# Protein carbamylation and cardiovascular disease

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**Carbamylation constitutes a posttranslational modification of proteins or amino acids and results from different pathways *in vivo*. First is the non-enzymatic reaction between isocyanic acid, a decomposition product of urea, and either the N-terminus or the ε-amino group of lysine residues. Isocyanic acid levels, while low *in vivo*, are in equilibrium with urea and are thus increased in chronic and end-stage renal diseases. An alternative pathway involves the leukocyte heme protein myeloperoxidase, which catalyzes the oxidation of thiocyanate in the presence of hydrogen peroxide, producing isocyanate at inflammation sites. Notably, plasma thiocyanate levels are increased in smokers, and leukocyte-driven protein carbamylation occurs both within human and animal atherosclerotic plaques, as well as on plasma proteins. Protein carbamylation is considered a hallmark of molecular aging and is implicated in many pathological conditions. Recently, it has been shown that carbamylated low-density lipoprotein (LDL) induces endothelial dysfunction via lectin-like-oxidized LDL receptor-1 activation and increased reactive oxygen species production, leading to endothelial nitric oxide synthase uncoupling. Moreover, carbamylated LDL harbors atherogenic activities, including both binding to macrophage scavenger receptors inducing cholesterol accumulation and foam-cell formation, as well as promoting vascular smooth muscle proliferation. In contrast, high-density lipoprotein loses its anti-apoptotic activity after carbamylation, contributing to endothelial cell death. In addition to involvement in atherogenesis, protein carbamylation levels have emerged as a particularly strong predictor of both prevalent and incident cardiovascular disease risk. Recent studies also suggest that protein carbamylation may serve as a potential therapeutic target for the prevention of atherosclerotic heart disease.**

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Traditional risk factors such as smoking, hypertension, diabetes, and hypercholesterolemia are major determinants of incident cardiovascular diseases (CVDs) in the general population. On the individual patient level, however, susceptibility to atherosclerosis varies considerably, and heterogeneity in disease burden remains insufficiently explained. Patients with chronic kidney disease (CKD), for instance, have a 10 to 30 times increase in CVD risk compared with an age- and gender-matched subjects with normal renal function, which cannot be attributed solely to traditional cardiovascular risk factors. Uremia, with the accumulation of toxic waste products inherent to a decline in the glomerular filtration rate, has been proposed to contribute to the enhanced CVD risks associated with CKD, and more recently there has been a growing understanding of potential underlying pathophysiological mechanisms. High urea levels facilitate posttranslational modification (PTM) of proteins through a process called protein carbamylation, altering their structure and function. Intriguingly, recent studies show that chronic inflammation and oxidative stress—both implicated in the process of atherogenesis—are also mechanistically linked to promotion of protein carbamylation.[1] Recent studies show that carbamylation of lipoproteins occurs *in vivo* and confers proatherogenic biological activities, such as endothelial dysfunction and cell death, macrophage foam-cell formation, and vascular smooth muscle proliferation.[1,2] Moreover, several clinical studies have recently shown that measurements of circulating levels of protein carbamylation predict incident cardiovascular risks, such as within the general population, among patients with CKD, and in patients with end-stage renal disease undergoing hemodialysis.[1,3–5] Protein carbamylation is thus becoming recognized as an additional contributory link in the pathophysiology of CVD, warranting further investigation to develop novel therapeutic strategies for CVD prevention and treatment.

## THE PROTEIN CARBAMYLATION PROCESS

Carbamylation (previously termed as carbamoylation) is a PTM of proteins or amino acids (Figure 1), resulting from the covalent adduction of the electrophilic isocyanic acid to specific nucleophilic functional groups. The major sites of carbamylation involve the $N^{\alpha}$-amino moiety of a protein N-terminus and the $N^{\varepsilon}$-amino moiety of protein lysine residues. However, carbamylation can also occur at the guanidine moiety of arginine and the reduced thiol moiety of

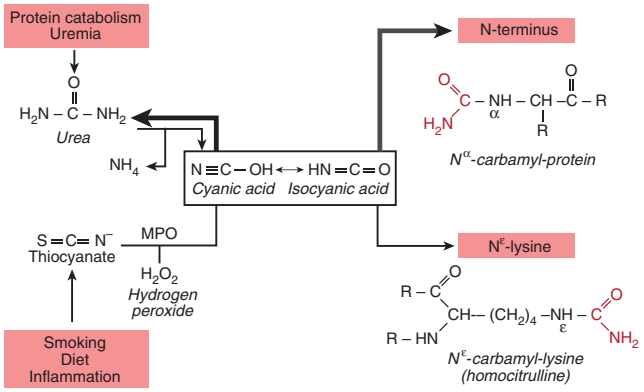


**Figure 1 | Pathways leading to protein carbamylation *in vivo*.** Protein carbamylation refers to the posttranslational modification of proteins or amino acids via adduction with isocyanic acid, on either the N-terminus of proteins or free amino acids ($N^{\alpha}$-carbamylation) or the $N^{\epsilon}$-amino group of protein lysine residues forming carbamyllysine (homocitrulline). Isocyanic acid is formed through either spontaneous decomposition of urea or myeloperoxidase (MPO)-catalyzed oxidation of thiocyanate at sites of inflammation, including atherosclerotic plaques.

