

Myeloperoxidase-Derived Chlorinating Species Induce Protein Carbamylation Through Decomposition of Thiocyanate and Urea: Novel Pathways Generating Dysfunctional High-Density Lipoprotein

Michael Holzer,¹ Klaus Zangger,² Dalia El-Gamal,¹ Veronika Binder,¹ Sanja Curcic,¹ Viktoria Konya,¹ Rufina Schuligoi,¹ Akos Heinemann,¹ and Gunther Marsche¹

Abstract

Aims: Protein carbamylation through cyanate is considered as playing a causal role in promoting cardiovascular disease. We recently observed that the phagocyte protein myeloperoxidase (MPO) specifically induces high-density lipoprotein (HDL) carbamylation, rather than chlorination, in human atherosclerotic lesions, raising the possibility that MPO-derived chlorinating species are involved in cyanate formation. **Results:** Here, we show that MPO-derived chlorinating species rapidly decompose the plasma components thiocyanate (SCN) and urea, thereby promoting (lipo)protein carbamylation. Strikingly, the presence of physiologic concentrations of SCN completely prevented MPO-induced 3-chlorotyrosine formation in HDL. SCN scavenged a 2.5-fold molar excess of hypochlorous acid, promoting HDL carbamylation, but not chlorination. Cyanate significantly impaired (i) HDL's ability to activate lecithin-cholesterol acyltransferase; (ii) the activity of paraoxonase, a major HDL-associated anti-inflammatory enzyme; and (iii) the antioxidative activity of HDL. **Innovation:** Here, we report that MPO-derived chlorinating species preferentially induce protein carbamylation—rather than chlorination—in the presence of physiologically relevant SCN concentrations. The carbamylation of HDL results in the loss of its anti-inflammatory and antioxidative activities. **Conclusion:** MPO-mediated decomposition of SCN and/or urea might be a relevant mechanism for generating dysfunctional HDL in human disease. *Antioxid. Redox Signal.* 17, 1043–1052.

Introduction

RECENT STUDIES SUPPORT the hypothesis that protein carbamylation is a pathway that is intrinsic to inflammation and the pathophysiology of atherosclerosis (15, 36). Proteins are carbamylated through cyanate, a reactive species that targets lysine residues to form ϵ -carbamyllysine (homocitrulline [HCit]). Cyanate can also interact with cysteine or histidine groups with an even higher reactivity, but in a reversible manner (33, 34). Humans may be exposed to exogenous cyanate, resulting from the pyrolysis/combustion of tobacco, coal, or biomass (28). Endogenously, cyanate is formed by the slow breakdown of urea and myeloperoxidase (MPO)-catalyzed oxidation of thiocyanate (SCN) (2, 36). SCN is a major physiological substrate of MPO that accounts for approximately 50% of hydrogen peroxide (H_2O_2) consumed by MPO (35). Plasma SCN levels vary according to dietary intake, with plasma levels in the range of 20–100 μM (25).

Plasma SCN levels in smokers are substantially higher (16). At plasma concentrations of SCN and chloride, MPO is far from being saturated; hence, MPO catalyzes the oxidation of SCN and chloride simultaneously (35).

Recent findings indicate that cyanate amplifies vascular inflammation, linking it to uremia and smoking (10). Leukocyte-derived MPO may serve as an important catalytic source for protein carbamylation at sites of inflammation, based on the observation that protein carbamylation during peritonitis was markedly reduced in MPO-deficient mice (36). Moreover, carbamylated epitopes were found to colocalize with MPO in human atherosclerotic lesions (36).

We have recently reported that high-density lipoprotein (HDL) is selectively carbamylated in human atherosclerotic lesions (15). The carbamyllysine content of lesion-derived HDL correlated with the MPO-specific oxidation product 3-chlorotyrosine (3-CT), strongly supporting the concept that leukocyte-derived MPO generates significant amounts of

¹Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Graz, Austria.

²Institute of Chemistry/Organic and Bioorganic Chemistry, University of Graz, Graz, Austria.

Innovation

The carbamylation of (lipo)proteins through cyanate generates novel risk factors for cardiovascular disease and is considered as playing a causal role in disease progression. We have recently observed that the phagocyte protein myeloperoxidase (MPO) preferentially induces high-density lipoprotein (HDL) carbamylation, rather than chlorination, in human atherosclerotic lesions, raising the possibility that MPO-derived chlorinating species are involved in cyanate formation.

Here, we report that MPO-derived reactive chlorinating species promote the carbamylation of proteins through the decomposition of urea and thiocyanate (SCN). Physiological concentrations of SCN prevented 3-chlorotyrosine (3-CT) formation in proteins, suggesting that MPO preferentially induces protein carbamylation—rather than chlorination—at sites of inflammation. Urea levels can reach 100 mM in patients with chronic renal failure; hence, an augmented decomposition of urea may generate high yields of cyanate. Significantly, elevated MPO activity was recently demonstrated in patients with inflammation undergoing hemodialysis, supporting the association between inflammation and cumulative oxidative stress. Moreover, here, we demonstrate that minimal carbamylation of HDL significantly impairs (i) HDL's ability to activate lecithin-cholesterol acyltransferase, an enzyme that is essential for cholesterol esterification and HDL maturation; (ii) the activity of the major HDL-associated anti-inflammatory enzyme paraoxonase; and (iii) the antioxidative capability of HDL. Thus, the carbamylation of HDL in the atherosclerotic intima might critically hamper the antioxidant and anti-inflammatory properties of HDL. These data raise the possibility that protein carbamylation links inflammation, uremia, and cardiovascular disease.

cyanate at sites of inflammation. These findings are in line with the observations that (i) MPO associates with HDL in atherosclerotic lesions (6, 39); (ii) MPO/HDL interaction increases on the oxidation of HDL; and (iii) association of MPO with HDL does not alter MPO activity (22). Interestingly, the carbamyllysine content of lesion-derived HDL is several-fold higher compared with 3-CT levels (15), raising the possibility that MPO-generated chlorinating species are involved in cyanate formation.

In the present study, we propose a role for MPO-derived chlorinating species in mediating the rapid decomposition of urea and SCN, as a novel mechanism inducing (lipo)protein carbamylation. Moreover, we describe that MPO-mediated cyanate formation might be an important mechanism in generating dysfunctional HDL.

Results

MPO predominantly generates carbamyllysine in the presence of SCN

HDL isolated from human atherosclerotic lesions is mainly carbamylated, rather than chlorinated (Fig. 1A), raising the possibility that in the presence of SCN, MPO-induced protein carbamylation is favored compared with chlorination. Carbamyllysine was detected on lesion-derived apolipoprotein

A-I (apoA-I) by immunoblotting using an anti-HCIt-antibody, but not on HDL isolated from peripheral blood (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ars).

We first sought to investigate whether SCN could alter MPO-induced 3-CT formation in HDL. The incubation of MPO in the presence of physiological or pathological concentrations of SCN (50 or 250 μ M, respectively), H₂O₂ (200 μ M), and chloride (100 mM) significantly induced carbamyllysine formation, whereas 3-CT formation was not observed (Fig. 1B). When a higher excess of H₂O₂ (400 μ M) than SCN (50 μ M) was used, carbamyllysine formation increased further and, as expected, the formation of 3-CT was also observed (Fig. 1C). When SCN was removed from the reaction mixtures, 3-CT formation was prominent (Fig. 1B, C). In the absence of MPO or H₂O₂, neither carbamyllysine nor 3-CT formation was observed.

