Myeloperoxidase-mediated lipoprotein carbamylation as a mechanistic pathway for atherosclerotic vascular disease

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ABSTRACT

There is an emerging and significant body of research that suggests that MPO (myeloperoxidase) may be a critical mediator in dysfunctional lipoprotein formation and, hence, atherogenic initiation and progression. MPO is a haem peroxidase found in leucocytes and is abundant in macrophages surrounding atherosclerotic lesions. Several lines of evidence support the role of MPO-mediated carbamylation of proteins in atherogenesis. The generic mechanism of MPO-mediated protein carbamylation has been elucidated recently and has been identified as a potentially crucial pathway that links smoking, inflammation and atherogenesis. HDL (high-density lipoprotein) exerts a physiologically beneficial effect of reducing arterial cholesterol deposition; however, there are considerable gaps in current understanding of the molecular basis of dysfunctional HDL formation. Especially deserving of attention is a contextual understanding of dysfunctional pro-atherogenic HDL formation in light of inflammatory changes in atheroma. The present review is especially timely in light of the solved structures of nascent and discoidal HDL and integrates the biochemical significance of MPO carbamylation in the context of these structures. Various avenues of experimental investigation are explored which will be crucial in understanding the vascular consequences of dysfunctional HDL formation and the identification of novel mechanistic pathways in vascular disease. It is anticipated that further knowledge on the intricacies of dysfunctional HDL formation, potentially by an MPO-driven pathway, will lead to considerable progress in identifying novel drug targets for atherosclerosis and characterization of the primary atherogenic process.

INTRODUCTION

Inflammation has been established as a key component of atherosclerosis and vascular disease. In support of this, several independent epidemiological studies have evaluated and confirmed that inflammatory markers, including CRP (C-reactive protein), various cytokines and adhesion molecules, are clinically useful in predicting the risk of vascular disease [1]. Previous investigations have suggested that protein carbamylation may be a novel inflammatory-mediated protein modification that yields effects pertinent to the development of atherosclerosis [2,3].

Carbamylation is a post-translational protein modification induced by urea-derived cyanate normally present in low concentrations in plasma and significantly

Key words: atherosclerosis, carbamylation, high-density lipoprotein (HDL), lipoprotein, myeloperoxidase, vascular disease.

Abbreviations: ABC, ATP-binding-cassette transporter; ACS, acute coronary syndrome; apo, apolipoprotein; ASVD, atherosclerotic vascular disease; CAD, coronary artery disease; CAM, cell adhesion molecule; CKD, chronic kidney disease; CRP, C-reactive protein; CVD, cardiovascular disease; ECM, extracellular matrix; HCl, homocitrulline; HDL, high-density lipoprotein; cHDL, carbamylated HDL; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HOCl, hypochlorous acid; ICAM-1, intercellular adhesion molecule-1; LDL, low-density lipoprotein; cLDL, carbamylated LDL; MI, myocardial infarction; MMP, matrix metalloproteinase; MPO, myeloperoxidase; PMN, polymorphonuclear cell; ROS, reactive oxygen species; SCN⁻, thiocyanate ion; SMC, smooth muscle cell; SR, scavenger receptor; VCAM-1, vascular cell adhesion molecule-1; VSMC, vascular SMC.

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Carbamylation is of historical significance in that it was one of the first post-translational modifications of proteins to be described, being astutely identified in denaturation-renaturation studies of proteins with urea as the causative mechanism for loss of protein function [4,5]. Notably, the formation of cyanate from urea is inversely associated with renal function and, hence, excess cyanate acts as a toxin in CKD [6]. Specifically, the active form of cyanate, isocyanic acid, is capable of interacting with \( \text{NH}_2 \) groups of proteins and especially with the \( \varepsilon \)-\( \text{NH}_2 \) groups of lysine residues, generating H\text{Cit} (homocitrulline; \( \varepsilon \)-carbamyllysine) (Figure 1).

CVDs (cardiovascular diseases), including CAD (coronary artery disease), hypertension, congestive heart failure, peripheral vascular disease and stroke, are worldwide medical and public health problems that together constitute the leading cause of death and disability in the Western world [7]. The majority of CVDs are attributable to atherosclerosis and its complications and, hence, atherogenesis is an important process deserving of special attention in dissecting the molecular underpinnings of CVD. A significant body of research has confirmed that the inflammatory process is a central tenet of atherosclerotic plaque initiation and progression. Inflammation indirectly implicates cellular mediators (i.e. neutrophils and macrophages) in the atherogenic process by the production of oxidative by-products, such as ROS (reactive oxygen species). These effects are essentially off-target negative ‘side effects’ of the primary protective roles of these cells.

The relationship between inflammation and atherosclerosis is so well-established that this leading cause of morbidity and mortality in Western societies has been dubbed ‘a chronic inflammatory disease’. Moreover, the ever increasing worldwide disease burden of ASVD (atherosclerotic vascular disease) as a consequence of commonly rooted inflammatory states, such as Type 2 diabetes and obesity, further establishes atherosclerosis as a disease on the brink of becoming a global epidemic. Much of our knowledge on established risk factors for vascular disease has focused on cholesterol metabolism and, especially, elevated LDL (low-density lipoprotein) levels. Although these findings are well-supported by sound clinicopathological and epidemiological studies, the healthy HDL (high-density lipoprotein) counterpart has remained an elusive component of our knowledge base of ASVD. Furthermore, the specific molecular contribution of lipid-laden foam cells derived from macrophages to atherogenesis has also remained nebulous, despite being commonly recognized as pathognomonic for atherosclerosis. The hypothesis that cellular inflammatory mediators functionally contribute to the atherogenic process is bolstered by recent evidence that macrophage-driven protein carbamylation is a critical atherogenic initiator and is capable of targeting both LDL and HDL.

