, smoking, obesity, etc., cannot explain in all instances the occurrence of cardiovascular disease events. Therefore, the interest of the scientific community is going towards new and potentially modifiable risk factors such as hyperhomocysteinemia. A large body of epidemiological evidence indicates the presence of an association between cardiovascular risk and hyperhomocysteinemia. In the general population, where cross-sectional and prospective studies were performed, even mild or moderate increases in blood homocysteine levels are associated with an increase in cardiovascular risk. This association is dose-related, and independent from other risk factors. However, some of the prospective large population studies were negative, so the matter is still under heated debate (Eikelboom, 1999). In a recent meta-analysis, including 30 studies and more than 6000 events, a 25% lower homocysteine level was associated with an 11% lower ischemic heart disease risk and a 19% lower stroke risk. It is concluded that homocysteine is a modest cardiovascular risk factor, in a healthy population. Nevertheless, the implications of lowering homocysteine levels could still be substantial (Homocysteine studies collaboration, 2002). In 1998, the International Task Force for Prevention of Coronary Heart Disease proposed, in a comprehensive document on cardiovascular risk factors (http://www.chd-taskforce.com), the criteria to be used to assess cause-effect relationships relevant to cardiovascular risk factors. These are: the availability of prospective studies, with the factor preceding the effect; strength and consistency of associations; independency, with a continuous effect; availability of basic science studies establishing a mechanism in the appropriate experimental models; positive intervention studies. Regarding homocysteine, several prospective studies are available, assessing independency, and the absence of a threshold. Basic science studies are available, even if conditions were not always appropriate; and several intervention trials are currently underway, both in the general population and in selected patient

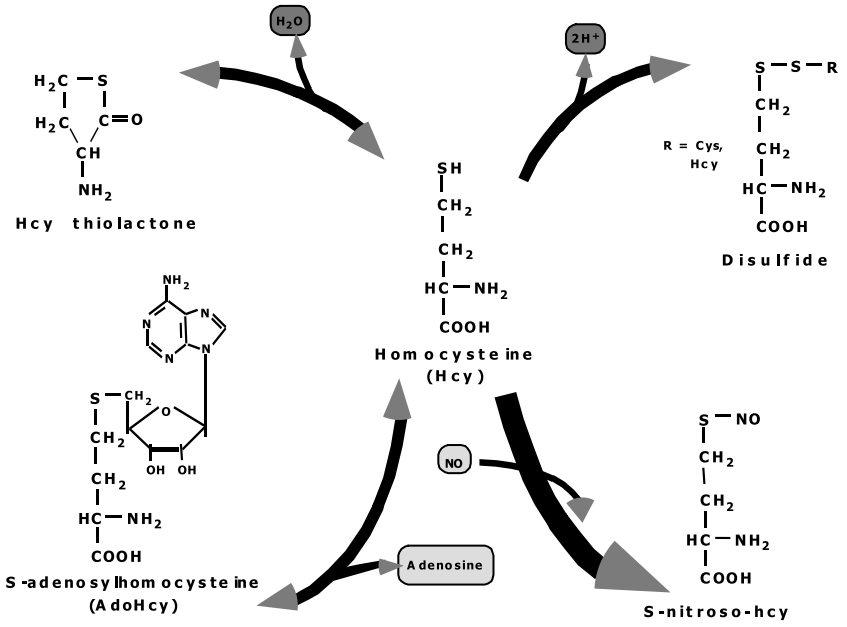


Fig. 1. The figure depicts the pathways through which homocysteine exerts its toxic effects. These are: the formation of homocysteine thiolactone, with consequent protein acylation; the formation of hydrogen peroxide through protein binding, and the oxidation of homocysteine to the mixed disulfide homocysteine-cysteine, and the homodimer homocysteine-homocysteine; the accumulation of S-adenosylhomocysteine; and the formation of S-nitroso-homocysteine

population groups, such as kidney transplant recipients, with results available in the near future (Bostom, 2001).

