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Review article

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THE PATHOPHYSIOLOGICAL HYPOTHESIS OF HOMOCYSTEINE THIOLACTONE-MEDIATED VASCULAR DISEASE

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Accumulating evidence suggests that homocysteine (Hcy) metabolite, the thioester Hcy-thiolactone, plays an important role in atherothrombosis. Hcy-thiolactone is a product of an error-editing reaction in protein biosynthesis which forms when Hcy is mistakenly selected by methionyl-tRNA synthetase. The thioester chemistry of Hcy-thiolactone underlies its ability to from isopeptide bonds with protein lysine residues, which impairs or alters protein's function. Protein targets for the modification by Hcy-thiolactone include fibrinogen, low-density lipoprotein, high-density lipoprotein, albumin, hemoglobin, and ferritin. Pathophysiological consequences of protein N-homocysteinylation include protein and cell damage, activation of an adaptive immune response and synthesis of auto-antibodies against *N*-Hcy-proteins, and enhanced thrombosis caused by *N*-Hcy-fibrinogen. Recent development of highly sensitive chemical and immunohistochemical assays has allowed verification of the hypothesis that the Hcy-thiolactone pathway contributes to pathophysiology of the vascular system, in particular of the prediction that conditions predisposing to atherosclerosis, such as genetic or dietary hyperhomocysteinemia, lead to elevation of Hcy-thiolactone and *N*-Hcy-protein. This prediction has been confirmed *in vivo* both in humans and in mice. For example, plasma Hcy-thiolactone was found to be elevated 59-72-fold in human patients with hyperhomocysteinemia secondary to mutations in methylenetetrahydrofolate reductase (MTHFR) or cystathionine β-synthase (CBS) genes. Plasma *N*-Hcy-protein levels are elevated 24-30-fold in MTHFR- or CBS-deficiency, both in human patients and in mice. Plasma and urinary Hcy-thiolactone and plasma *N*-Hcy-protein levels are also elevated up to 30-fold in mice fed a hyperhomocysteinemic (1.5% methionine) diet. Furthermore, plasma levels of pro-thromobogenic *N*-Hcy-fibrinogen were elevated in human CBS deficiency, which explains increased atherothrombosis observed in CBS-deficient patients. We also observed increased immunohistochemical staining for *N*-Hcy-protein in aortic lesions from ApoE-deficient mice with hyperhomocysteinemia induced by a high methionine diet, relative to the mice fed a normal chow diet. We conclude that genetic or dietary hyperhomocysteinemia significantly elevates proatherothrombotic metabolites Hcy-thiolactone and *N*-Hcy-proteins in humans and mice.

Key words: *autoantibodies, atherosclerosis, CBS, fibrinogen, hyperhomocysteinemia, homocysteine thiolactone hypothesis, immune activation, MTHFR, protein N-homocysteinylation, thrombosis*

HOMOCYSTEINE METABOLISM – AN OVERVIEW

In mammals homocysteine (Hcy) is formed from methionine (Met) as a result of cellular methylation reactions (1). In this pathway Met is first activated by ATP to yield S-adenosylmethionine (AdoMet). As a result of the transfer of its methyl group to an acceptor, AdoMet is converted to S-adenosylhomocysteine (AdoHcy). *Enzymatic hydrolysis of AdoHcy is the only known source of Hcy in the human body*. Levels of Hcy are regulated by remethylation to Met, catalyzed by Met synthase (MS), and transsulfuration to cysteine, the first step of which is catalyzed by cystathionine β-synthase (CBS). The remethylation requires vitamin B_{12} and 5,10-methyl-tetrahydrofolate (CH_3-THF), generated by 5,10-methylene-THF reductase (MTHFR). The transsulfuration requires vitamin B_6.

Hcy is also metabolized to a thioester, Hcy-thiolactone, by methionyl-tRNA synthetase (MetRS) (*Fig.1*) in an error-editing reaction in protein biosynthesis when Hcy is mistakenly selected in place of Met (2-9). The flow through the Hcy-thiolactone pathway increases when re-methylation or trans-sulfuration reaction is impaired by genetic alterations of enzymes, such as CBS (10-13), MS (10, 11), and MTHFR (14), or by inadequate supply of CH_3-THF (5, 12, 15).