Evidence suggests that lipoprotein carbamylation may serve as a novel mechanistic pathway for atherogenesis via the formation of dysfunctional LDL and HDL.

Aberrant lipoprotein metabolism has been identified as a crucial step in the atherogenic process. Goldstein et al. [8] reported that native LDL is incapable of converting normal cultured macrophages into foam cells in vitro, providing critical evidence that modified lipoproteins are key to the promotion of vascular disease. Since then, several other modifications of LDL have been identified that are linked to atherosclerotic plaque formation and progression, such as oxidative pathways. Indeed, evidence that structurally unrelated antioxidants inhibit atherosclerosis in animal models of hypercholesterolaemia [9,10] substantiate the importance of oxidative modifications of LDL in ASVD. Recent evidence, however, suggests that the quantitatively dominant form of modified LDL is its carbamylated form that occurs either non-enzymatically by urea-derived cyanate or via a reaction catalysed by the enzyme MPO (myeloperoxidase) [3] generated in bone marrow by granulocytes of PMN (polymorphonuclear cell) and monocyte lineages.

MPO is a haem protein highly expressed in neutrophils, monocytes and some populations of human macrophages, such as those in foam cells at the site of an atheroma [11,12]. Additionally, immunohistochemical and MS studies have detected the presence of MPO oxidation products in human atherosclerotic lesions and in lesion LDL [13–18]. For instance, Thukkani et al. [18] demonstrated that plasmalogens are attacked by MPO-derived reactive chlorinating species within human atheroma and the resultant species formed possess potent pro-atherogenic properties. These experimental findings strongly suggest that MPO is present in an enzymatically active form in human atherosclerotic tissue and that LDL is one of its targets.

Although macrophages do not generate mRNA for MPO and therefore do not technically ‘express’ MPO, a subset of macrophages has been shown to have MPO protein associated with them by immunohistochemical methods. The cytokine/growth factor environment in which the monocyte-to-macrophage differentiation occurs is critical in determining this association, whereby the presence of GM-CSF (granulocyte/macrophage colony-stimulating factor) allows the continual association of MPO with a differentiated macrophage population [11]. MPO expression is employed as a protective host defence mechanism and uses an \( \text{H}_2\text{O}_2 \) substrate to generate a wide array of reactive intermediates [10,12]. These reactive products can interact and damage surrounding host inflamed tissue [19]. Both LDL and HDL have been identified as targets for the MPO/peroxide system.

Epidemiological studies have correlated levels of MPO with the risk of vascular disease in patients with established CAD. In such patients, blood
MPO levels are elevated and are predictive of the risk of MI (myocardial infarction) in subjects with unstable angina [20–22]. A landmark study examining subjects with suspected ACSs (acute coronary syndromes) showed that a single initial measurement of plasma MPO independently predicts the early risk of MI, as well as the risk of major adverse cardiac events in the ensuing 30-day and 6-month periods in patients presenting to the emergency department with chest pain [21]. The first large-scale community-based screen, the EPIC-Norfolk (European Prospective Investigation into Cancer-Norfolk) study, demonstrated for the first time that elevated MPO levels predict the future risk of CAD in apparently healthy individuals, indicating that MPO-catalysed inflammatory activation precedes the onset of overt CAD by many years [22].

Population genetic studies have identified a promoter polymorphism in the MPO gene that results in reduced MPO expression and an associated decrease in the risk of clinical events in patients with CAD [23–25]. An observational study by Kutter et al. [25] examined the largest cohort of MPO-deficient subjects reported to date. In that study, a group of 100 totally or sub-totally MPO-deficient patients were compared with 118 randomly selected reference probands. The results demonstrated that MPO deficiency may protect against cardiovascular damage, but is also associated with a significantly higher occurrence of severe infections and chronic inflammatory processes [25].

The risk association of MPO and CAD suggests a possible functional role for MPO in the atherogenic process. In addition to studies demonstrating direct modification and formation of aberrant LDL by MPO-generated products, MPO contributes to endothelial dysfunction [26–28]. Studies on the effects of MPO on endothelial function have their origins in the mechanistic studies of Abu-Soud and Hazen [27], who first reported that MPO uses NO as a substrate and, thus, could serve as a catalytic sink for NO in vivo. Additionally, Vita et al. [28] performed clinical studies linking MPO levels to in vivo measures of endothelial dysfunction. In addition to its effects on endothelial function, MPO targets and thereby oxidizes HDL [29–32], resulting in the formation of dysfunctional or pro-atherogenic HDL.

PROTEIN CARBAMYLATION

Carbamylation of proteins, even a single-residue modification via any mechanism, can yield both functional and structural changes in the target protein that have clinically relevant effects. The irreversible reaction of isocyanic acid with free NH2 groups on a polypeptide can bring about protein conformational changes by altering charge distribution, both locally and globally, resulting in a consequent loss of function. This is evidenced by studies of the attenuation of functional enzymatic activity in target proteins, such as MMP-2 (matrix metalloproteinase-2) or 6-phospho-D-glucuronate dehydrogenase, upon carbamylation [33,34]. Additionally, the normal physiological role of insulin to facilitate cellular glucose uptake is disrupted by carbamylation [35]. Similarly, a single modification on the α-NH2 of the N-terminal cysteine residue of TIMP-2 (tissue inhibitor of metalloproteinases-2) results in a loss of its inhibitory activity [36]. Clinically, the development of cataracts has been linked to carbamylation of α-crystallins, resulting in conformational changes leading to lens opacity [37,38].