Hypochlorous acid induces decomposition of SCN and causes protein carbamylation

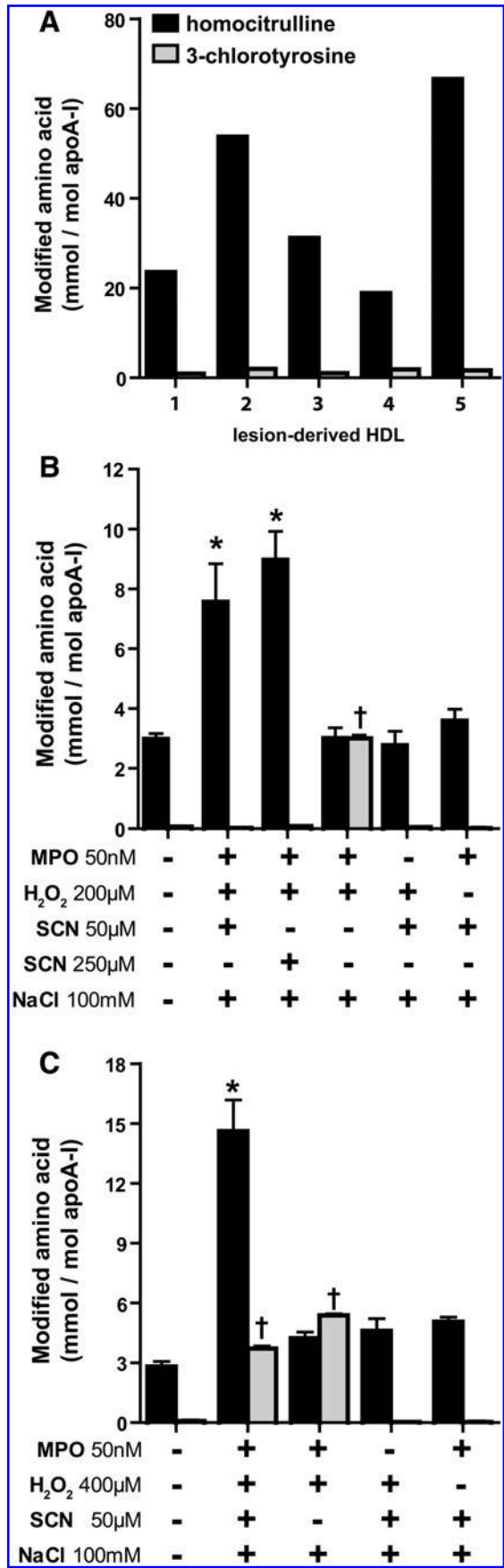
The lack of significant 3-CT formation indicates that SCN is a preferred substrate for MPO and is particularly effective in scavenging reactive chlorinating species, as demonstrated in previous studies (3, 24, 27, 37). Remarkably, when 100 μ M hypochlorous acid (HOCl) was added to 30% plasma containing SCN (interstitial fluids contain about 30%–40% of plasma proteins) (30, 32), carbamyllysine formation was observed, whereas 3-CT formation was not increased (Fig. 2A). When HOCl was added at a higher concentration, the carbamyllysine formation further increased, depending on the SCN concentration. Of note, HOCl added at higher concentrations induced 3-CT formation, even in the presence of SCN (Fig. 2A).

Similar results were obtained when HDL was incubated with SCN and HOCl in the absence of serum. Under these experimental conditions, 3-CT formation was completely prevented by SCN (Fig. 2B). The addition of HOCl in the absence of SCN induced 3-CT formation, as expected.

To estimate the HOCl-scavenging potential of SCN, a fixed amount of SCN was incubated with increasing concentrations of HOCl. We observed that SCN completely scavenged a 2.5-fold molar excess of HOCl (Fig. 2C). This indicates that over-oxidized products such as cyanosulfite or sulphate are formed on the oxidation of SCN with an excess of HOCl or chloroamines, as suggested in a recent study (37). These data indicate that only in the absence of adequate SCN, the overproduction of HOCl by MPO might result in relevant protein chlorination.

Cyanate formation through HOCl-induced decomposition of urea

Besides the formation of cyanate from SCN oxidation, cyanate is known to be formed by the slow decomposition of urea. It is well known that excess HOCl decomposes urea, forming nitrogen and carbon dioxide. We tested whether cyanate is formed as a byproduct. For that purpose, we investigated the decomposition of urea by HOCl using solution-state nuclear magnetic resonance (NMR) spectroscopy, using a 1:0.5 molar ratio of ¹³C, ¹⁵N-labeled urea: HOCl. The reaction was monitored by inverse gated decoupling ¹³C NMR spectra. On the addition of HOCl, the signal of urea (at 162.8 ppm)



decreased within the dead time of the experiment (3 min), and peaks for carbonate and cyanate appeared at 168.5 and 128.7 ppm, respectively (Fig. 3A). Longer incubations did not further increase the signal intensity of cyanate, indicating the rapid formation of cyanate. About 25% of urea is converted to cyanate, demonstrating that high yields of cyanate are formed on the HOCl-induced decomposition of urea. The addition of HOCl (100 μ M) to urea levels observed in control subjects (5 mM) induced significant protein carbamylation and 3-CT formation. Notably, in the presence of high urea concentrations (50 mM, observed in renal patients), the addition of HOCl significantly amplified protein carbamylation, but did not result in 3-CT formation, whereas the addition of HOCl alone induced only 3-CT formation (Fig. 3B). High urea concentrations on their own tended to induce protein carbamylation, but the extent of carbamylation significantly increased only in the presence of HOCl. Based on these results, it can be concluded that MPO-derived chlorinating species induce (lipo)protein carbamylation through the oxidation of SCN and/or the rapid decomposition of urea.