Homocysteine (Fig. 1) is a sulfhydryl amino acid metabolized to cysteine in the *transsulfuration* pathway, where cystathionine-beta-synthase (CBS) is the rate-limiting enzyme. The *remethylation* pathway instead leads to methionine formation from homocysteine, which receives a methyl group from methyltetrahydrofolate. Methionine, contained either in the diet or originating from protein breakdown, is condensed with ATP to form S-adenosylmethionine (AdoMet). AdoMet in turn donates its methyl group in the *transmethylation* pathway to various methyl acceptors, and its demethylated product is S-adenosylhomocysteine (AdoHcy). AdoHcy is hydrolyzed to adenosine and homocysteine in a reversible reaction, which is inhibited by AdoHcy itself (competitive product inhibition).

The inherited enzymatic defect of CBS represents the most common form of homocystinuria, in which affected patients, who display very high homocysteine levels in blood, and a variety of clinically relevant derangements attributable to homocysteine accumulation, used to die of premature cardiovascular disease (McCully, 1969). Therefore, homocystinuria is the first described human model of hyperhomocysteinemia, in which the latter causes high mortality levels, and therapy leads to a significant increase in survival (Wilcken, 1997). Animal models are consistent with the view that homocysteine causes cardiovascular disease: knock-out mice for the methylenetetrahydrofolate gene, which display hyperhomocysteinemia, show developmental retardation and abnormal lipid deposition in the aorta (Chen, 2001). Recently, induction of hyperhomocysteinemia in ApoE-null mice, a model of genetic susceptibility to atherosclerosis, accelerates the development of atherosclerotic lesions (Hofmann, 2001).

As said above, epidemiological data are important, but *in vitro* molecular biology and cellular studies, in which conditions can be controlled, are of equal importance in establishing cause-effect relationships. Homocysteine may act as a toxin with respect to endothelial cells, can enhance vascular smooth muscle cell proliferation, increase platelet aggregation, and act on the coagulation cascade and fibrinolysis, thus directly inducing or acting in a synergistic manner with other factors in determining the appearance of atherosclerosis. In particular, it activates coagulation factors V, X, and XII, along with decreased activation of protein C and cell-surface thrombomodulin, and modulation of tissue plasminogen activator binding to its endothelial receptor, annexin II, thus creating a prothrombotic environment (Thambyrajah, 2000). In addition, it determines specific alterations of endothelial cells. For example, homocysteine induces alterations of the arterial endothelial barrier, but only when added in combination with copper, that is when there is generation of hydrogen peroxide (Berman, 1993). Homocysteine inhibits, through AdoHcy accumulation, methylation of protein $p21^{ras}$ in cultured vascular endothelial cells. $P21^{ras}$ hypomethylation leads to reduced membrane association of this important regulator of cell cycle, thus it may have important effects on atherosclerotic

lesion formation through reduced endothelial cell proliferation (Wang, 1997). Endothelial cells may be particularly prone to homocysteine damage, also because they cannot rely on the transsulfuration pathway to detoxify homocysteine. The relevant enzymes, in fact, are lacking in these cells, which depend exclusively on the remethylation pathway to metabolize homocysteine (Jacobsen, 1995).

These mentioned above are just a few of the studies available on homocysteine toxicity. However, it should be stressed that several studies performed in the past utilized millimolar concentrations of homocysteine, much higher than those present in any human pathological state, which are in the micromolar range. Moreover, rarely the specificity of homocysteine toxic action was verified utilizing as control other sulfydryl compounds, such as cysteine. How does this toxic effect occur? Is it a direct effect of the molecule or it is mediated by one of its derivatives?

In normal individuals, plasma total homocysteine concentration range from 5 to 12 μM, and over 97% is in its oxidized form. Hyperhomocysteinemia of moderate degree is defined between 16 and 30 μM, intermediate between 31 and 100 μM, and severe as $>100\,\mu$M (Kang, 1992). In homocystinuria, levels are in the severe range, while in chronic renal failure levels are in the moderate-intermediate range, and in the general population mild increases (between 12 and 16 μM) can still provide for an increase in cardiovascular risk (Clarke, 1991).