Circulating proteins and ECM (extracellular matrix) proteins are common targets for post-translational carbamylation, as a consequence of their wide presence and long half-life respectively [33,39,40]. In fact, in uremic patients, anti-HCt antibodies have demonstrated the carbamylation of haemoglobin and ECM proteins, i.e. collagens [39,40], altering critical cellular functions (i.e. cell adhesion and migration, and gene regulation) that are controlled by interactions between cells and ECM [41,42]. Carbamylation of ECM proteins is also thought to be functionally linked to the inflammatory process itself, given their importance in positively regulating the pathogenic defence mechanism, i.e. respiratory burst, employed by human PMNs [43,44]. In support, carbamylated type I collagen has been shown to induce the adhesion of human monocytes as well as their release of MMP-9 [43], and protein carbamylation has been hypothesized as a mechanism that may underlie the repeated infections that constitute one of the major causes of morbidity and mortality in uremic patients [44].

The relative specificity for carbamylation of ε-lysine residues in a protein is a consequence of the high frequency and comparably decreased pKa value of the ε-NH2 moiety compared with other nucleophilic targets. Although lysine side chains are the main amino acid target for carbamylation on proteins, all N-terminal α-NH2 groups can be carbamylated at a physiological pH [45], but this infrequently leads to changes in protein function.

Uraemia secondary to renal disease is capable of producing a chemical environment hospitable for protein carbamylation, and several lines of evidence indicate that kidney disease is an independent risk factor for the development of cardiovascular disease [46], mediated via uraemia-induced carbamylation of proteins. Until recently, protein carbamylation has been largely considered quantitatively important solely in the context of kidney disease; however, the recent elucidation of a novel MPO-dependent pathway for protein carbamylation suggests that a uraemia-independent carbamylation mechanism may dominate in the absence of renal impairment [3].
Protein carbamylation has largely been viewed as a consequence of excess cyanate production in uraemic patients, whereby urea spontaneously decomposes to cyanate and ammonium ions (A). MPO has been elucidated to serve as the catalyst for the quantitatively predominant pathway for cyanate production in patients without CKD, whereby thiocyanic acid reacts with $H_2O_2$ (MPO product) to form cyanate (cyanic acid) (B). Cyanic acid thus formed by either pathway can then react with a nucleophilic $\varepsilon$-lysine residue on target proteins to form a carbamylated HCit-bound protein target (C).

**BIOCHEMICAL REACTION OF PROTEIN CARBAMYLATION**

The uraemia-mediated biochemical mechanism of protein carbamylation has been well known for some time. In particular, the traditional understanding of post-translational protein carbamylation involves the reaction of isocyanic acid with a nucleophilic base, i.e. any N-terminus residue, or, preferably, an amino acid residue such as lysine. Protein carbamylation often occurs in isoelectric focusing as an unintended effect of the by-products of urea degradation. In this procedure, urea acts as a chaotrope that is in solution and in equilibrium with isocyanic acid that reacts with nucleophilic protein amino acid groups, such as lysine and arginine side chains, which are usually deprotonated at alkaline pH. The specific reaction that occurs at room temperature is that isocyanic acid, produced from urea, reacts with free bases on a polypeptide (Figure 1).

Nucleophilic amino acid residue targets of isocyanic acid, when carbamylated, cause resultant changes in protein structure and function, as described above. Additionally, protein carbamylation makes the resulting protein unsuitable for enzymatic digestion and results in peptides with unexpected retention times and masses in MS experiments. This fact has been exploited in the experimental detection of carbamylated peptides [3].

**CKD AND PROTEIN CARBAMYLATION**

CKD is a global health problem affecting approx. 10% of the worldwide population [47–49] and is also an independent risk factor for CVD [46]. In fact, CVD is a leading cause of death in patients with chronic renal insufficiency and, even after statistical control for other CVD risk factors (such as age, gender, race and diabetes),
there is a 10–20-fold greater risk of cardiovascular mortality in CKD patients when compared with healthy control subjects [46]. The inability to fully account for this CVD risk by traditional risk factors has led some to hypothesize that the unique biochemical context of renal impairment plays a significant role.

Renal disease is characterized by nitrogenous retention and is manifest in high blood urea levels, i.e. uraemia. The high urea characteristic of CKD is expected to rearrange under physiological conditions to form ammonia and cyanate [50]. The cyanate thus formed can become protonated and form isocyanic acid, which reacts with available α- and ε-NH₂ groups of proteins, resulting in the formation of a carbamylated polypeptide [51]. The strong correlation between protein carbamylation and renal disease is intuitive from this perspective and, in fact, several targets of carbamylation have been demonstrated in patients with renal failure, including free amino acids, plasma proteins, leucocyte proteins and haemoglobin [52–62]; however, the carbamylation of lipoproteins is most relevant to atherogenesis. In CKD patients, carbamylation of LDL has been identified as a novel risk factor for CVD and a biomarker for atherogenic progression [2].