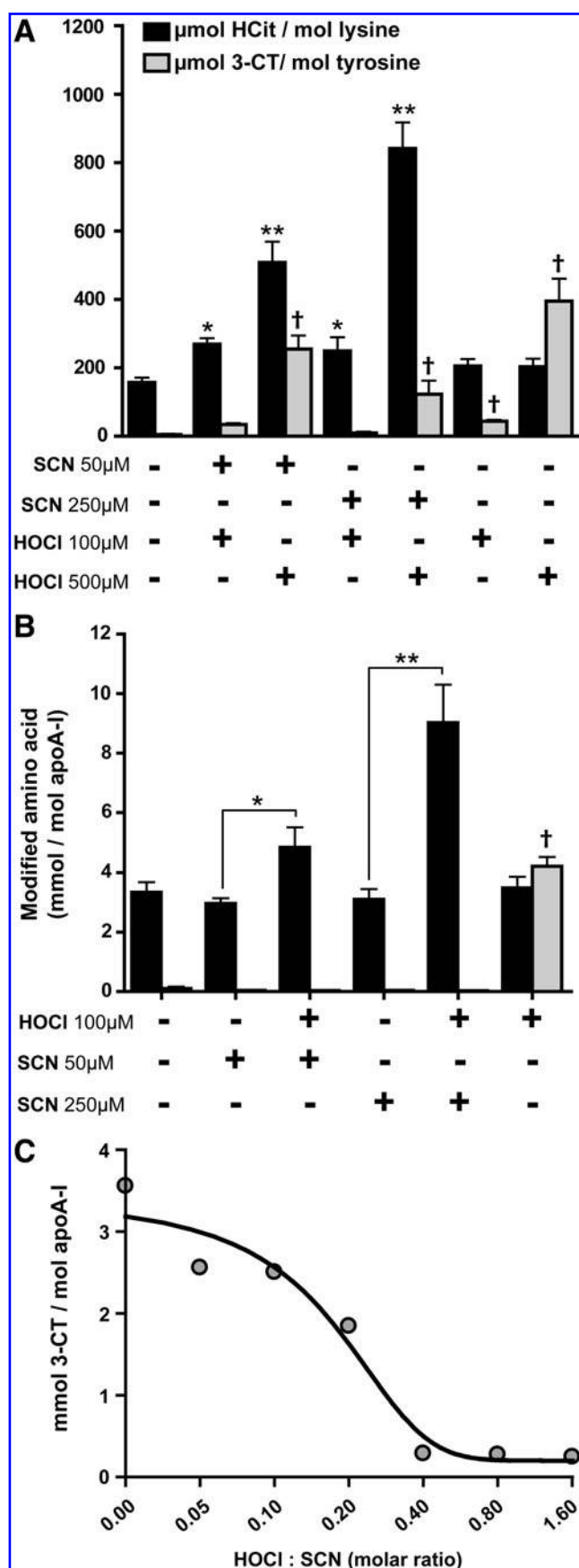
Carbamylated HDL is a less potent substrate for lecithin-cholesterol acyltransferase

Lecithin-cholesterol acyltransferase (LCAT) drives the maturation of nascent HDL into spherical HDL, an essential step in reverse cholesterol transport. To assess whether carbamylation of HDL alters LCAT activity, a commercially available assay for serum/plasma LCAT activity was used to measure the efficiency of different HDL preparations to activate LCAT. For that purpose, HDL was labeled with a fluorescent LCAT substrate, which changes its fluorescence emission wavelength from 390 to 460 nm on hydrolysis by LCAT. The labeled substrate was rapidly incorporated into HDL (Supplementary Fig. S2). The labeled HDL preparations were incubated with a fixed amount of lipoprotein-deficient serum (LPDS) as a source for LCAT. As seen in Figure 4, carbamylation dose dependently decreased the ability of HDL to activate LCAT.

Carbamylation reduces the antioxidant properties of HDL and decreases the activity of HDL-associated paraoxonase

Besides the important role of HDL in lipid metabolism, it also exhibits unique antioxidative activity. We investigated the ability of HDL to inhibit radical-induced low-density lipoprotein (LDL) oxidation. LDL was incubated with the

FIG. 1. Myeloperoxidase (MPO)-promoted thiocyanate (SCN) decomposition induces high-density lipoprotein (HDL) carbamylation, but not 3-chlorotyrosine (3-CT) formation. (A) HDL isolated from five atherosclerotic lesions was analyzed for its homocitrulline (HCit) and 3-CT content using liquid chromatography tandem mass spectrometry (LC-MS/MS). (B) Reaction samples contained 2.5 mg/ml HDL, 50 nM MPO, 200 μ M hydrogen peroxide (H₂O₂), 100 mM NaCl, and SCN at 50 or 250 μ M. (C) Reaction samples contained 2.5 mg/ml HDL, 50 nM MPO, 400 μ M H₂O₂, 100 mM NaCl, and 50 μ M SCN. Reactions were allowed to take place for 48 h at 37°C. Unreacted compounds were removed by gel filtration. HCit and 3-CT were assessed using LC-MS/MS. * p < 0.05, versus HCit; † p < 0.01; versus 3-CT.



radical inducer 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH), and a characteristic oxidation curve with lag time and propagation phase was obtained (Fig. 5A). The oxidation of LDL could be markedly inhibited by the addition of native HDL. However, the ability of HDL to inhibit radical-induced oxidation was significantly decreased on carbamylation (Fig. 5B), which indicates that carbamylation impairs the antioxidative properties of HDL.

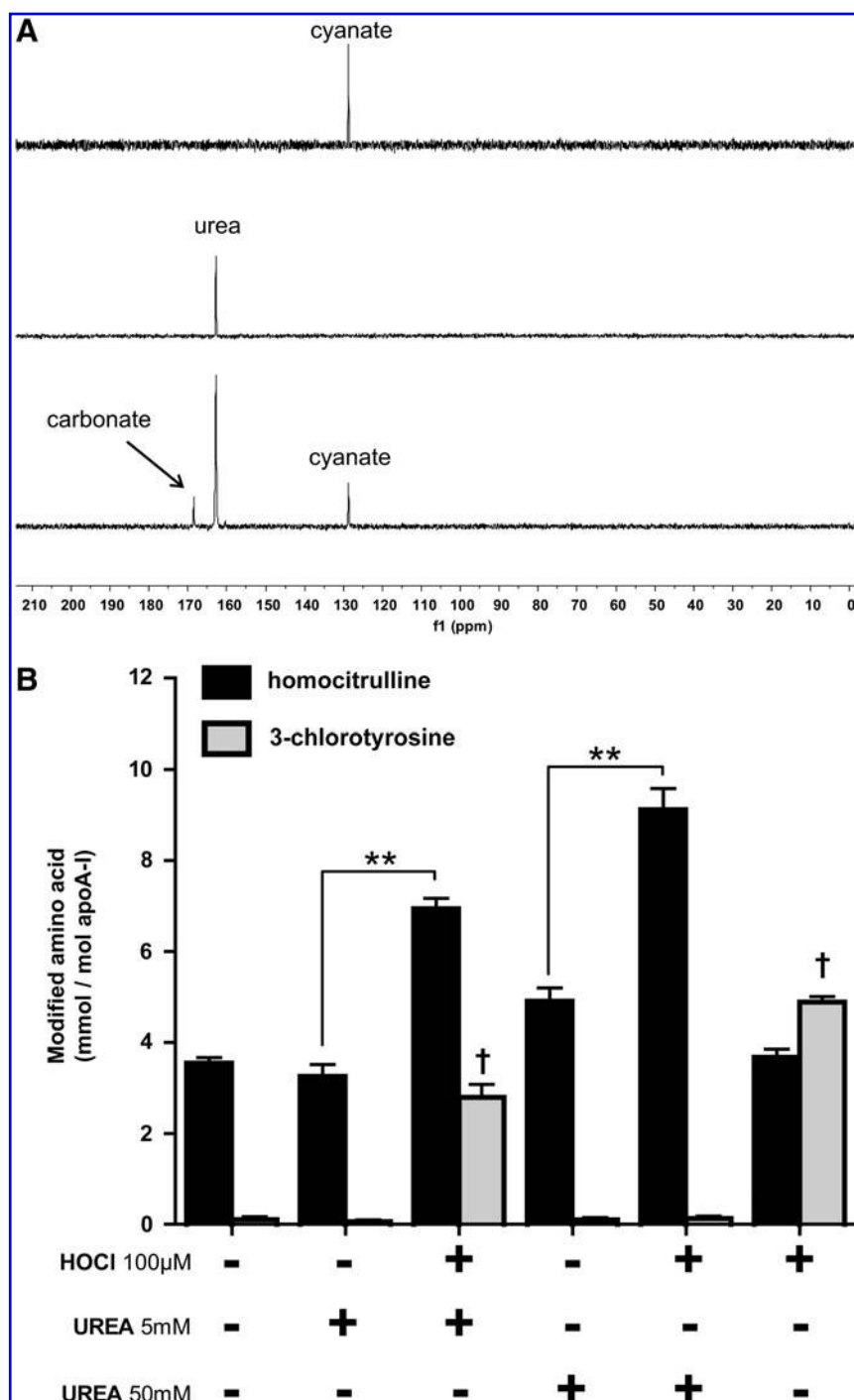
Recent data suggest that the HDL-associated enzymes paraoxonase (PON) and LCAT are involved in the antioxidant activity of HDL (4, 5, 13). We analyzed the impact of protein carbamylation on PON-associated PON and arylesterase activity. Strikingly, when HDL was exposed to cyanate-inducing minimal carbamylation, PON (Fig. 6A) and arylesterase activities (Fig. 6B) of PON were significantly reduced. PON activity further decreased when HDL was modified with higher cyanate concentrations.