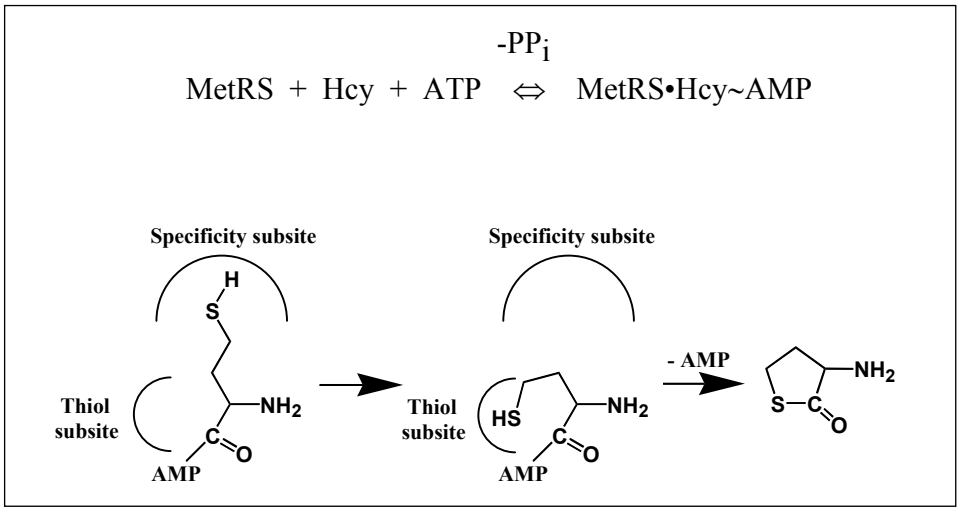

Fig. 1. The formation of Hcy-thiolactone catalyzed by MetRS. During protein biosynthesis Hcy is often mistakenly selected in place of Met by methionyl-tRNA synthetase (MetRS) and activated with ATP to form Hcy-AMP (*upper panel*). The misactivated Hcy is not transferred to tRNA but converted to Hcy-thiolactone in an error-editing reaction (*lower panel*) (adapted from ref. (3)).

PATHOPHYSIOLOGY OF HYPERHOMOCYSTEINEMIA

Among pathological manifestations of genetic hyperhomocysteinemia, which include mental retardation, ectopia lentis and osteoporosis, vascular complications remain the major cause of morbidity and mortality in untreated patients (16-19) . McCully observed advanced arterial lesions in children with inborn errors in Hcy metabolism and proposed a hypothesis that Hcy causes vascular disease (20). Even mild hyperhomocysteinemia, quite prevalent in the general population, is associated with an increased risk of vascular events (21). The findings that Hcy-lowering by vitamin-B supplementation improves vascular outcomes in CBS-deficient patients show that Hcy plays a causal role in atherothrombosis. For example, untreated CBS-deficient patients suffer 1 vascular event per 25 patient-years (16) while treated CBS-deficient patients suffer only 1 vascular event per 263 patient-years (relative risk 0.091, $p<0.001$) (17). Hcy-lowering therapy started early in life prevents also brain disease from severe MTHFR deficiency (19, 22). Lowering plasma Hcy by vitamin-B supplementation improves cognitive function also in the general population (23). High risk stroke (24, 25) but not myocardial infarction patients (25, 26) benefit from lowering of plasma Hcy by B-vitamin supplementation. These findings suggest that Hcy plays a greater role in stroke than in myocardial infarction, a suggestion consistent with the observations that in untreated CBS-deficient patients cerebrovascular incidents are 8-times more frequent than myocardial infarctions (16). Furthermore, studies of genetic and nutritional hyperhomocysteinemia in animal models provide additional support for a causal role of Hcy in atherothrombosis (27).

THE HCY-THIOLACTONE HYPOTHESIS

A preponderance of biochemical and genetic data suggest that elevated Hcy promotes a proatherothrombotic phenotype. Potential mechanisms include modification of proteins by homocysteinylation, oxidative stress, inflammation, endothelial dysfunction, and thrombosis (7, 13, 27).