Protein disulfide-bound homocysteine accounts for $>70\%$ of total homocysteine (where homocysteine binds to a cysteine residue in position 34 of the albumin) and the remaining is found as free low molecular weight disulfide forms including homocystine (the homocysteine homodimer) and the homocysteine-cysteine mixed disulfide. About 1.5–4% of homocysteine in circulation is present in its reduced form (Ueland, 1996). Post-biosynthetic acylation of free amino groups, in particular the epsilon amino group of lysine residues and the terminal amino group of proteins (mediated by homocysteine thiolactone, a homocysteine derivative) is also present (Fig. 1, Jakubowski, 2000a, b). In blood, other thiols are present, such as cysteine, which is several fold more concentrated than homocysteine, and cysteine-glycine, while in the red cells glutathione is particularly highly concentrated. Also high blood levels of cysteine have been proposed as a cardiovascular risk factor, although the association is less strong (El-Khairy, 2001). It has been argued that the presence of large amounts of reduced homocysteine in homocystinuric patients and relatively small amounts in healthy subjects is in accord with the idea that this species in particular is the one playing a role in atherogenesis (Ueland, 1996; Mansoor, 1993a, b). In this respect, homocysteine protein binding occurs in plasma with binding sites being saturable above 140 μM (Togawa, 2000). Above this level, reduced homocysteine would be no longer free for protein binding and increases in plasma, as in homocystinuria. Another consideration is that normal albumin concentration is about 650 μM, so the expected binding capacity of plasma, according to a theoretical 1:1 binding stoichiometry, would be higher than that actually found. Concurrently, displacement of bound cysteine from proteins occurs with protein binding (Togawa, 2000). Conversely, inside the cell homocysteine is thought to be present in its reduced form, due probably to the presence of reducing power due to abundant glutathione (Blom, 2000).

Most thiols autoxidize in the presence of transition metal catalysts and molecular oxygen, and this leads to the formation of reactive oxygen species (ROS). Homocysteine, as a thiol molecule, can undergo similar autoxidative chemistry. Homocysteine can induce *in vitro* a pro-oxidant action through the production of hydrogen peroxide during metal-catalyzed oxidation, and, in the presence of nitric oxide, the superoxide anion can form the powerful oxidant peroxynitrite (Starkebaum, 1986; Loscalzo, 1996). In addition, it has been thought that oxidant stress is induced *in vivo* by hyperhomocysteinemia, as indicated by the decrease in plasma cysteine when homocysteine is added to plasma, with a concomitant increase in plasma cystine concentration (Loscalzo, 1996).

Oxidative modifications of low-density lipoproteins

Formation of ROS could lead to lipid peroxidation, which initiate an inflammatory response and is involved in the establishment of foam cells, a key atherosclerotic lesion component. Studies performed *in vitro* have shown both a pro-oxidant and an antioxidant effect of homocysteine on LDL oxidation. Blom et al. and Dudman et al. found no increase, in homocystinuric patients, of markers of LDL oxidation (Blom, 1992; Dudman, 1993). Some later studies have reported a pro-oxidant effect of thiols on LDL oxidation in cell-free systems containing free Fe^{++} (Lynch, 1997), while other investigators demonstrated an antioxidant effect of thiols on LDL oxidation by a physiological source of Fe^{3+} (*i.e.* hemin, Lynch, 2000). Hemin promotes oxidation of the core lipoprotein lipids,

while free Fe^{3+} that of surface lipids. Therefore, thiols can function both as anti- or pro-oxidants depending on the physical location of the oxidant damage occurring to lipoproteins. Voutilainen and collaborators have shown high plasma levels of F2-isoprostanes, stable markers of *in vivo* lipid peroxidation, in men with mild hyperhomocysteinemia (Voutilainen, 1999). Halvorsen and co-workers instead have shown that while low concentrations of homocysteine (< 6 micromolar) tended to promote LDL oxidation *in vitro*, high concentrations were protective (Halvorsen, 1996). Accordingly, it seems that depending on the experimental condition, homocysteine can both inhibit and promote LDL oxidation, and it has been speculated that its concentration (low = pro-oxidant, high = anti-oxidant) determines which activity will prevail (Lynch, 2000). However, in a recent study, phosphatidylcholine hydroperoxide, a major product of lipid peroxidation, and P-selectin, a cell adhesion molecule and a marker of endothelial damage, were measured after a methionine load. These compounds were found to be unchanged after the load and it was suggested that the effects of homocysteine on the endothelium are not mediated by oxidative stress mechanisms (Chao, 2000). Clearly, more studies are necessary to elucidate the role of homocysteine on lipoprotein oxidation.