Discussion

Carbamylated (lipo)proteins are risk factors for cardiovascular disease and seem to play a causal role in disease progression. It was long thought that cyanate formation occurs to a significant extent only during renal dysfunction through the slow decomposition of urea. Approximately, 0.8% of urea decomposes to cyanate within one week in aqueous solution (8). However, recent studies have unambiguously demonstrated that humans are exposed to additional significant amounts of cyanate formed by the pyrolysis/combustion of coal, biomass, or tobacco (28), and that cyanate formation is catalyzed by the leukocyte hemeperoxidase MPO (2, 36). Here, we provide novel evidence of an alternative mechanism for cyanate formation. MPO-derived chlorinating species rapidly decompose SCN and/or urea, forming cyanate, thereby promoting (lipo)protein carbamylation (Fig. 7). Physiologic concentrations of SCN prevented 3-CT formation in HDL, suggesting that MPO preferentially induces protein carbamylation—rather than chlorination—at sites of inflammation. This is in good agreement with the finding that the carbamyllysine content of lesion-derived HDL was more than 20-fold higher in comparison to 3-CT levels, a specific oxidation product of MPO (15). We quantified that apoA-I recovered from atherosclerotic tissue contains approximately 70 mmol HCit/mol apoA-I (Fig. 1A), indicating that approximately 7% of lesion-derived apoA-I possess at least one HCit residue. Our *in vitro* experiments revealed that one HCit-residue/apoA-I renders HDL dysfunctional; therefore, particularly those HDL in

FIG. 2. Hypochlorous acid (HOCl)-induced decomposition of SCN promotes HDL carbamylation. (A) Human plasma (30%) was incubated in the presence or absence of indicated concentrations of SCN and HOCl for 16 h at 37°C. (B) HDL (2.5 mg/ml) was incubated with 50 or 250 μM SCN in the presence or absence of HOCl (100 μM) for 16 h at 37°C. **p* < 0.05 versus HCit; ***p* < 0.01 versus HCit; †*p* < 0.01; ‡*p* < 0.05 versus 3-CT. (C) Increasing amounts of SCN (5–180 μM) were incubated with 100 μM HOCl for 16 h at 37°C. Unreacted compounds were removed by gel filtration. HDL was hydrolyzed, and HCit and 3-CT content was assessed using LC-MS/MS.

FIG. 3. HOCl-induced rapid decomposition of urea generates cyanate. (A) One-dimensional ^{13}C -nuclear magnetic resonance (NMR) spectra of potassium cyanate (upper panel), of ^{13}C , ^{15}N -labeled urea (162 ppm) (middle panel) and ^{13}C , and ^{15}N -labeled urea incubated with 0.5 molar equivalents of HOCl for 3 min (lower panel). Besides the main product cyanate (128 ppm), carbonate was also detected (160 ppm) by inverse gated decoupling ^{13}C NMR spectra. (B) HOCl-induced decomposition of urea results in protein carbamylation. HDL was incubated with 5 or 50 mM urea in the presence or absence of HOCl (100 μM) for 16 h at 37°C. Unreacted compounds were removed by gel filtration, HCit and 3-CT were assessed using LC-MS/MS. $**p < 0.01$, versus HCit; $^{\dagger}p < 0.01$, versus 3-CT.



macrophage-rich compartments with high MPO activity—but not all HDL in the lesion—are likely to be rendered dysfunctional through carbamylation. It should be noted that more than 10-fold higher 3-CT levels of apoA-I—as observed in our study—were reported when human atherosclerotic tissue was stripped of adventitia before isolating HDL (39), raising the possibility that HDL in subendothelial compartments with high MPO activity may be even more extensively carbamylated.

In line with our observations, it was recently demonstrated that SCN effectively scavenges HOCl and protein-associated chloramines, forming cyanate and sulphate (37). This is in agreement with a study showing that the rate constant for the

reaction of SCN with chloramines is comparable to the rate constants for the best proteinaceous nucleophiles cysteine and methionine (26). Interestingly, hypothiocyanous acid (HOSCN) itself was reported to react even faster with chloramines than SCN (37), likely explaining that in the present study, low concentrations of SCN completely blocked 3-CT formation, as lysine-chloramine formation is reported to be involved in 3-CT formation (9).

Another important observation in the present study is that the major MPO product HOCl decomposes urea, generating high yields of cyanate. Our findings may be of particular relevance, as urea levels can reach 100 mM in patients with chronic renal failure, and elevated MPO activity was

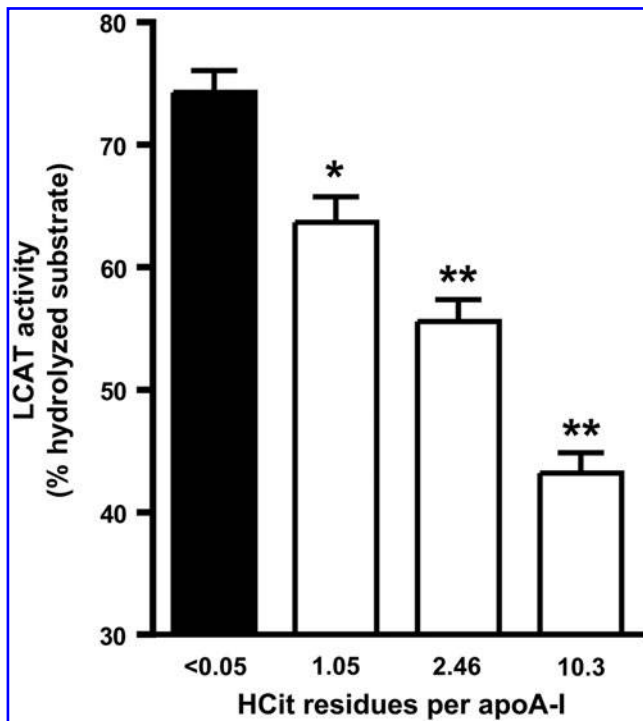


FIG. 4. Carbamylation decreases the ability of HDL to activate lecithin-cholesterol acyltransferase (LCAT). Native or carbamylated HDL preparations (100 $\mu\text{g}/\text{ml}$) were labeled with a fluorescent LCAT substrate and incubated for 5 h at 37°C with lipoprotein-deficient serum as a source of LCAT. The change in LCAT substrate fluorescence was used to assess LCAT activity. Results represent the mean of duplicate determinations \pm SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