Hcy-thiolactone hypothesis, originally formulated in 1997 (12) states that a pathway initiated by Hcy conversion to Hcy-thiolactone contributes to Hcy pathobiology (*Fig. 2*) (13, 14). Consistent with this hypothesis, plasma Hcy-thiolactone is elevated under conditions predisposing to atheroscthrombosis, such as hyper-homocysteinemia caused by mutations in *CBS* or *MTHFR* gene in humans or a high-Met diet in mice (28). Hcy-thiolactone is a reactive metabolite that causes protein *N*-homocysteinylation through the formation of amide bonds with protein lysine residues (*Fig. 3*), which impairs or alters the protein's function (29). *N*-linked protein Hcy (*N*-Hcy-protein), originally discovered *in vitro* in human fibroblasts and endothelial cells (5, 12, 15, 29), occurs in the human body

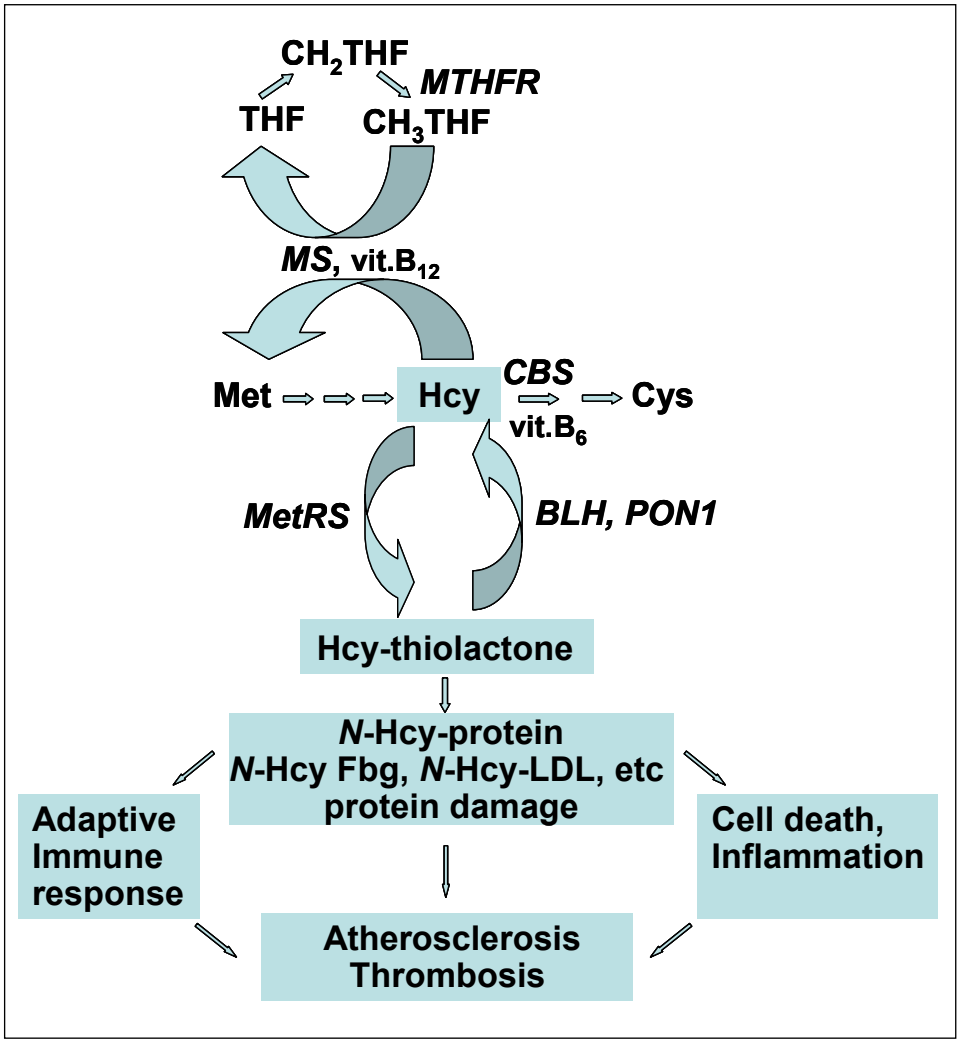

Fig. 2. The pathophysiologic hypothesis of Hcy-thiolactone-mediataed vascular disease. *N*-Hcy-Fbg, *N*-Hcy-LDL - N-homocysteinylated forms of fibrinogen and low density lipoprotein, respectively (adapted from ref. (28)). See text for discussion.