**FUNCTIONAL CONSEQUENCES OF LIPOPROTEIN CARBAMYLATION**

LDL and HDL are frequently the target of carbamylation, especially at the site of atherosclerotic lesions. Although a significant body of literature has focused on cLDL (carbamylated LDL), studies on the targeting of HDL are decidedly deficient. cLDL formation occurs via the irreversible chemical reaction of apoB (apolipoprotein B), the protein component of the LDL particle, with urea-derived isocyanic acid present in the plasma of patients with renal impairment [6]. cLDL was shown to be positively correlated with atherosclerosis and is, in fact, the most abundant LDL isoform in human plasma by sandwich ELISA, and even more predominant in uraemic patients [63]. cLDL has also been shown to be functionally linked to atherosclerosis as it is capable of inducing pro-atherogenic events such as cLDL incorporation into endothelial cells, VSMC [vascular SMC (smooth muscle cell)] proliferation and endothelial cell cytotoxicity [2].

cLDL

LDL apoB carbamylation is functionally correlated with the pathophysiological development of atheroma at levels of both atherogenic initiation and progression. Specifically, it was found that cLDL induces monocyte adhesion to endothelial cells through ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) [64]. This finding is especially relevant in the context of monocyte adhesion and atherosclerotic plaque initiation. In response to vascular injury, inflammatory signals and chemokines are expressed in the vascular wall along with a concomitant up-regulation of CAMs (cell adhesion molecules) on endothelial cells. The increased expression of chemotactic factors attracts monocytes to the vascular wall, whereby monocyte adhesion to endothelial cells is mediated by Ig-like CAMs, selectins and integrins. Hence cLDL may activate monocyte adhesion via the up-regulation of the expression of adhesion molecules involved in monocyte–endothelial cell adhesion, i.e. ICAM-1 and VCAM-1 on endothelial cells. This is important in understanding the functional implications of protein carbamylation and, more significantly, the potential of carbamylated lipoproteins as pathogenic atherosclerotic markers or therapeutic targets.

A recent study has shown that cLDL also has multiple downstream effects that potentiate atherosclerotic plaque formation and progression [3]. To test the role of MPO-mediated arterial LDL apoB carbamylation in atherosclerosis, Wang et al. [3] examined whether cLDL formation either by the MPO/H₂O₂/SCN⁻ (thiocyanate ion) system or as a function of uraemia in CKD patients affected specific steps in the atherogenesis pathway. They found that both forms of cLDL similarly attenuated LDLR (LDL receptor) recognition of the modified lipoprotein [3,65], providing strong mechanistic support for the observation that cLDL is positively correlated with ASVD severity. cLDL, so retained in the arterial wall, also has other pro-atherogenic biological activities. Specifically, it was shown that the cLDL particles have increased uptake by macrophage scavenger receptors, independent of scavenger receptor CD36, resulting in cholesterol accumulation and foam-cell formation, pathognomonic for atherosclerotic plaque formation. Separate studies with CD36- and SR-A1 (scavenger receptor-A1)-deficient mice along with *in vitro* studies using the SR-A1 inhibitor fucoidin on stably cells transfected with SR-A1 or control parental cells demonstrated that SR-A1 was, in fact, almost wholly involved in cLDL recognition by macrophages [3]. The interaction of cLDL with SR-A1 on the macrophage surface has other downstream effects.

Both uraemia-induced as well as MPO-mediated cLDL have also been shown to have pro-atherosclerotic effects on vascular wall tissue. Specifically, cLDL can induce VSMC proliferation as well as endothelial cell apoptosis, both critical features of atherosclerosis. Additionally, cLDL, but not native LDL, when exposed to aortic SMCs (either human or bovine), induces marked SMC proliferation. The VSMC proliferation induced by cLDL is dependent on SR-A1, since fucoidin in the culture medium markedly suppresses the proliferation induced by the addition of MPO-cLDL [3].

**cHDL (carbamylated HDL)**

In contrast with LDL, surprisingly little is known on the pro-atherogenic effects of HDL carbamylation. One study [3], however, has demonstrated that cHDL
can promote aortic endothelial cell apoptosis, a critical hallmark of atherosclerotic lesions, as measured by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assays and by caspase activation. Exposure of bovine aortic endothelial cells to physiologically relevant levels of cHDL induced apoptosis. Although native HDL has a protective antiatherogenic and anti-apoptotic effect, cHDL was found to promote atherosclerosis by inducing endothelial cell death, both in bovine and human cell models. The anti-apoptotic activity of HDL is dependent on HDL binding to its cognate receptor SR-B1 as shown by experiments wherein siRNA (small interfering RNA) directed towards the gene encoding SR-B1 attenuated the anti-apoptotic activity of HDL [3].

The HDL carbamylation can also be achieved by an MPO-dependent pathway. Specifically, addition of SCN\(^{-}\) to culture medium along with catalytic (nanomolar) concentrations of MPO were shown to be sufficient to induce HDL carbamylation and the subsequent dose-dependent increases in endothelial cell caspase 3/7 activation [3].

Hence the retention of modified LDL in the arterial wall may serve as the nidus for atherosclerotic plaque initiation by triggering multiple pro-atherogenic steps (Figure 2). In particular, cLDL retention: (i) increases the expression of factors that induce monocyte adhesion to endothelial cells, (ii) induces modified LDL uptake by macrophages, resulting in foam cell formation, and (iii) stimulates the proliferation of VSMCs, all important steps in the formation of fatty atheroma. HDL is also a target for carbamylation and recent evidence [3] suggests that cHDL is in fact dysfunctional HDL and lacks the protective anti-apoptotic and metabolic effects of HDL.