Endothelial-dependent vasodilation

Endothelium-dependent vasodilation is utilized as a non-invasive method to study endothelial function and therefore to document pre-atherosclerotic alterations in predisposed subjects. This method consists in applying a sphygmomanometer at the level of the brachial artery at a pressure greater than the systolic blood pressure for 5 min, and then releasing it. Hyperemia ensues, which will last about 3 min. After previous sublingual nitroglycerin administration, an endothelium-dependent vasodilation is prompted during hyperemia. This response is therefore related to $NO^{\bullet}$ release. Several studies have shown that homocysteine can impair endothelial cell function, which can be monitored by the reduction in endothelium-derived flow-mediated vasodilation, by either a methionine load in healthy individuals or in hyperhomocysteinemic patients (Chambers, 1998; Bellamy, 1998). Kanani et al. have shown that vitamin C prevents induction of endothelial dysfunction by hyperhomocysteinemia induced by a methionine load (Kanani, 1999). Vitamin C is a potent antioxidant scavenger of ROS, but may also prevent direct inactivation of nitric oxide by superoxide or increase intracellular reduced glutathione concentration. In addition, Usui et al. (Usui, 1999) reported that folic acid, which reduces superoxide anion generation from nitric oxide synthase and xanthine oxidase, administered before a methionine load in humans prevents endothelial dysfunction, measured by the flow-mediated vasodilation, while it did not attenuate methionine-induced hyperhomocysteinemia (therefore a mechanism independent of homocysteine). Vitamin C + E administration to healthy individuals receiving a methionine load is able to prevent the alterations in endothelial cell function and coagulation caused by acute hyperhomocysteinemia (Nappo, 1999). Because of these effects exerted by antioxidant administration, and because of the cell culture studies showing that homocysteine leads to considerable toxic effects mediated by the formation of ROS, it has been therefore suggested that the effects of homocysteine on the endothelium occur via oxidative stress. Nevertheless, it cannot be ruled out that antioxidants may act by preventing folate oxidation, thus increasing availability of the most reduced form of folate (*i.e.* methyltetrahydrofolate), which is required for homocysteine remethylation to methionine (Kohnen, 2001).

Nitric oxide ($NO^{\bullet}$)

It has been reported that endothelial cells can detoxify homocysteine by stimulating the release of nitric oxide, with an accompanying increase in *Nos3* mRNA levels (Stamler, 1993). Therefore, from the oxidation of homocysteine with nitric oxide, S-nitroso-homocysteine formation ensues. Thus, homocysteine can actually reduce $NO^{\bullet}$ bioavailability. However, S-nitroso-homocysteine has also vasodilatory and platelet antiaggregation properties, and does not support hydrogen peroxide generation. This represents a protective mechanism against the adverse effects of homocysteine. However, this scavenging effect of homocysteine is eventually overcome by chronic exposure to high homocysteine, leading to a reduced production or availability of $NO^{\bullet}$. This effect leads to unopposed oxidative-injury mediated by homocysteine and formation of peroxynitrite.

On the other hand, it has been also reported that homocysteine can function as an antagonist of $NO^{\bullet}$ endogenously (De Groote, 1996). In addition, electrochemical detection of $NO^{\bullet}$ release from endothelial cells exposed to homocysteine and stimulated with bradykinin, calcium ionophore, or L-arginine, showed that homocysteine produces an indirect suppression of endothelial $NO^{\bullet}$ synthase (eNOS) activity, and therefore $NO^{\bullet}$ production, without affecting its expression. Folates, superoxide ions and peroxynitrite scavengers restore the $NO^{\bullet}$ generating activity of eNOS (Zhang, 2000).

Some evidence exists that homocysteine may affect glutathione peroxidase activity, thus altering the microenvironment in the propagation of ROS (Upchurch, 1997). Endothelial glutathione peroxidase catalyzes the reduction of hydrogen and lipid peroxides to the corresponding alcohols, and it also prevents the oxidative inactivation of $NO^{\bullet}$. Homocysteine reduces also mRNA levels of glutathione peroxidase, indicating that the expression of the enzyme is inhibited or downregulated. These findings were confirmed in heterozygous CBS deficient mice, where treatment with a cysteine donor led to an increase in reduced glutathione peroxidase and restored reactivity of the mesenteric microvasculature to methacholine and bradykinin (Weiss, 2002).

