Protein Carbamylation and Cardiovascular Disease

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Abstract

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 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 haem protein myeloperoxidase, which catalyses 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 harbours 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.
Keywords
vascular calcification; uremia; inflammation

Introduction
Traditional risk factors such as smoking, hypertension, diabetes, and hypercholesterolemia are major determinants of incident cardiovascular diseases (CVD) 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 to an age- and gender-matched subjects with normal renal function, which cannot be attributed solely to traditional cardiovascular risk factors. Uraemia, with the accumulation of toxic waste products inherent to a decline in 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 facilitates 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.† Recent studies show 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 measurement of circulating levels of protein carbamylation predicts incident cardiovascular risks, such and within the general population, among patients with CKD, and in patients with end-stage renal disease undergoing haemodialysis.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 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 Nα-amino moiety of a protein N-terminus and the Nε-amino moiety of protein lysine residues. However, carbamylation can also occur at the guanidine moiety of arginine, and the reduced thiol moiety of 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 favouring 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 in patients with CKD. Recent studies demonstrate that cyanate may also be generated via enzyme-catalysed 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 catalyses 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 a-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 protein carbamylation occurs at increased levels within atherosclerotic plaques$^{1,8-13}$, and alternative studies suggest that protein carbamylation may play a role in Alzheimer 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 LDL has been demonstrated to induce endothelial dysfunction through uncoupling of endothelial nitric oxide synthase (eNOS).$^2$ eNOS normally acts as a nitric oxide producing enzyme, but emerges as a source of reactive oxygen species (ROS) when its dimer becomes uncoupled. S-glutathionylation is suggested as one potential underlying molecular mechanism contributing to eNOS 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 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, while silencing LOX-1 abrogated the effect.$^2$ In addition, carbamylated LDL-induced endothelial ROS production was almost completely prevented by the administration of captopril or the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase inhibitor diphenylene iodium, suggesting that NADPH-oxidase induced ROS 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 to native LDL.\textsuperscript{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 signalling.\textsuperscript{1} Moreover, carbamylated LDL promotes adhesion of monocytes to endothelial cells, and induces endothelial cell apoptosis through the mitogen-activated protein kinase pathway.\textsuperscript{19,22} Furthermore, carbamylated LDL is a potent stimulator of vascular smooth muscle proliferation.\textsuperscript{23} In contrast, carbamylation inhibits atheroprotective functions of high-density lipoprotein, producing a “dysfunctional” form of high-density lipoprotein both at sites of inflammation and under conditions with elevated urea levels such as within subjects with renal disease.\textsuperscript{1,24,25} In addition to effects on circulating lipoproteins, carbamylation also impacts the extracellular matrix, making collagen type I more exposed to collagenases, a PTM that is suggested to potentially foster enhanced plaque vulnerability.\textsuperscript{14} Recent animal studies have also shown that direct provision of cyanate in vivo induces protein carbamylation. Specifically, when introduced in mice to generate levels of protein carbamylation observed in uremic patients, reduction in aortic ring vasorelaxation in response to acetylcholine was observed, along with reduction in endothelial nitric oxide production, and coincident increases of tissue factor and plasminogen activator inhibitor-1 protein levels in aortas of cyanate-treated mice. These results suggest that cyanate, whether produced during renal insufficiency as a result of uremia, or locally at a site of vascular inflammation, can impair endothelial function, and thus potentially participate in enhanced cardiovascular risk.\textsuperscript{26}

Protein Carbamylation as a Biomarker

Carbamylated proteins are of particular interest to use as biomarkers, because they may quantitatively reflect the burden of pathological conditions (inflammation and uremia) and are present in plasma or whole blood. Apart from carbamylated lipoproteins discussed above, carbamylated haemoglobin results from the covalent binding of isocyanic acid to the N-terminal valine residue of globin (3 chains. In patients with end-stage renal disease undergoing haemodialysis, carbamylated haemoglobin has been suggested to better reflect the cumulative uremic burden and dialysis efficiency when compared to Kt/V (i.e., the standard measurement of dialysis efficiency dependent of the dialyzer clearance of urea, dialysis time, and the patients estimated total body water) or the urea reduction rate.\textsuperscript{27} In addition, human albumin is a plasma protein with half-life of ~3 weeks, and possesses a predominant carbamylation site on Lys549.\textsuperscript{3} As a result, the proportion of carbamylated albumin may serve as a biomarker for overall carbamylation levels. Alternatively, total protein-bound homocitrulline (carbamylated lysine) levels can be quantified with high accuracy using stable isotope dilution high-performance liquid chromatography coupled with on-line tandem mass spectrometry to a similar purpose with clinical prognostic value.\textsuperscript{1}

Protein Carbamylation and Clinical Studies of Cardiovascular Disease

Protein carbamylation has been linked to prevalent and incident CVD risks. In an age- and gender-matched case-control study (n=300/150), subjects with CVD (i.e., angiographic
evidence of coronary artery disease, peripheral artery disease, myocardial infarction, stroke or previous revascularization) had significantly higher concentrations of plasma protein-bound homocitrulline than healthy controls.\(^1\) Moreover, there was a dose-dependent relationship between protein-bound homocitrulline levels and the risk of prevalent CVD. In an independent case-control study (n=275/275), subjects who experienced a major adverse cardiovascular event within 3 years after enrolment also had significantly higher levels of plasma protein-bound homocitrulline than those without interim events.\(^1\) Importantly, protein-bound homocitrulline levels predicted incident cardiovascular disease even after extensive adjustments for traditional cardiovascular risk factors, renal function, and both MPO levels and high sensitivity C-reactive protein concentrations.