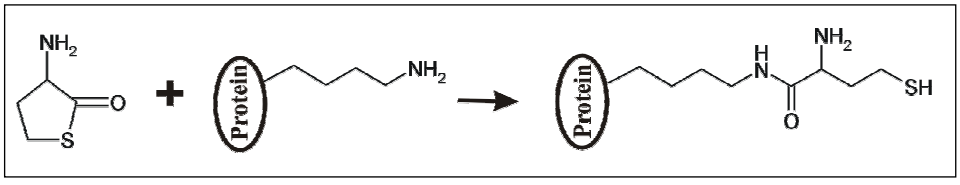

Fig. 3. Schematic illustration of chemical modification of a protein lysine residue by Hcy-thiolactone.

(5), is elevated in hyperhomocysteinemia (3, 30-32), and accumulates in atherosclerotic lesions in mice (33).

TOXICITY OF HCY-THIOLACTONE

Consistent with the Hcy-thiolactone hypothesis, chronic treatments of animals with Hcy-thiolactone cause pathophysiological changes similar to those observed in human genetic hyperhomocysteinemia. For example, Hcy-thiolactone infusions or Hcy-thiolactone-supplemented diet produce atherosclerosis in baboons (34) or rats (35) whereas treatment with Hcy-thiolactone causes developmental abnormalities in chick embryos, including optic lens dislocation (36), characteristic of the CBS-deficient human patients (1, 16).

Hcy-thiolactone induces apoptotic death in cultured human vascular endothelial (37, 38) and promyeloid cells (39), placental trophoblasts (40), and inhibits insulin signaling in rat hepatoma cells (41). Hcy-thiolactone also induces endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in retinal epithelial cells (42). Furthermore, *Hcy-thiolactone is more toxic to cultured cells than Hcy itself* (37-40, 42).

CONSEQUENCES OF PROTEIN *N*-HOMOCYSTEINYLATION BY HCY-THIOLACTONE

Through many routes, cellular physiology can be impacted by incorporation of Hcy into protein, which introduces global changes in primary protein sequence. These routes include disruption of protein folding, creation of altered proteins with newly acquired interactions, or induction of autoimmune responses. During the folding process, proteins form their globular native states in a manner determined by their primary amino acid sequence. Thus, *small changes in amino acid sequence caused by Hcy incorporation have the potential to create misfolded protein aggregates*. Indeed, *N*-Hcy-proteins do have a propensity to form protein aggregates (29). The appearance of misfolded/aggregated proteins in the ER activates a signaling pathway, the UPR, that, when overwhelmed, leads to cell death *via* apoptosis (27, 43). These pathways are induced by treatments of cultured cells and mice with excess Hcy (44), which is metabolized to Hcy-thiolactone (13). Hcy-thiolactone is more effective that Hcy in inducing ER and UPR (42). *In this scenario the formation of N-Hcy-proteins leads to the UPR and induction of the apoptotic pathway.* In humans, Hcy incorporation into proteins triggers an auto-immune response (45) and increases vascular inflammation (46) (*Fig. 2*), known modulators of atherogenesis (47).

Like Hcy-thiolactone synthesis, protein N-homocysteinylation is enhanced by CBS or MTHFR deficiency (32), antifolate drugs such as aminopterin (12), and inhibited by supplementation with folic acid, which lowers the Hcy/Met ratio (15). Hcy incorporation into protein is also inhibited by the Hcy-thiolactonase

activity of PON1 (*Fig. 2*) carried in the circulation on high-density lipoproteins (HDL) (15, 48).

As predicted by the Hcy-thiolactone hypothesis, *N*-linked protein Hcy is correlated with plasma total Hcy in humans (30, 32). *N*-linked Hcy is present essentially in each protein examined so far, including hemoglobin (Hb), albumin, γ-globulin, transferrin, HDL, low-density lipoprotein (LDL), α1-antitrypsin, and fibrinogen (49). *N-Hcy-Hb, present at 12.7 μM (or 0.9 mg/ml), constitutes the largest known Hcy pool in human blood* (30). The highest levels of *N*-linked Hcy, about 0.5 mol/mol protein, occur in human and equine ferritins (49). The inability to detect *N*-linked Hcy in transthyretin (50) is most likely due to inadequate assay sensitivity.