**MPO AND PROTEIN CARBAMYLATION**

Recent experiments have elegantly demonstrated that MPO-mediated protein carbamylation can serve as a uraemia-independent mechanism for this post-translational modification [3]. Synthesis of MPO is initiated in the bone marrow during myeloid differentiation and is completed within granulocytes prior to their release into the circulation [66]. The enzyme is stored within primary leucocytic granules that are released upon leucocyte activation and subsequent degranulation.

MPO is protective in the context of a pathogenic insult in that it forms free radicals and diffusible oxidants with antimicrobial activity. Concomitantly, MPO promotes oxidative damage of host tissues at sites of inflammation, including atherosclerotic lesions [67]. Macrophages may contribute to the inflammatory process and initiate atherogenic processes by producing ROS such as superoxide and \(H_2O_2\). These macrophage products are subsequently chemically converted into stronger oxidants.

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**Figure 2  Multiple pro-atherogenic consequences of lipoprotein carbamylation**

In atherosclerosis, monocytes in the arterial lumen enter the subendothelial space where, as macrophages, they contribute significantly to the atherogenic process. Macrophage-released products catalyse the carbamylation of both LDL and HDL causing (1) an increased expression of CAMs that induce monocyte adhesion to endothelial cells, (2) modified LDL uptake by macrophages, resulting in lipid-laden foam cell formation, (3) proliferation of VSMCs, and (4) endothelial cell apoptosis.
such as HOCI (hypochlorous acid; ‘cellular bleach’) and peroxy-nitrite, and are employed to destroy invading micro-organisms [68,69]. MPO released by macrophages generates HOCI by the reaction of H₂O₂ and Cl⁻ in the acidic milieu of the inflamed region [12,14].

Both of these immunologically protective products of MPO catalysis have been shown to be potent atherogenic mediators. In particular, human atherosclerotic lesions are characterized by the presence of enzymatically active MPO [12] and, in such lesions, lipoproteins have been detected that have been modified by HOCI [70]. Additionally, the peroxynitrite also formed by the macrophage is an RNS (reactive nitrogen species) that can target lipoproteins for nitrosylation [71].

MPO has been identified to play a pivotal role in the formation of dysfunctional lipoproteins at the site of atherosclerotic lesions. Immunostaining experiments have demonstrated the presence of MPO in human atherosclerotic lesions [31], and studies have shown that oxidation products generated by MPO are richly abundant in human atheroma and in LDL recovered from diseased tissue [14,16]. Additionally, reactive intermediates generated by MPO are capable of transforming HDL to a dysfunctional pro-atherogenic form [31], although the exact molecular significance of such a modification is contested. In particular, although detection of tyrosine modification serves as a marker of ‘dysfunctional HDL’ [dysfunctional with respect to ABCA1 (ATP-binding-cassette transporter A1) efflux activity], tyrosine modification is not causally linked to an impairment in cholesterol efflux activity [72]. Indeed, it has recently been demonstrated that it is one or more tryptophan residues within HDL, and not a tyrosine residue, that serves as the oxidative ‘on/off’ switch for impaired ABCA1-mediated efflux activity [73]. Site-specific tyrosine oxidation (Tyr^166) has been convincingly shown to impair a different process within the reverse cholesterol transport pathway/HDL maturation via LCAT (lecithin:cholesterol acyltransferase) activity [31]. Although MPO-mediated dysfunctional lipoprotein formation via multiple oxidative mechanisms has been well-established, the role of MPO-mediated carbamylation has only been recently elucidated.

The mechanism for MPO-dependent protein carbamylation uses its physiological substrate the pseudohalide SCN⁻ [3]. Specifically, SCN⁻ and H₂O₂ serve as co-substrates for protein carbamylation via MPO (Figures 1B and 1C). Plasma SCN⁻ levels can differ and are highly dependent on environmental factors, including dietary intake and tobacco exposure, as well as existing vascular disease states [74]. The mechanism established for MPO-mediated carbamylation suggests that protein carbamylation by cyanate may be driven not only by uraemia, but also by inflammation. Recent studies have convincingly implicated MPO as the uraemia-independent enzymatic catalyst of LDL carbamylation in vivo, transforming it into a high-uptake form for macrophages leading to numerous atherogenic consequences [3,75].

In contrast with a significant body of research that documents the pro-atherogenic consequences of LDL modification, i.e. carbamylation or oxidation, there is a notable gap in current understanding of the molecular basis of dysfunctional HDL formation.

**HDL as a Target for MPO**

The anti-atherogenic effects of HDL in cholesterol metabolism and transport are well-established [76,77], in which HDL accepts cholesterol from lipid-laden macrophage foam cells in the arterial wall and transports it back to the liver for excretion, a process termed ‘reverse cholesterol transport’. Cholesterol efflux occurs via ABCA1, a membrane-associated transporter, and is facilitated by lipid-free apoAI, the major HDL protein [76]. In contrast, ABCG4 and ABCG1 mediate cholesterol trafficking from cells to mature HDL particles.

A great deal of experimental evidence supports the role of MPO-induced HDL chlorination and oxidation in the (i) impairment of cholesterol efflux by the ABCA1 pathway, and (ii) subsequent formation of dysfunctional pro-inflammatory HDL [31]. Although these findings suggest a pathway for dysfunctional HDL formation, understanding of this complex process is decidedly superficial, owing to, until recently, the lack of crystal or solution structures for the full-length lipid-free form of apoAI [78,79].