In a separate study encompassing 96 patients with CKD, elevated carbamylated LDL concentrations (higher than the median) were associated with significantly higher all-cause mortality, as well as shorter cardiovascular event-free survival.\(^2\) Further in support of a mechanistic link between protein carbamylation and CVD is a prospective cohort study of 347 patients with end-stage renal disease undergoing haemodialysis.\(^3\) Protein carbamylation assessed by plasma protein-bound homocitrulline level was significantly higher in subjects with end stage renal disease compared to healthy controls. Moreover, subjects in the highest fertile of protein-bound homocitrulline levels had significantly increased all-cause mortality. Remarkably, addition of protein carbamylation (homocitrulline) levels to traditional cardiovascular risk factors resulted in a significant 14% reclassification of study subjects.

Berg and colleagues retrospectively assessed the fraction of carbamylated albumin in the Accelerated Mortality on Renal Replacement (ArMORR) study and Deutsche Diabetes Dialyse Studie (4D) hemodialysis trial, together encompassing 1,348 patients on haemodialysis.\(^3\) Again, increasing fertiles of carbamylated albumin fractions were associated with significantly elevated risk of all-cause mortality. However, no cardiac-specific endpoints were assessed.

**Future Therapeutic Implications**

Thus, a substantial and growing body of evidence indicates that systemic measures of protein-bound carbamylation are strongly associated with CVD risks, and mechanistic and animal model studies suggest a link between protein carbamylation and atherogenesis. Accordingly, therapeutic approaches aimed at intercepting or preventing protein carbamylation in vivo have been suggested as a novel pharmacological approach for the prevention and treatment of CVD, particularly among subjects with CKD or end-stage renal disease. Consistent with this, studies sought to test the hypothesis that provision of excess free amino acids might serve as a relatively safe approach to compete with proteins for reaction with isocyanic acid, protecting them from PTM via carbamylation. Owing to the difference in pKa of the N\(^{\alpha}\)- versus N\(^{\varepsilon}\)-amino moieties at physiological pH, the nucleophilicity (reactivity) with isocyanic acid strongly favours a-amino moieties. Consequently, at physiological pH, the N\(^{\alpha}\)-amino group of free amino acids reacts \(\sim\)100 times greater than for the lysine N\(^{\varepsilon}\)-groups on proteins, potentially enabling the free amino acid pool to act as natural ambient scavengers.\(^5\) Although all 20 amino acids can potentially be carbamylated via their primary (or secondary in case of proline) N\(^{\alpha}\)-amino group, *in vitro*
studies have shown that the side groups of cysteine, histidine, arginine and lysine bear the strongest potential for reaction with isocyanate. Additionally, the dipeptide glycyglycine was even more strongly carbamylated after incubation with cyanate when compared to any amino acid in its unmodified form, suggesting that simple dipeptides might possess therapeutic potential. Indeed, it was shown that glycyglycine was able to reduce albumin carbamylation by 64% in vitro when abundantly present, with taurine, the most abundant intracellular amine, also an effective scavenger of cyanate. Moreover, mice fed with a low-protein diet to induce amino acid deficiencies were shown to be more prone to either acute or chronic albumin carbamylation through injection of cyanate or addition of dietary urea, respectively. A recently conducted first in-human pilot study encompassing 23 haemodialysis patients has shown promising potential for amino acid therapy to reduce carbamylation in vivo Subjects who received treatment with 14 g doses of essential amino acids after each dialysis session had an 8.4% reduction in carbamylated albumin levels after 4 weeks, compared to a 4.3% increase in controls. Moreover, the effects seemed to accumulate over time, with an even greater difference between both groups after 8 weeks (15% versus 1% decrease). Alternatively, some vitamins with antioxidant properties such as ascorbic acid, a-tocopherol, and lycopene, have also been demonstrated to reduce LDL carbamylation in vitro However, further investigations are needed to confirm their efficacy in reducing carbamylation in an in vivo setting.

Conclusion

Protein carbamylation, a PTM of proteins or amino acids involved in a variety of disease states including molecular aging, inflammation, and impairment in renal function such as during CKD and end stage renal disease, has been mechanistically linked to atherosclerosis. Sitting at the nexus between uraemia, inflammation and vascular disease, lipoprotein carbamylation is of particular interest, and has shown clinical efficacy in explaining cardiovascular risks not captured by traditional risk factors. Strategies designed to prevent protein carbamylation are under investigation as novel approaches for the treatment and prevention of atherosclerosis.

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Protein carbamylation refers to the posttranslational modification of proteins or amino acids via adduction with isocyanic acid, such as on either the N-terminus of proteins or free amino acids (Nα-carbamylation) or the Nε-amino group of protein lysine residues forming carbamllysine (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.
Lipoprotein carbamylation is mechanistically linked to atherogenesis through multiple mechanisms. For example, carbamylated low-density lipoprotein (cLDL), through lectin-like-oxidized low-density lipoprotein receptor-1 (LOX-1) stimulation, promotes NADPH-oxidase induced production of reactive oxygen species (ROS) and uncoupling of endothelial nitric oxide synthase (eNOS). cLDL has a reduced affinity for the hepatic LDL receptor, causing decreased clearance from the circulation in subjects with end stage renal disease. cLDL conversely shows enhanced affinity for macrophage scavenger receptors, facilitating cholesterol accumulation and macrophage foam-cell formation. cLDL incubation with endothelial cells facilitates adherence of monocytes to endothelial cells, enhances endothelial cell death, and foster both macrophage inflammatory signalling and vascular smooth muscle proliferation. In contrast, carbamylation of high-density lipoprotein is one mechanism the lipoprotein appears to be rendered dysfunctional, losing its athero-protective and anti-apoptotic biological activities.

Means stimulated by cLDL