cysteine. Two major biochemical pathways have been elucidated to result in protein carbamylation *in vivo* (Figure 1). Urea, which is present abundantly throughout the human body as a waste product of protein catabolism, slowly decomposes spontaneously in aqueous solutions forming cyanic acid (and its conjugate base, cyanate) according to an equilibrium favoring urea >99%.[6,7] Cyanic acid is in rapid equilibrium with its reactive form, isocyanic acid.[7] The plasma concentration of isocyanic acid in healthy individuals is estimated to be ~50 nmol/l but can reach 150 nmol/l in patients with CKD. Recent studies demonstrate that cyanate may also be generated via enzyme-catalyzed oxidation of the pseudo-halide thiocyanate ($SCN^-$) by myeloperoxidase (MPO).[1,8–13] MPO is the most abundant protein in leukocytes (both neutrophils and monocytes) and is both enriched within and catalytically active in atherosclerotic lesions.[8–13] Moreover, MPO has been mechanistically linked to the development of atherosclerosis and vulnerable plaques in humans.[1,11–13] Studies with MPO knock-out and MPO transgenic mice both confirm that MPO catalyzes protein carbamylation *in vivo*.[1]

## INVOLVEMENT OF PROTEIN CARBAMYLATION IN PATHOPHYSIOLOGY

Several proteins have been demonstrated to undergo carbamylation in different pathophysiological conditions, often altering their structure and rendering them dysfunctional. Long-lived proteins are particularly prone to PTMs such as carbamylation, which are considered the hallmark of molecular aging. Carbamylation of α-crystallins induces conformational changes responsible for lens opacities in cataract. In addition, carbamylation disturbs the triple-helix structure of collagen type I, leading to a decreased ability to polymerize into normal fibrils and increased susceptibility to collagenases.[14] Furthermore, enzymatic activity of insulin and erythropoietin are substantially diminished after carbamylation.[15,16] Interestingly, carbamylation has also been shown to be potentially involved in the pathogenesis of rheumatoid arthritis, where in animal models carbamylated peptides were shown to serve as a potent neo-antigen for production of autoantibodies and an erosive arthritis phenotype.[17] Importantly, recent studies also show that protein carbamylation occurs at increased levels within atherosclerotic plaques,[1,8–13] and alternative studies suggest that protein carbamylation may have a role in Alzheimer's disease development through the generation of abnormal tau protein deposits in the brain.[18]

## EFFECTS OF PROTEIN CARBAMYLATION ON LIPOPROTEIN METABOLISM AND FUNCTION

### Carbamylated low-density lipoprotein and endothelial dysfunction

Increasing evidence implicates lipoprotein carbamylation as a potentially pivotal mediator of atherogenesis (Figure 2). Carbamylated low-density lipoprotein (LDL) has been demonstrated to induce endothelial dysfunction through uncoupling of endothelial nitric oxide synthase.[2] Endothelial nitric oxide synthase normally acts as a nitric oxide producing enzyme but emerges as a source of reactive oxygen species when its dimer becomes uncoupled. S-glutathionylation is suggested as one potential underlying molecular mechanism contributing to endothelial nitric oxide synthase uncoupling and is increased in human aortic endothelial cells after exposure to carbamylated LDL.[2] Recent evidence also suggests that carbamylated LDL may interact with the endothelial lectin-like-oxidized LDL receptor-1 (LOX-1) in a manner similar to oxidized LDL or other agents induced by oxidative stress.[19] Indeed, overexpression of LOX-1 enhanced endothelial dysfunction caused by carbamylated LDL exposure, whereas silencing LOX-1 abrogated the effect.[2] In addition, carbamylated LDL-induced endothelial reactive oxygen species production was almost completely prevented by the administration of captopril or the NADPH (nicotinamide adenine dinucleotide phosphate)-oxidase inhibitor diphenylene iodinium, suggesting that NADPH-oxidase-induced reactive oxygen species production is a downstream effect of LOX-1 activation.[2]

### Carbamylated lipoproteins and atherosclerotic plaque formation

Carbamylated LDL exhibits decreased clearance from the circulation because of a lower affinity for the hepatic LDL receptor when compared with native LDL.[1,20,21] In addition, macrophage scavenger receptor recognition of carbamylated LDL facilitates cholesterol accumulation and macrophage foam-cell formation, as well as pro-inflammatory signaling.[1] Moreover, carbamylated LDL promotes adhesion of monocytes to endothelial cells and induces endothelial cell apoptosis through the mitogen-activated protein kinase pathway.[19,22] Furthermore, carbamylated LDL is a potent