Solutions to the crystal structure of full-length lipid-free apoAI and nascent discoidal HDL [78,79] provide novel insights into potential processes involved in the transformation of HDL into a dysfunctional form that has markedly pro-inflammatory effects. ApoAI is remarkable in that the overall structure contains two negatively charged patches on the protein’s surface and two helical domains: an N-terminal four-helix bundle (three-quarters of apoAI) and a C-terminal domain that is a two-helix bundle.

Previous studies have shown that acrolein, a reactive α,β-unsaturated carbonyl generated by MPO, renders HDL dysfunctional by the potent inhibition of cholesterol efflux via the ABCA1 pathway [80]. Inhibition of activity was strongly associated with modification of Lys^236 of apoAI, thereby putatively disordering the negative patch at the C-terminus of apoAI. On the basis of the structure of apoAI, it is postulated that alterations in the distribution of surface charge are significant because negatively charged regions of apoAI promote interactions between itself and ABCA1 [81]. Interestingly, the binding of isocyanic acid to ε-lysyl residues can lead to a loss of protein function by altering the overall surface charge distribution [6]. Furthermore, it is noteworthy that Lys^236 resides in loop 10 of lipid-free apoAI and...
plays a vital role in the lipid-mediated conformational switch in the C-terminus of apoAI [79].

In the context of recent structural information on HDL, the lipoprotein has numerous potential targets for carbamylation by an MPO-driven pathway. On the basis of experimental findings demonstrating Lys226 of apoAI as a target for MPO products [80], structural data on the accessibility and conformational role of the residue [79,81] and its susceptibility as an ideal nucleophilic amino acid residue target of isocyanic acid [3,6], it is hypothesized that MPO-induced carbamylation of apoAI, especially at Lys226, alters the remodelling pathway of apoAI, generating dysfunctional HDL that has inflammatory effects.

**CLINICAL RELEVANCE**

The direct and indirect consequences of MPO expression by macrophages at the site of atherosclerotic lesions can be explored for both therapeutic and diagnostic uses. At the present time, most studies have focused on the diagnostic predictive value of MPO and MPO-generated reactive intermediates. Serum levels of MPO are commonly used as quantitative indicators of inflammation and have been correlated with CVD risk [82,83]. Several studies now support the measurement of MPO levels in the risk assessment and stratification of patients with ACS [82]. Compared with healthy controls, plasma MPO concentrations are significantly increased in patients presenting with ACS, with or without evidence of myonecrosis at initial presentation. Clinical studies also demonstrate that patients with higher MPO levels early after an ACS event have an associated greater morbidity and mortality [20,21], highlighting the strong relationship between MPO and the risk of ischaemic recurrence that is independent of other risk factors. MPO measurements as an early diagnostic tool in ACS patients has also surpassed more traditional clinical biomarkers, such as BNP (brain natriuretic peptide) and CRP, which are linked more to risk of mortality and not ischaemic recurrence. In one study, an MPO value above a specified normal threshold was significantly predictive of the risk of death or recurrent ischaemic events at 30 days [odds ratio, 1.7 (95% confidence interval, 1.2–2.3); \( P = 0.003 \)] [83]. However, much work is still needed to optimize the prognostic utility of MPO in terms of long-term clinical outcome. In addition to the measurement of MPO levels, the reactive intermediates produced by MPO or their modified targets themselves can also be of diagnostic significance. For instance, pHA (p-hydroxyphenylacetaldehyde) is an aldehyde formed by exposure of free tyrosine residues to the MPO/H\(_2\)O\(_2\)/Cl\(^-\) system and is being explored in tissues as a marker of MPO-catalysed amino acid oxidation in vivo [84–87].

Modified lipoproteins have also been employed as biomarkers that are positively correlated with the development of atherosclerosis and CVDs. Carbamylated lipoproteins are being investigated for their diagnostic and prognostic utility. cLDL can be quantified by colorimetric techniques; however, this requires isolation by ultracentrifugation and is needlessly expensive and time consuming [88]. Immunoassays using specific antibodies in a sandwich ELISA have been developed that can be performed in a clinical laboratory [89] and are capable of measuring carbamylated lipoproteins in human serum. Additionally, serum levels of the pseudohalide anion SCN\(^-\), a co-substrate of the MPO/H\(_2\)O\(_2\)/SCN\(^-\) system, are positively correlated with smoking and are also dependent on diet. SCN\(^-\) levels are, in fact, often used to measure primary and secondary exposure to smoke in situations where cotinine levels are not dependable [90]. Despite its indispensable role in the ureaemia-independent carbamylation of proteins, however, elevated SCN\(^-\) levels alone are not predictive of atherosclerosis [3], implying a localized requirement of the entire MPO/H\(_2\)O\(_2\)/SCN\(^-\) system, i.e. in the vessel wall. In support, clinical studies have demonstrated that protein-bound HCit concentrations are positively predictive of CAD or a future major adverse cardiac event \( (P < 0.001) \), highlighting the potential prognostic value of protein carbamylation to predict atherosclerotic CVD risks in both smokers and non-smokers [3]. This strong association between CVD risk and elevated protein-bound HCit levels is independent of other CVD risk factors and renal disease, since the relationship remained after multi-logistic regression analysis following adjustments for traditional CVD risk factors, estimated glomerular filtration rate and CRP levels.