Protective effects of homocysteine

Extracellular superoxide dismutase, an important antioxidant in vascular tissue, was measured along with homocysteine in homocystinuric patients, and found to be positively associated with homocysteine levels. This strong relationship can be envisioned as a protective antioxidant response to homocysteine-induced oxidative damage. This could partially explain why the risk of a vascular event in treated homocystinuric patients is not extremely high, even if their plasma levels remain higher than those seen in other forms of hyperhomocysteinemia (Wilcken, 2000).

Another study performed with coltured endothelial cells demonstrated that homocysteine reduces endothelin-1 production, through oxidative products (Drunat, 2001). Endothelin-1 is a well-known potent vasoconstrictor, and this study could be related to these protective responses elicited by homocysteine.

In a recent paper, Zappacosta et al. found that homocysteine produces only negligible quantities of hydrogen peroxide (1:4000 mole of hydrogen peroxide/mole of homocysteine) and only when catalysts metal ions are present (Zappacosta, 2001). Hydrogen peroxide was measured with a sensitive fluorometric method. In addition, these authors found that homocysteine does not enhance peroxynitrite formation, but conversely inhibits dihydrorhodamine oxidation by peroxynitrite. Therefore, it seems that homocysteine at micromolar concentrations does not act as a pro-oxidant but displays an antioxidant effect on cellular and chemical systems (Zappacosta, 2000).

Mechanisms of albumin binding

How does the binding of homocysteine to proteins actually take place? This issue has been only recently addressed, even if the protein bound form of homocysteine is actually the most represented in blood. Albumin makes up for more than 50% of total plasma proteins. It contains 17 intrachain disulfide bonds, and one additional cysteine residue at Cys_{34} that is not involved in intrachain disulfide bonding. This Cys_{34} accounts for the majority of free thiols in plasma. The disulfide bond of homocysteine to Cys_{34} of albumin forms itself through the displacement of cysteine and formation of an albumin thiolate anion, which then reacts with the homocysteine-cysteine mixed disulfide or homocystine in blood to form albumin-bound homocysteine. Albumin is formed and secreted from the liver in its free thiol form and the cysteine residue in albumin reacts with plasma homocysteine. In addition, human albumin mediates the conversion of homocysteine to its low molecular weight disulfide forms by thiol/disulfide exchange reactions. Only a small fraction of homocystine is formed through an oxidative process in which copper bound to albumin, but not ceruloplasmin, mediates the reaction. It has been reported that ceruloplasmin catalyzes the autoxidation of homocysteine to homocystine with hydrogen peroxide production. However, recent results show that ceruloplasmin is important only in driving cysteine oxidative chemistry, but not homocysteine. Therefore, it has been concluded that the role of copper-catalyzed or ceruloplasmin-catalyzed autoxidation of homocysteine is probably a minor process in circulation (Sengupta, 2001a, b).

In light of these recent findings, the likelihood of the unifying ''oxidation hypothesis'' has been seriously questioned and more attention is currently being paid to protein-bound homocysteine, which is the most abundant form of circulating homocysteine. In particular, oxidative stress may be certainly generated within vascular cells, but not necessarily as a result of thiol oxidation (Jacobsen, 2000). In addition, perhaps folates, other B vitamins, and vitamin C and E are protective via mechanisms independent of their homocysteine-lowering or antioxidant effects. For example, it has been shown that folates stimulate regeneration of endogenous tetrahydropterin from dihydropterin (Matthews, 1980). Tetrahydropterin is a cofactor of NOS. In addition, folates protect against oxidative modifications of human LDL independently of homocysteine levels in an *ex vivo* system (a suggested free radical scavenging activity, Nakano, 2001).

Alternative hypotheses

Alternative hypotheses with respect to oxidation, regarding homocysteine toxicity (called as a whole ''molecular

target hypothesis'', Jacobsen, 2001) include protein homocysteinylation, modulation of the activity of the glutamate receptor, which binds also N-methyl-D-aspartate, and hypomethylation.