demonstrated in patients undergoing hemodialysis with high-grade persistent inflammation (29). Therefore, the increased decomposition of urea may generate high yields of cyanate at sites of inflammation. In line with our data, it was previously reported that MPO-containing neutrophils and monocytes of patients with chronic renal disease are highly enriched with carbamylated proteins compared with control subjects (20). The localization of phagocytes in the immediate vicinity of endothelial cells at sites of inflammation may contribute to the cyanate-induced expression of endothelial intercellular cell adhesion molecule-1, as recently observed (10). This raises the possibility that cyanate increases endothelial-leukocyte interaction, thus forming a vicious circle. In this regard, it was shown that serum MPO levels correlate with levels of inflammatory markers and with mortality in patients undergoing hemodialysis (17).

In line with our previous results (15), we observed that carbamyllysine levels are markedly elevated in HDL isolated from human atherosclerotic tissue. These results are in line with previous findings that MPO binds to HDL within human atherosclerotic lesions and might produce reactive species in closest proximity to HDL (6, 15, 23, 39). Interestingly, HDL derived from atherosclerotic lesions was mainly carbamylated—rather than chlorinated—suggesting that MPO-generated chlorinating species are involved in cyanate formation.

In the present study, we report that the carbamylation of HDL significantly impairs its ability to activate LCAT, an enzyme that is essential for cholesterol esterification and maturation of HDL. apoA-I is recognized as a major activator of LCAT in HDL; however, the mechanism by which apoA-I facilitates cholesterol esterification and particle maturation remains unclear. Previous studies have shown that the mutation of charged residues in apoA-I results in marked reductions of LCAT activity, indicating that the elimination of

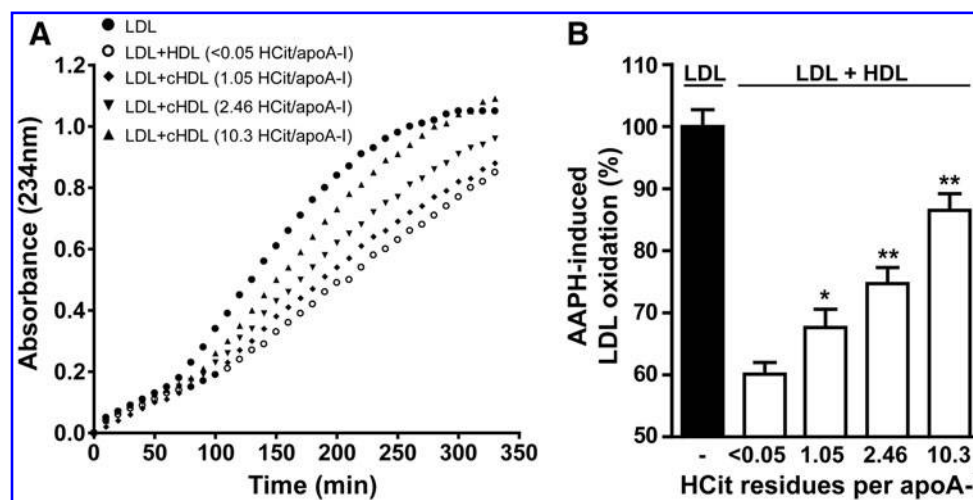
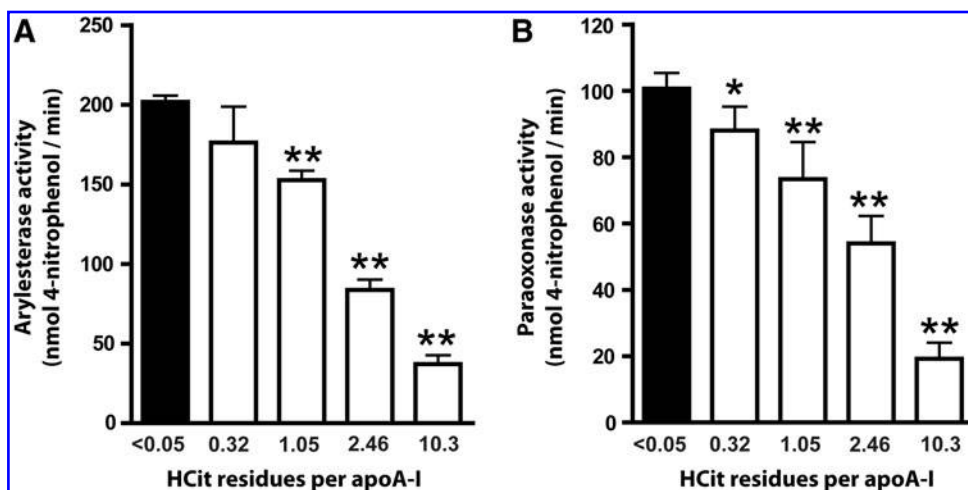


FIG. 5. Carbamylation decreases the ability of HDL to prevent low-density lipoprotein (LDL) oxidation. LDL (100 $\mu\text{g}/\text{ml}$) was incubated with 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) (a free radical inducer) in the presence or absence of HDL (100 $\mu\text{g}/\text{ml}$). (A) A representative time course of an AAPH-induced oxidation process is monitored at 234 nm for 350 min. (B) One time point in the propagation phase (200 min) was selected for quantification. Oxidation in the presence of HDL was calculated as a relative value to the oxidation of LDL alone, which was set to 100%. Results represent the mean of triplicate determinations \pm SEM of three independent experiments.

FIG. 6. Carbamylation decreases activity of HDL-associated paraoxonase (PON). (A) Arylesterase activity of HDL-associated PON was measured using phenylacetate as substrate (HDL, 20 $\mu\text{g}/\text{ml}$). (B) PON activity of HDL-associated PON was measured using paraoxon as substrate (HDL, 50 $\mu\text{g}/\text{ml}$). The PON and arylesterase activities of PON were calculated from the slopes of three independent experiments and were expressed as means \pm SEM. * $p < 0.05$; ** $p < 0.01$.



positive charge through the carbamylation of apoA-I lysine residues may cause reduced LCAT activity (1). Significantly, cyanate targets not only lysine residues, but also phosphatidylethanolamine (15) and interacts with cysteine groups with an even higher reactivity (34). Therefore, the decrease in the LCAT-activating capability of cyanate-treated HDL might be the result of the modification of several reactive groups in HDL-associated apoproteins, enzymes, and phospholipids.