**SMOKING AND DIET–ENVIRONMENTAL INFLUENCES ON MPO-CATALYSED PROTEIN CARBAMYLLATION**

Diet and smoking are significant determinants of plasma SCN\(^-\) concentration and, hence, serve as modifiable risk factors for impaired lipoprotein metabolism and atherosclerosis via increased MPO-mediated protein carbamylation [3]. Neutrophil MPO utilizes the co-substrates SCN\(^-\) and H\(_2\)O\(_2\) in the cyanate-forming reaction that triggers inflammatory site-specific protein carbamylation, i.e. in the atherosclerotic plaque. The interaction of environmental risk factors in a defined chemical reaction offers unique mechanistic insight into the proposed association between smoking, diet, and inflammation.

Dietary influences on plasma SCN\(^-\) concentration include dairy products, almonds, cruciferous vegetables and certain fruits. Cruciferous vegetables are rich in glucosinolates, sulfur-containing compounds that are hydrolysed by myrosinase plant enzymes, resulting in the formation of biologically active indoles and isothiocyanates [74,91].
Smoking has long been regarded as a risk factor for cardiovascular events and is corroborated by results that indicate a positive correlation between smoking and cardiovascular morbidity, including increased risk of MI and sudden cardiac death [92]. Nonetheless, the precise mechanism by which smoking contributes to CVD has remained elusive. Some of the proposed mechanisms have implicated coronary endothelial dysfunction [93] and increased inflammatory responses as key consequences, without identifying a unique molecular pathway. For instance, one study hypothesized that smoking may be associated with decreased NO biosynthesis [94]. A recent study [95] demonstrated that smoking is associated with epicardial coronary endothelial dysfunction and increased levels of inflammatory biomarkers and oxidative stress, including MPO (156 ± 19 ng/ml in smokers compared with 89 ± 8 ng/ml in non-smokers). Endothelial dysfunction is considered an early atherogenic event that compromises the integrity of the barrier between VSMCs and the blood, which can thereby precipitate plaque formation and progression (Figure 2) [96–98]. Smoking has been linked to endothelial dysfunction in both the peripheral [99] and coronary [100] circulation, and is a positive predictor of long-term cardiovascular events [101,102].

A potentially unifying molecular understanding of the association between smoking, MPO and atherosclerosis has recently emerged [3]. Other studies have shown that both MPO and smoking have each been independently linked to coronary artery endothelial cell apoptosis, a critical event in the pathogenesis of atherosclerosis and an identified consequence of lipoprotein carbamylation (Figure 2) [103,104]. The hypothesis that MPO-mediated protein carbamylation may serve as the underlying pathway that mechanistically links smoking with atherosclerosis is supported further by recent clinical epidemiological studies that demonstrate that plasma levels of protein-bound HCit serve as independent predictors of increased CVD risk [3].

Specifically, in two clinical trials (n = 1000), subjects with CVD, compared with controls, had significantly higher concentrations of plasma protein-bound HCit and, reciprocally, levels of protein-bound HCit were significantly and independently associated with the frequency of CVD, including both CAD and peripheral arterial disease. Furthermore, subjects with plasma protein-bound HCit ≥ 300 μmol of HCit/mol of lysine had almost an order of magnitude greater risk of clinical/angiographic evidence of CVD compared with subjects with ≤ 30 μmol of HCit/mol of lysine. Smokers had a significant 3-fold increase in plasma SCN⁻ levels compared with non-smokers (median levels, 158.4 compared with 46.9 μmol/l respectively) [3].

Alternative theories for the vascular inflammatory consequences of smoking have focused on findings that smoking is associated with increased oxidative stress [105], blood thrombogenicity [106] and inflammatory responses [107], which form the trifecta for endothelial dysfunction [108]. An increased WBC (white blood cell) count, a general indicator of systemic inflammation, was observed in smokers with progressive atherosclerosis [109], and was independently associated with a greater risk of cardiovascular events [110], lending further credence to the oxidative stress/inflammation hypothesis.

Interestingly, an MPO genetic polymorphism that results in reduced MPO activity, i.e. 463G → A, results in attenuated smoking-induced inflammatory and carcinogenic sequelae [111,112]. Smoking causes a local inflammatory response in the lung, resulting in the massive recruitment and activation of neutrophils [113], leading to the release of MPO [114]. Several epidemiological studies have demonstrated that decreased MPO expression results in a reduced risk in several human cancers [115], including lung [116–118], ovarian [119], bladder [120], liver [121] and breast [122] cancer in some populations. Taken cumulatively, these findings suggest that the inflammatory consequences of smoking are, at least in part, due to the SCN⁻ by-product of tobacco metabolism that is used by MPO to carbamylate normal proteins and, thereby, effect their transformation into ‘corrupted’ proatherogenic and inflammatory/carcinogenic mediators.

### PHARMACOLOGICAL REGULATION OF MPO

Previous studies have demonstrated that statins strongly inhibit MPO gene expression levels in human and murine macrophages by inhibiting the production of isoprenoid intermediates of the HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) pathway [123]. Statins are inhibitors of HMG-CoA reductase, thereby preventing the conversion of HMG-CoA into mevalonate, the rate-limiting step leading to cholesterol synthesis. Geranylgeranylation of proteins, inhibited by statins, has been demonstrated to be important in MPO gene expression [123]. That study showed that both natural and synthetic statins strongly suppress human and murine MPO gene expression, with a reduction in mRNA levels of approx. 20–200-fold in monocyte/macrophages and bone marrow precursors. This observation suggests that statin-mediated suppression of MPO expression is mediated by common promoter elements. Additionally, other studies have demonstrated that the effects of statins are not solely limited to altered leucocyte expression of MPO at the promoter level. Specifically, Shishehbor et al. [124,125] have shown that, following statin therapy, there is a corresponding decrease in systemic levels of MPO-generated protein oxidative products.