Regarding protein homocysteinylation, this occurs through various mechanisms:

- oxidation of thiol groups, mediated by homocysteine in its free form (in particular cysteine residues), which occurs through the formation of a thiolate anion, as discussed above;
- the misincorporation of S-nitroso-homocysteine in place of methionine in the covalent backbone of proteins during protein biosynthesis (Jakubowski, 2000c);
- post-biosynthetic acylation of free amino groups, in particular the epsilon amino group of lysine residues and the terminal amino group of proteins (mediated by homocysteine thiolactone, a homocysteine derivative).

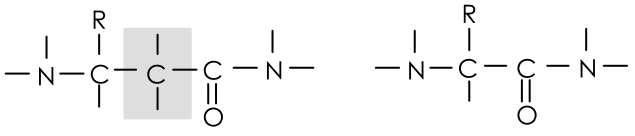
Considering the third mechanism relative to protein homocysteinylation, the formation of homocysteine thiolactone is consequent to a *proof-reading* reaction which prevents the post-translational incorporation of homocysteine into proteins, or it can appear as a result of a reaction that binds homocysteine to initiator tRNA. This complex is then methylated to methionine by a methylating factor. When hypomethylation is present, the homocysteine-tRNA complex is hydrolyzed to form homocysteine thiolactone.

Taking as a model plasma proteins incubated in the presence of homocysteine thiolactone, the formation of homocysteinylated proteins occurs spontaneously, through a non-enzymatic mechanism, and rapidly, with complete disappearance of thiolactone from the medium after 3 hours. Consequences of protein homocysteinylation are protein damage with an altered electrophoretic mobility, and loss of enzymatic activity (with protein denaturation), in several model systems, such as plasma proteins, methionyl tRNA synthetase, tripsyn, lysin oxidase, etc. Normally, it is not possible to measure homocysteine thiolactone in blood, probably because of its high reactivity. However, when there is an increase in blood homocysteine, an increase in homocysteinylated proteins can be observed. Therefore, protein homocysteinylation could be one of the principal mediators of homocysteine toxicity, contributing to determine structural and functional alterations at the molecular and cellular level (Jakubowski, 1993, 1997, 1999, 2000b).

We have focused on one particular aspect of homocysteine toxicity, that is the link between homocysteine and hypomethylation, and additional evidence coming from other investigators in the field of uremia, and other models of hyperhomocysteinemia, is confirming its importance.

In fact, a consequence of the increased plasma levels of homocysteine in uremic patients is a rise in the intracellular concentration of AdoHcy (Perna, 1993b, 1995). This thioether is the only homocysteine precursor and the natural inhibitor of all AdoMet-dependent transmethylation reactions. The ratio [AdoMet]/[AdoHcy] is a good indicator of the normal flow of methyl groups transferred from the methyl donor to methyl acceptors within the cell. The rise of AdoHcy concentration in erythrocytes of uremic patients, which is not paralleled by a rise of AdoMet concentration, gives way to a significant reduction of the [AdoMet]/[AdoHcy] ratio. This, in turn causes a significant impairment of AdoMet-dependent membrane protein carboxyl methylation reaction, catalyzed by protein carboxyl methyltransferase (PCMT, EC 2.1.1.77). The reaction catalyzed by PCMT is the only one represented in erythrocytes, that is no other methylation reaction is present in erythrocytes to a significant extent, although PCMT is present in all cell types.

This ubiquitous methylation reaction is involved in the repair of molecular damage, represented by L-isoaspartyl residues, spontaneously occurring in proteins through deamidation of labile asparagine residues. In the L-isoaspartyl residue, the regular alternation of nitrogen and carbon atoms in the peptide backbone is interrupted by the presence of an extra methylene group, since the aspartyl is linked through its β-carbonyl to the subsequent residue in the peptide chain, which can result in the destabilization of protein local conformation:



The repair mechanism involves PCMT, and repeated cycles of methylation and demethylation allow the quantitative conversion of damaged L-isoaspartyl residues arising from asparagine degradation into normal L-aspartyl ones. Several pieces of evidence confirmed the ability of this combined pathway to restore the biological function of proteins thus inactivated. For example, enzymatic methyl esterification is able to restore, *in vitro*, a significant portion of the biological activity of deamidated calmodulin. Importantly, PCMT knock out mice die prematurely and large amounts of L-isoaspartyl containing, isomerized proteins accumulate in several tissues (Kim, 1997).