In a previous study, we observed that carbamylation increases the binding affinity of HDL to its physiological receptor, scavenger receptor B-I (SR-BI), thereby causing intracellular cholesterol accumulation and lipid-droplet formation in macrophages (15). According to our present study, these data raise the possibility that the loss of functional HDL/SR-BI interaction and LCAT-activating ability—as induced by carbamylation—results in impaired reverse-cholesterol transfer capacity.

The antiatherogenic effect of HDL has been attributed to several mechanisms, including the prevention of LDL oxidation. HDL-mediated inactivation of LDL-associated phospholipid-hydroperoxides involves initial transfer from LDL to HDL, which is governed by the rigidity of the surface monolayer of HDL, and the subsequent reduction of hydroperoxides by redox-active methionine residues of apolipoproteins (12, 19, 38). HDL-associated enzymes may, in turn,

contribute to the hydrolytic inactivation of short-chain oxidized phospholipids. PON is a HDL-associated enzyme hydrolyzing various lactones and organophosphates and was shown to metabolize lipoprotein-associated oxidized lipids (5, 21). Significantly, PON was shown to selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions (4) and to protect against atherosclerosis and coronary artery diseases (31). Therefore, the decrease in antioxidant activity of carbamylated HDL may be the result of decreased PON activity. However, other cyanate-induced modifications of the protein and phospholipid moiety of HDL may contribute to the impaired antioxidative capacity.

In summary, the present study has identified novel mechanisms of protein carbamylation, highlighting the link between inflammation and cardiovascular disease with uremia, smoking, and air pollution. The carbamylation of HDL in the atherosclerotic intima might critically impair antioxidant and anti-inflammatory properties of HDL, thereby promoting the development of atherosclerosis.

Materials and Methods

^{13}C , ^{15}N -labeled urea was purchased from Cambridge Isotope Laboratories (Andover, MA). HCIt (carbamyllysine) was purchased from Bachem (Weil am Rhein, Germany), and $^{13}\text{C}_6$ -

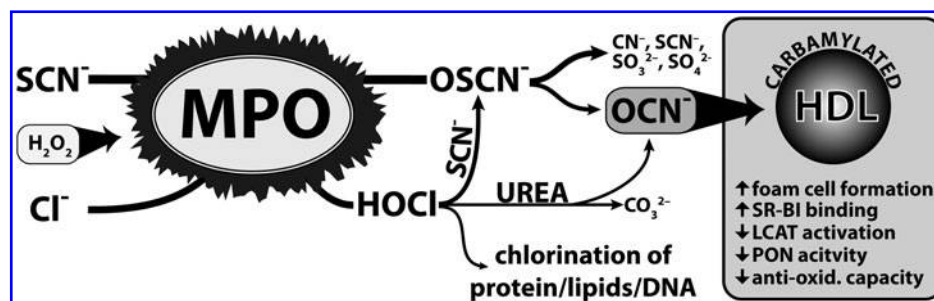


FIG. 7. Cyanate generation by MPO-derived reactive species. A schematic illustration summarizing MPO-mediated pathways generating cyanate (OCN^-). MPO oxidizes SCN^- and chloride (Cl^-) in the presence of H_2O_2 to hypochlorite (OSCN^-) and HOCl. OSCN^- decomposes to cyanate (OCN^-) and to a variety of other compounds such as cyanide (CN^-), sulfite (SO_3^{2-}), sulfate (SO_4^{2-}), or back to SCN^- (18). HOCl oxidizes SCN^- to OSCN^- (3), thereby increasing the formation of OCN^- . Decomposition of urea through HOCl generates OCN^- and carbonate. Cyanate-induced carbamylation results in a reduction of the potential athero-protective functions of HDL.

HCit was purchased from Ascent Scientific (Bristol, United Kingdom). The LCAT activity kit was purchased from Merck (Darmstadt, Germany). MPO was supplied by Enzo Life Science (Vienna, Austria). All other reagents were obtained from Sigma (Vienna, Austria), unless otherwise specified.

Blood collection

Blood was sampled from healthy volunteers after an informed consent, according to a protocol approved by the Institutional Review Board of the Medical University of Graz (14).

Isolation of lipoproteins

For lipoprotein isolation, serum density from normolipidemic blood donors was adjusted with KBr to 1.24 g/ml. To optimize lipoprotein separation, long centrifuge tubes (16 × 76 mm; Beckman Instruments, Krefeld, Germany) were used. A two-layer density gradient was generated by layering the density-adjusted serum (1.24 g/ml) underneath phosphate-buffered saline (PBS; 1.006 g/ml). The tubes were sealed and centrifuged at 90,000 rpm for 4 h in a 90TI fixed-angle rotor (Beckman Instruments). After centrifugation, the two clearly separated HDL- and LDL-containing bands were collected, desalted *via* a PD10 column (GE Healthcare, Vienna, Austria), and directly used for experiments. HDL-like particles from human atherosclerotic tissue were isolated as previously described (15).

Modification of HDL

HDL was carbamylated with potassium cyanate as previously described (15). MPO-mediated modification of HDL was performed in 50 mM phosphate buffer (pH 7.0). Reaction samples contained 2.5 mg/ml HDL, 50 nM MPO, 100 mM NaCl, 200 or 400 μ M H₂O₂, and 50 or 250 μ M SCN. The reactions were allowed to take place for 48 h at 37°C. Afterward, the samples were placed on ice to stop the reaction, and unreacted compounds were removed by gel filtration. Carbamylation was assessed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

HOCl-induced decomposition of SCN/urea

SCN (50–250 μ M) or urea (5–50 mM) in PBS were mixed with HOCl (100 μ M) and immediately added to a 2.5 mg/ml HDL solution. The reaction mixture was incubated for 16 h at 37°C. For experiments with human plasma, total plasma was diluted with PBS to 30%. The diluted plasma was incubated in the absence or presence of 50 or 250 μ M SCN and 100 or 500 μ M HOCl for 16 h at 37°C. Afterward, the unreacted compounds were removed by gel filtration, and protein carbamylation was assessed by LC-MS/MS.

NMR spectroscopy

All spectra were recorded on a Bruker 700 MHz Avance III NMR spectrometer, equipped with a 5 mm TCI cryo probe. Inverse gated proton-decoupled ¹³C-spectra were acquired with 128 scans and an interscan delay of 2 s. The pulse width corresponded to a flip angle of ~30° to reduce any saturation in the spectra. A solution of 50 mM ¹³C, ¹⁵N-labeled urea was mixed with a 750 mM stock solution of HOCl, yielding a final

concentration of 25 mM HOCl. The experiments were carried out in a PBS buffer (pH 7.4) at 298 K.

Quantification of carbamyllysine and 3-CT

Proteins were hydrolyzed with a fast, low-volume hydrolysis method as described (7). To quantify carbamyllysine and chlorotyrosine formation in HDL, electrospray ionization LC-MS/MS was used as previously described (15).