Therefore the anti-inflammatory pleiotropic benefits of statins, independent of their effect on LDL levels, may be attributable to the suppression of MPO expression. Building on these observations, several studies are...
MPO-mediated protein carbamylation, driven by cyanate formation from thiocyanic acid and $H_2O_2$, represents a potential molecular pathway that links smoking, diet, vascular inflammation and CVD risk. Several areas of investigation are required to fully understand the significance of this reaction, including: (1) understanding the structural and conformational consequences of HDL carbamylation (cHDL formation), (2) demonstrating that cHDL is formed in vivo in atherosclerotic lesions, (3) exploring the molecular functional significance of apoAI carbamylation, and (4) dissecting the molecular mechanisms linking enhanced CVD risk with tobacco use and specific diets. RCT, reverse cholesterol transport.

underway focusing on determining whether statins are capable of inhibiting the formation of dysfunctional HDL and its deleterious effects.

CONCLUSIONS AND OUTLOOK

Evidence suggests that MPO-induced HDL chlorination, oxidation and, possibly, carbamylation are critical in the (i) impairment of cholesterol efflux by the ABCA1 pathway, and (ii) subsequent formation of dysfunctional pro-inflammatory HDL. On the basis of exciting findings that LDL carbamylation by MPO is a mechanistically as well as quantitatively dominant form of modified LDL physiologically [3], it is anticipated that MPO may also serve as a novel mechanistic paradigm for dysfunctional HDL formation. In order to clearly establish the clinical relevance and functional molecular consequences of HDL carbamylation, however, several critical developments must take place (Figure 3). First, it is essential to establish the molecular context for apoAI carbamylation by the MPO/$H_2O_2$/SCN$^-$ system. Although Wang [3] demonstrated that MPO/carbamylation of apoAI in vivo and in atherosclerotic lesions

4) Dissect the molecular mechanisms linking enhanced CVD risks with tobacco use and specific diets.

2) Demonstrate MPO carbamylation of apoAI in vivo and in atherosclerotic lesions

3) Functional significance of apoAI carbamylation

Vascular Smooth Muscle Cells (VSMCs)
et al. [3] quantified in vitro MPO/H\textsubscript{2}O\textsubscript{2}/SCN\textsuperscript{−}-mediated carbamylation of apoAI of HDL using LC (liquid chromatography)/ESI (electrospray ionization)/MS/MS (tandem MS)-based methods, the functional significance of the reaction should be examined in the context of the recently elucidated structure of HDL. Specifically, in order to establish the importance of MPO carbamylation in dysfunctional HDL formation, it is necessary to directly test the hypothesis that MPO induces the carbamylation of apoAI at lysine residues of known functional importance. Finally, to establish the clinical relevance of apoAI carbamylation, apoAI should also be identified as a carbamylated protein in serum from patients with CVD.

The second developmental task is to examine whether apoAI carbamylation is dependent on MPO in atherosclerosis. Such an experiment, however, is prefaced on the hypothesis that MPO-induced carbamylation of apoAI is mechanistically linked to the development of observable atherosclerotic lesions. As multiple proteins are subject to MPO-induced carbamylation, further studies will be essential to address the specific functional consequences of apoAI carbamylation.

The third critical developmental task is to fully characterize the functional significance of MPO-driven apoAI carbamylation. To substantiate a claim of the atherogenic importance of apoAI carbamylation, it is crucial to demonstrate that MPO-induced apoAI carbamylation impairs ABCA1-dependent cholesterol efflux and potentiates other pro-atherogenic events, i.e. vascular smooth muscle proliferation and aortic endothelial cell apoptosis.

The fourth and final task is to scrupulously examine the impact of smoking and dietary influences on MPO-mediated protein carbamylation. Wang et al. [3] set the stage for such future work by demonstrating the potential prognostic utility of systemic protein carbamylation measures as a gauge of atherosclerotic CAD risks in both smokers and non-smokers. Clinical epidemiological studies should address the predictive utility of measures of systemic protein-bound H\textsubscript{2}Cit concentrations as an indicator of overall reductions in vascular inflammation and CVD risks accompanying smoking cessation. It is expected that future work in this area will dissect the underlying mechanisms that contribute to the enhanced cardiovascular risks associated with tobacco use and specific diets.

MPO-mediated protein modification has already been identified as a significant mechanism for LDL carbamylation and oxidation, and its subsequent transformation into potent pro-atherogenic molecules under physiologically relevant conditions. In the near future, it is likely that HDL and, more specifically, dysfunctional HDL formation will play an increasingly important role in our understanding of the complexities of atherogenesis and will yield extensive insight into the development of future HDL-targeted therapeutics. Additionally, the potential dual role of MPO carbamylation of both LDL and HDL, resulting in a synergistic promotion of atherosclerosis, may lead to a better understanding of current drugs (i.e. statins) and the development of therapeutics specifically targeted toward decreasing the deleterious effects of MPO expression.

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