Characterization of methyl accepting membrane protein species in chronic renal failure and hemodialysis patients show that the erythrocyte cytoskeletal component ankyrin (band 2.1), which connects spectrin and the integral membrane protein band 3 (AE1), is methylated (repaired) to a significantly lesser extent compared to normal.

We found that the reduction of the [AdoMet]/[AdoHcy] levels, measured by straightforward analytical procedures, is in good agreement with the degree of impairment of membrane protein repair. We in fact measured the racemized and isomerized aspartyl residues (D-Asp+D-isoAsp), which appear as side products of the repair reaction. Proteins are cleaved by mild chemical and enzymatic digestion at 37 °C, using specific proteases. Accumulation of these residues in ESRD patients is about one third of controls, which according to a computer simulation model, corresponds to a residual PCMT activity of about 11% of normal. This degree of inhibition can lead to an up to 600-fold increase in L-isoaspartyl residues in erythrocyte membrane proteins (Perna, 1997a).

The final outcome is the inadequate repair of such structural alterations in erythrocyte membrane proteins, with the attending accumulation of damaged residues, in chronic renal failure patients. Several crucial transmethylation-dependent processes, in addition to protein repair, in cells different from erythrocytes, can be affected by a reduction in the [AdoMet]/[AdoHcy] ratio. For example, it has been shown that an increase in plasma homocysteine is associated with parallel increases in plasma AdoHcy and to lymphocyte DNA hypomethylation, evaluated by HpaII digestion and cytosine-extension assay, in women. Disruption of nonrandom DNA methylation pattern can lead to inappropriate gene expression and promotion of disease (Chen, 1998; Friso, 2002). It has been recently shown that folate treatment reverts impairment in DNA methylation and alterations of gene expression in uremic/hyperhomocysteinemic patients (Ingrosso, 2003).

In plasma of uremic patients, levels of damaged proteins, that is proteins containing the L-isoaspartyl residue mentioned above, are increased in uremia almost twofold. L-isoaspartyl residues in plasma proteins were quantitated using human recombinant PCMT. The major protein involved comigrated with serum albumin. Although hyperhomocysteinemia caused a redistribution of thiols bound to plasma proteins, this mechanism did not significantly contribute to the increase in isoaspartyl residues. Folate treatment can lower, but not significantly, levels of damaged plasma proteins, meaning that other toxins beside homocysteine have a role in protein damage (Perna, 2001).

Van Guldener et al., using the powerful tool of stable isotope labeling of methionine, measured whole body rates of methionine and homocysteine metabolism in fasting hemodialysis patients, and found significantly lower remethylation and transmethylation rates compared to control (Van Guldener, 1999).

Metabolic repercussions of folate administration, 15 mg/day *per os* for 2 months, are to increase the erythrocyte [AdoMet]/[AdoHcy] ratio to levels not significantly different from those detected in normal individuals (Perna, 1997b). In fact, the folate-induced increase in methionine is handled by the cell through an increase in AdoMet biosynthesis.

It has been proposed that AdoHcy is a more sensitive indicator of cardiovascular risk than plasma homocysteine itself (Kerins, 2001). Uremia can be considered a model of accelerated arteriosclerosis, where the high mortality rate is due to cardiovascular disease (Baigent, 2000). These high rates cannot be entirely explained by the presence of traditional and non-traditional (pertaining to uremia, e.g. hyperparathyroidism, hypertryglyceridemia) risk factors in these patients. In relation to the oxidation hypothesis, it has been shown that inflammation, but not hyperhomocysteinemia, is related to oxidative stress in uremia (Mezzano, 2001). Therefore, that hypomethylation due to high AdoHcy is a consequence of high homocysteine in uremia, transmethylation rates are lower in the body as a whole, and protein repair is impaired, could be important in light of these recent findings and may represent the missing link between cardiovascular disease and uremia.

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