Hcy incorporation creates altered proteins with newly acquired interactions, detrimental to their function. For example, lysine oxidase (51), trypsin (29), MetRS (29), and PON1 (52) are inactivated by *N*-homocysteinylation. *N*-homocysteinylation of albumin (31) and cytochrome c (53) impairs their red-ox function. *N*-Hcy-proteins (29)(5), including N-Hcy-LDL (54) tend to form aggregates *in vitro*. *N*-Hcy-LDL, but not native LDL, induces cell death in human endothelial cells (55); a finding consistent with the inherent toxicity of protein aggregates (56).

PROTHROMBOTIC PROPERTIES OF *N*-HCY-FIBRINOGEN

Fibrinogen is known to undergo facile *N*-homocysteinylation by Hcy-thiolactone *in vitro* (5, 29) and *N*-Hcy-fibrinogen is present *in vivo* in humans (30). Sauls *et al.* (57) showed that clots formed from Hcy-thiolactone-treated normal human plasma or fibrinogen lyse slower than clots from untreated controls. Some of lysine residues susceptible to *N*-homocysteinylation are close to tissue plasminogen activator and plasminogen binding, or plasmin cleavage, sites, which can explain abnormal characteristics of clots formed from *N*-Hcy-fibrinogen (57). The detrimental effects of elevated plasma tHcy on clot permeability and resistance to lysis in humans are consistent with a mechanism involving fibrinogen modification by Hcy-thiolactone (58). Furthermore, CBS-deficient patients, who suffer from increased atherothrombosis (16) have significantly elevated plasma levels of prothrombotic *N*-Hcy-fibrinogen (32). These findings suggest that fibrinogen *N*-homocysteinylation leads to abnormal resistance of fibrin clots to lysis and contributes to increased risk of thrombosis (*Fig. 2*).

AUTOIMMUNOGENICITY OF *N*-HCY-PROTEIN

Atherosclerosis is now wildly recognized as a chronic inflammatory disease that involves both innate and adaptive immunity (47). Like other post-translationally modified proteins, *N*-Hcy-proteins elicit an auto-immune response

in humans, manifested by the induction of IgG autoantibodies directed against *N*ε-Hcy-Lys epitopes. This response is enhanced in stroke and coronary artery disease (CAD) patients, suggesting that it is a general feature of atherosclerosis (59-61). Elevated levels of anti-*N*-Hcy-protein IgG auto-antibodies are a consequence of elevated levels of *N*-Hcy-protein observed in CAD patients (62). Anti-*N*-Hcy-protein IgG auto-antibodies (59) and *N*-Hcy-protein levels [34] vary considerably among individuals and are strongly correlated with plasma tHcy, but not with Cys or Met. Such correlation is explained by direct mechanistic links between Hcy-related species, predicted by the Hcy-thiolactone hypothesis (*Fig. 2*): elevation in Hcy leads to inadvertent elevation in Hcy-thiolactone (*Fig. 1*), observed in human endothelial cells (10, 15), as well as in human and mouse plasma (10, 28, 63, 64). Hcy-thiolactone mediates Hcy incorporation into proteins (5, 12, 15, 29-31) and thus the formation of neo-self antigens, *N*-Hcy-protein (*Fig. 3*). Raising levels of these antigens trigger an auto-immune response (*Fig. 2*). Auto-antibodies recognizing the *N*ε-Hcy-Lys epitope react with any *N*-Hcy-protein (59, 65) in many tissues, contributing to known deleterious effects of hyper-homocysteinemia on many organs (1, 16). If the neo-self *N*ε-Hcy-Lys epitopes were present on endothelial cell membrane proteins, anti-*N*-Hcy-protein autoantibody would form antigen-antibody complexes on the surface of the vascular vessel. Endothelial cells coated with anti-*N*-Hcy-protein auto-antibodies would be taken up by the macrophage *via* the Fc receptor, resulting in injury to the vascular surface. If the *N*-Hcy-proteins were present chronically, repeating attempts to repair the damaged vascular wall would lead to a lesion (33).