PON/arylesterase activity assay

Ca²⁺-dependent PON activity was determined as the rate of hydrolysis of paraoxon into 4-nitrophenol, which can be monitored by an increase in absorbance at 405 nm (11). Native or carbamylated HDL (10 μ g protein) was placed into a 96-well plate, and the reaction was initiated by the addition of 200 μ L buffer containing 100 mM Tris, 2 mM CaCl₂, and 1 mM paraoxon (pH 8.0). The assay was performed at 37°C, and readings were taken every 5 min at 405 nm on a plate reader to generate a kinetic plot. The slope from the kinetic chart was used to determine Δ Abs_{405nm}/min. Enzymatic activity was calculated with the Beer–Lambert Law from the molar extinction coefficient of 17,100 L·mol⁻¹·cm⁻¹. PON activities were expressed as nM 4-nitrophenol formed per minute.

Ca²⁺-dependent arylesterase activity was determined with a photometric assay using phenylacetate as the substrate (11). Native or carbamylated HDL (2 μ g protein) was added to 200 μ L buffer containing 100 mM Tris, 2 mM CaCl₂ (pH 8.0), and phenylacetate (1 mM). The rate of hydrolysis of phenylacetate was monitored by the increase of absorbance at 270 nm, and readings were taken every 30 s at room temperature to generate a kinetic plot. The slope from the kinetic chart was used to determine Δ Abs_{270nm}/min. Enzymatic activity was calculated with the Beer–Lambert Law from the molar extinction coefficient of 1310 L·mol⁻¹·cm⁻¹ for phenylacetate.

Carbamylation of HDL and assessment of LCAT activity

LCAT activity was measured using a commercially available kit (Merck), which was adapted to measure the substrate efficiency of HDL preparations. For that purpose, native or carbamylated HDL was preincubated with LCAT substrate for 90 min at 37°C, and the unbound substrate was removed using gel filtration. The binding efficiency was ~95% of the added substrate, determined by fluorescence measurement of the substrate at 470 nm after gel filtration. The labeled HDL preparations were used to determine the substrate efficiency in response to LCAT enzymatic activity. LPDS was used as the source for LCAT.

The experimental setting was as follows: 100 μ g labeled HDL and 10 μ L LPDS were completed with LCAT buffer to 200 μ L. LCAT buffer contained 150 mM NaCl, 10 mM TRIS, 4 mM mercaptoethanol, and 1 mM EDTA.

Endpoint readouts were obtained after incubation for 5 h at 37°C. Results are expressed as the percent of substrate hydrolyzed.

Determination of the antioxidative capacity of HDL

The antioxidative activity of HDL was determined as previously described (69, 120). LDL (100 μ g/ml protein) was oxidized *in vitro* with the free radical initiator AAPH (1 mM in

PBS) in the presence or absence of HDL (100 μ g/ml protein) at 37°C. The oxidation was monitored at 234 nm. A time point in the propagation phase (at 200 min) was selected to assess the antioxidant activity of HDL. Oxidation in the presence of HDL was calculated as relative to the oxidation of LDL alone, which was set to 100%.

Statistical analyses

Data are shown as mean \pm SEM for n observations unless otherwise stated. Comparison between 2 groups was performed with Student's t-test and between multiple groups using one-way ANOVA with Bonferroni *post hoc* test. Significance was accepted at probability levels of $p < 0.05$ and $p < 0.01$. Statistical analyses were performed with GraphPad Prism version 4.03.

Sources of Funding

Michael Holzer and Sanja Curcic were funded by the Ph.D. Program Molecular Medicine of the Medical University of Graz. This work was supported by the Austrian Science Fund FWF (Grants P21004-B02, P22521-B18, P22976-B18, P20020, and P22630).

Author Disclosure Statement

No competing financial interests exist.

References

- Alexander ET, Bhat S, Thomas MJ, Weinberg RB, Cook VR, Bharadwaj MS, and Sorci-Thomas M. Apolipoprotein A-I helix 6 negatively charged residues attenuate lecithin-cholesterol acyltransferase (LCAT) reactivity. *Biochemistry* 44: 5409–5419, 2005.
- Arlandson M, Decker T, Roongta VA, Bonilla L, Mayo KH, MacPherson JC, Hazen SL, and Slungaard A. Eosinophil peroxidase oxidation of thiocyanate. Characterization of major reaction products and a potential sulfhydryl-targeted cytotoxicity system. *J Biol Chem* 276: 215–224, 2001.
- Ashby MT, Carlson AC, and Scott MJ. Redox buffering of hypochlorous acid by thiocyanate in physiologic fluids. *J Am Chem Soc* 126: 15976–15977, 2004.
- Aviram M, Hardak E, Vaya J, Mahmood S, Milo S, Hoffman A, Billicke S, Draganov D, and Rosenblat M. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation* 101: 2510–2517, 2000.
- Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL, and La Du BN. Paraonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraonase. *J Clin Invest* 101: 1581–1590, 1998.
- Bergt C, Pennathur S, Fu X, Byun J, O'Brien K, McDonald TO, Singh P, Anantharamaiah GM, Chait A, Brunzell J, Geary RL, Oram JF, and Heinecke JW. The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proc Natl Acad Sci U S A* 101: 13032–13037, 2004.
- Damm M, Holzer M, Radschler G, Marsche G, and Kappe CO. Microwave-assisted high-throughput acid hydrolysis in silicon carbide microtiter platforms—a rapid and low volume sample preparation technique for total amino acid analysis in proteins and peptides. *J Chromatogr A* 1217: 7826–7832, 2010.
- Dirnhuber P and Schutz F. The isomeric transformation of urea into ammonium cyanate in aqueous solutions. *Biochem J* 42: 628–632, 1948.
- Domigan NM, Charlton TS, Duncan MW, Winterbourn CC, and Kettle AJ. Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils. *J Biol Chem* 270: 16542–16548, 1995.
- El-Gamal D, Holzer M, Gauster M, Schicho R, Binder V, Konya V, Wadsack C, Schuligoi R, Heinemann A, and Marsche G. Cyanate is a novel inducer of endothelial icam-1 expression. *Antioxid Redox Signal* 16: 129–137, 2012.
- Gan KN, Smolen A, Eckerson HW, and La Du BN. Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. *Drug Metab Dispos* 19: 100–106, 1991.
- Garner B, Waldeck AR, Witting PK, Rye KA, and Stocker R. Oxidation of high density lipoproteins. II. Evidence for direct reduction of lipid hydroperoxides by methionine residues of apolipoproteins AI and AII. *J Biol Chem* 273: 6088–6095, 1998.
- Goyal J, Wang K, Liu M, and Subbaiah PV. Novel function of lecithin-cholesterol acyltransferase. Hydrolysis of oxidized polar phospholipids generated during lipoprotein oxidation. *J Biol Chem* 272: 16231–16239, 1997.
- Holzer M, Birner-Gruenberger R, Stojakovic T, El-Gamal D, Binder V, Wadsack C, Heinemann A, and Marsche G. Uremia Alters HDL Composition and Function. *J Am Soc Nephrol* 22: 1631–1641, 2011.
- Holzer M, Gauster M, Pfeifer T, Wadsack C, Fauler G, Stiegler P, Koefeler H, Beubler E, Schuligoi R, Heinemann A, and Marsche G. Protein carbamylation renders high-density lipoprotein dysfunctional. *Antioxid Redox Signal* 14: 2337–2346, 2011.
- Husgafvel-Pursiainen K, Sorsa M, Engstrom K, and Einisto P. Passive smoking at work: biochemical and biological measures of exposure to environmental tobacco smoke. *Int Arch Occup Environ Health* 59: 337–345, 1987.
- Kalantar-Zadeh K, Brennan ML, and Hazen SL. Serum myeloperoxidase and mortality in maintenance hemodialysis patients. *Am J Kidney Dis* 48: 59–68, 2006.
- Kalmar J, Woldegiorgis KL, Biri B, and Ashby MT. Mechanism of decomposition of the human defense factor hypochlorite near physiological pH. *J Am Chem Soc* 133: 19911–19921, 2011.
- Kontush A and Chapman MJ. Antiatherogenic function of HDL particle subpopulations: focus on antioxidative activities. *Curr Opin Lipidol* 21: 312–318, 2010.
- Kraus LM, Elberger AJ, Handorf CR, Pabst MJ, and Kraus AP, Jr. Urea-derived cyanate forms epsilon-amino-carbamoyl-lysine (homocitrulline) in leukocyte proteins in patients with end-stage renal disease on peritoneal dialysis. *J Lab Clin Med* 123: 882–891, 1994.
- Mackness MI, Arrol S, and Durrington PN. Paraonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett* 286: 152–154, 1991.
- Marsche G, Furtmuller PG, Obinger C, Sattler W, and Malle E. Hypochlorite-modified high-density lipoprotein acts as a sink for myeloperoxidase *in vitro*. *Cardiovasc Res* 79: 187–194, 2008.
- Marsche G, Hammer A, Oskolkova O, Kozarsky KF, Sattler W, and Malle E. Hypochlorite-modified high density lipoprotein, a high affinity ligand to scavenger receptor class B, type I, impairs high density lipoprotein-dependent selective