The involvement of an autoimmune response against *N*-Hcy-protein in CAD is supported by the findings that lowering plasma Hcy by folic acid supplementation lowers anti-*N*-Hcy-protein autoantibodies levels in control subjects but not in patients with CAD (61). These findings suggest that once accumulated, the antigens causing the antibody response, i.e. *N*-Hcy proteins persist and that chronic protein damage caused by N-homocysteinylation cannot be easily reversed in CAD patients. Furthermore, these findings also suggest that while primary Hcy-lowering intervention by vitamin supplementation is beneficial, secondary intervention may be ineffective, and may explain at least in part the failure of vitamin therapy to lower cardiovascular events in myocardial infarction patients (25, 26).

ELIMINATION OF HCY-THIOLACTONE

Two enzymes are known that have the ability to hydrolyze the toxic metabolite Hcy-thiolactone: extracellular (serum) Hcy-thiolactonase/paraoxonase 1 (PON1) (48, 66-68) and intracellular Hcy-thiolactonase/bleomycin hydrolase (BLH) (69).

PON1, named for its ability to hydrolyze the organophosphate paraoxon, is synthesized exclusively in the liver and carried on HDL in the circulation. PON1-

deficient mice are more susceptible to a high-fat diet-induced atherosclerosis than wild type littermates, but do not develop atherosclerosis on a normal chow diet (70). PON1 transgenic mice (carrying 3 copies of the human *PON1*) are less susceptible to atherosclerosis (71). *In vitro* studies indicate that HDL from PON1 deficient animals does not prevent LDL oxidation, whereas HDL from PON1 transgenic animals protects LDL against oxidation more effectively than HDL from wild type mice. PON1 is an Hcy-thiolactonase which is able to protect proteins against N-homocysteinylation, at least *in vitro* (15, 66). Human *PON1* has genetic polymorphisms, *e.g. PON1-M55L*, *PON1-R192Q*, which affect PON1 function (72), including Hcy-thiolactonase activity (66-68). In mice, Hcy is a negative regulator of PON1 expression (73). In humans, Hcy-thiolactonase activity of PON1 is negatively correlated with tHcy (67) and predicts cardiovascular disease (68).

BLH, named for its ability to hydrolyze the anticancer drug bleomycin, has been studied in the context of cancer therapy. More recently, BLH has been implicated in Alzheimer's disease. For example, a *BLH* polymorphism, Ile443Val, is associated with an increased risk for Alzheimer's disease, making BLH a potential target for a drug that can prevent or slow the progression of the disease (74). Higher BLH levels accumulate in diseased brains from Alzheimer's patients than in controls (75). BLH has the ability to process amyloid precursor protein and amyloid-β *in vitro* (76). BLH deficient mice are more sensitive to bleomycin toxicity than wild type animals and prone to tail dermatitis (77).

BLH, in contrast to PON1, is ubiquitous in various mammalian tissues (78) and also in other species (79). Human and yeast BLH have almost identical molecular structure, similar to the 20S proteasome and belong to a family of self-compartmentalizing intracellular cysteine proteases (79, 80). Although its evolutionary conservation and wide distribution suggested a conserved cellular function, BLH's function was unknown until 2006 when we found that BLH is a major Hcy-thiolactonase in humans and yeast, which protects against Hcy toxicity (69). Possible roles of BLH and PON1 in Hcy-thiolactone detoxification (*Fig. 2*) in humans remain to be investigated.

CONCLUSION

Despite advances in our understanding of cardiovascular disease, coronary heart disease is still the major cause of mortality in industrial nations. Traditional risk factors such as hypertension, diabetes, hyperlipidemia, and smoking do not accurately predict cardiovascular events. Thus identification of novel risk factors and their mechanisms of action has important public health implications. Hcy is a novel risk factor for the development of cardiovascular disease (81). Studies of severe genetic hyperhomocysteinemia in humans and genetic and nutritional hyperhomocysteinemia in animal models show that Hcy plays a causal role in

atherothrombosis. As reviewed briefly above, the involvement of Hcy in proatherogenic changes in vascular endothelium can be explained for by its metabolism to Hcy-thiolactone, which in turn spontaneously modifies proteins in the human body. Protein N-homocysteinylation induces pathophysiological responses, such as an autoimmune activation and increased susceptibility to thrombosis. Chronic activation of these processes can lead to vascular disease.

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