- lipid uptake and reverse cholesterol transport. *J Biol Chem* 277: 32172–32179, 2002.
24. Morgan PE, Pattison DI, Talib J, Summers FA, Harmer JA, Celermajer DS, Hawkins CL, and Davies MJ. High plasma thiocyanate levels in smokers are a key determinant of thiol oxidation induced by myeloperoxidase. *Free Radic Biol Med* 51: 1815–1822, 2011.
 25. Olea F and Parras P. Determination of serum levels of dietary thiocyanate. *J Anal Toxicol* 16: 258–260, 1992.
 26. Pattison DI and Davies MJ. Reactions of myeloperoxidase-derived oxidants with biological substrates: gaining chemical insight into human inflammatory diseases. *Curr Med Chem* 13: 3271–3290, 2006.
 27. Pattison DI, Hawkins CL, and Davies MJ. What are the plasma targets of the oxidant hypochlorous acid? A kinetic modeling approach. *Chem Res Toxicol* 22: 807–817, 2009.
 28. Roberts JM, Veres PR, Cochran AK, Warneke C, Burling IR, Yokelson RJ, Lerner B, Gilman JB, Kuster WC, Fall R, and de Gouw J. Isocyanic acid in the atmosphere and its possible link to smoke-related health effects. *Proc Natl Acad Sci U S A* 108: 8966–8971, 2011.
 29. Rodriguez-Ayala E, Anderstam B, Suliman ME, Seeberger A, Heimbürger O, Lindholm B, and Stenvinkel P. Enhanced RAGE-mediated NFκB stimulation in inflamed hemodialysis patients. *Atherosclerosis* 180: 333–340, 2005.
 30. Rutili G and Arfors KE. Protein concentration in interstitial and lymphatic fluids from the subcutaneous tissue. *Acta Physiol Scand* 99: 1–8, 1977.
 31. She ZG, Chen HZ, Yan Y, Li H, and Liu DP. The human paraoxonase gene cluster as a target in the treatment of atherosclerosis. *Antioxid Redox Signal* 16: 597–632, 2012.
 32. Smith EB and Staples EM. Distribution of plasma proteins across the human aortic wall—barrier functions of endothelium and internal elastic lamina. *Atherosclerosis* 37: 579–590, 1980.
 33. Stark GR. Reactions of cyanate with functional groups of proteins. II. Formation, decomposition, and properties of N-carbamylimidazole. *Biochemistry* 4: 588–595, 1965.
 34. Stark GR. On the reversible reaction of cyanate with sulfhydryl groups and the determination of N^h2-terminal cysteine and cystine in proteins. *J Biol Chem* 239: 1411–1414, 1964.
 35. van Dalen CJ, Whitehouse MW, Winterbourn CC, and Kettle AJ. Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem J* 327 (Pt 2): 487–492, 1997.
 36. Wang Z, Nicholls SJ, Rodriguez ER, Kumm O, Horkko S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, and Hazen SL. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* 13: 1176–1184, 2007.
 37. Xulu BA and Ashby MT. Small molecular, macromolecular, and cellular chloramines react with thiocyanate to give the human defense factor hypothiocyanite. *Biochemistry* 49: 2068–2074, 2010.
 38. Zerrad-Saadi A, Therond P, Chantepie S, Couturier M, Rye KA, Chapman MJ, and Kontush A. HDL3-mediated inactivation of LDL-associated phospholipid hydroperoxides is determined by the redox status of apolipoprotein A-I and HDL particle surface lipid rigidity: relevance to inflammation and atherogenesis. *Arterioscler Thromb Vasc Biol* 29: 2169–2175, 2009.
 39. Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, Schmitt D, Fu X, Thomson L, Fox PL, Ischiropoulos H, Smith JD, Kinter M, and Hazen SL. Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest* 114: 529–541, 2004.

Address correspondence to:

Dr. Gunther Marsche

Institute for Experimental and Clinical Pharmacology

Medical University of Graz

Universitätsplatz 4

8010 Graz

Austria

E-mail: gunther.marsche@medunigraz.at

Date of first submission to ARS Central, November 9, 2011; date of final revised submission, March 30, 2012; date of acceptance, March 31, 2012.

Abbreviations Used

3-CT	= 3-chlorotyrosine
AAPH	= 2,2'-azobis-2-methyl-propanimidamide dihydrochloride
apoA-I	= apolipoprotein A-I
BSA	= bovine serum albumin
cHDL	= carbamylated high-density lipoprotein
HCit	= homocitrulline (carbamyllysine)
HDL	= high-density lipoprotein
H ₂ O ₂	= hydrogen peroxide
HOCl	= hypochlorous acid
LCAT	= lecithin-cholesterol acyltransferase
LC-MS/MS	= liquid chromatography tandem mass spectrometry
LDL	= low-density lipoprotein
LPDS	= lipoprotein deficient serum
Lys	= lysine
m.ox-HDL	= minimal oxidized HDL
MPO	= myeloperoxidase
NMR	= nuclear magnetic resonance
OSCN ⁻	= hypothiocyanate
PBS	= phosphate-buffered saline
PON	= paraoxonase
SCN	= thiocyanate
SDS-PAGE	= sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	= Tris-buffered saline