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Thiocyanate supplementation decreases atherosclerotic plaque in mice expressing human myeloperoxidase

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Abstract

Elevated levels of the heme enzyme myeloperoxidase (MPO) are associated with adverse cardiovascular outcomes. MPO predominantly catalyzes formation of the oxidants hypochlorous acid (HOCl) from Cl^- , and hypothiocyanous acid (HOSCN) from SCN^- , with these anions acting as competitive substrates. HOSCN is a less powerful and more specific oxidant than HOCl, and selectively targets thiols; such damage is largely reversible, unlike much HOCl-induced damage. We hypothesized that increased plasma SCN^- , and hence HOSCN formation instead of HOCl, may decrease artery wall damage. This was examined using high-fat fed atherosclerosis-prone $\text{LDLR}^{-/-}$ mice transgenic for human MPO, with and without SCN^- (10 mM) added to drinking water. Serum samples, collected fortnightly, were analyzed for cholesterol, triglycerides, thiols, MPO and SCN^- ; study-long exposure was calculated by area under the curve (AUC). Mean serum SCN^- concentrations were elevated in the supplemented mice (200–320 μM) relative to controls (<120 μM). Normalized aortic root plaque areas at sacrifice were 26% lower in the SCN^- -supplemented mice compared to controls ($P=0.0417$), but plaque morphology was not appreciably altered. Serum MPO levels steadily increased in mice on the high-fat diet, however, comparison of SCN^- -supplemented vs. control mice showed no significant changes in MPO protein, cholesterol or triglyceride levels; thiol levels were decreased in supplemented mice at one time-point. Plaque areas increased with higher cholesterol AUC ($r=0.4742$; $P=0.0468$), and decreased with increasing SCN^- AUC ($r=-0.5693$; $P=0.0134$). These data suggest that increased serum SCN^- levels, which can be achieved in humans by dietary manipulation, may decrease atherosclerosis burden.

Keywords

myeloperoxidase; thiocyanate; hypothiocyanous acid; hypochlorous acid; atherosclerosis; thiol oxidation

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of this paper.

Introduction

Myeloperoxidase (MPO) is a heme enzyme released predominantly by neutrophils and monocytes at sites of inflammation [1]. MPO catalyzes the formation of the oxidants hypochlorous acid (HOCl), hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN), from hydrogen peroxide (H_2O_2) and the halide / pseudohalide ions chloride (Cl^-), bromide (Br^-), and thiocyanate (SCN^-) respectively [2]. MPO can also utilize nitrite (NO_2^-) to nitrate tyrosine residues [3]. There is strong experimental and epidemiological evidence linking increased MPO levels with human inflammatory pathologies including cardiovascular disease, neurodegenerative disorders, rheumatoid arthritis, cystic fibrosis, asthma, and some cancers (reviewed [4,5]). This has led to considerable interest in mechanisms that might diminish MPO-mediated damage associated with misplaced and/or elevated MPO expression [6].

HOSCN is a less powerful oxidant than HOCl or HOBr, and reacts predominantly with thiols [7,8]. Although Cl^- is more abundant in human serum than SCN^- (100–140 mM; Br^- , 20–100 μM ; SCN^- , 20–250 μM), MPO has a much greater specificity constant for SCN^- than either Cl^- (~730-fold), or Br^- (~60-fold) [2]. Accordingly, increased SCN^- concentrations can significantly increase HOSCN formation, chiefly at the expense of HOCl. Up to 50% of the H_2O_2 consumed by MPO has been predicted to be converted to HOSCN at physiological ion concentrations [9,10], and serum levels of the HOCl-dependent marker 3-chlorotyrosine present on proteins have been shown to inversely correlate with SCN^- concentrations in humans [11].

There is debate in the literature regarding the effects of elevated SCN^- and possibly higher levels of HOSCN on human disease. A number of studies have reported beneficial effects, such in the oral cavity [12], in the lungs [13,14] and in subjects who have suffered a myocardial infarction [15], whereas others have reported that exposure to HOSCN induces detrimental effects by, for example, increasing lipoprotein modification [16,17], damaging key cellular enzymes [18–20] and altering plasma thiol status [10]). It is well established that lactoperoxidase (LPO) and salivary peroxidase (SPO) use the high (millimolar) concentrations of SCN^- present in milk, tears and saliva to produce HOSCN as an antibacterial defense (reviewed [21]). However, whether increased formation of HOSCN in the artery wall is beneficial or detrimental is unclear, though a recent study examining all-cause mortality in subjects who have suffered a myocardial infarction has provided evidence that high SCN^- levels are protective [15]. Previous *in vivo* studies have been hampered by the use of smokers as a model of elevated SCN^- (e.g. [10,17]), a system confounded by the many other compounds present in cigarette smoke.

In this study we have examined the effect of SCN^- supplementation (in drinking water) on the burden of atherosclerosis (measured as plaque volume) in atherosclerosis-prone $\text{LDLR}^{-/-}$ mice transgenic for human MPO (–463G allele). This enzyme has been shown to be expressed in such mice in the artery wall and atherosclerotic lesions, as occurs in humans [22,23] and results in increased atherosclerosis on high-fat feeding, particularly in male mice [22]. The –463G allele has also been associated with increased atherosclerosis in humans [24,25].

Materials and Methods

Materials

Chemicals were from Sigma–Aldrich (Castle Hill, NSW, Australia and St Louis MO, USA) unless otherwise stated. Water was from a Millipore (North Ryde, NSW, Australia) Milli Q Advantage A10 system.

Animal studies

All animal work was carried out at the Sanford-Burnham Medical Research Institute. The care of the mice, and all the procedures performed were approved by the institutional animal care and use committee, and complied with National Institutes of Health animal care guidelines. Two batches of male mice transgenic for human MPO (–463G allele) on an atherosclerosis-prone LDL receptor-deficient (LDLR^{–/–}) C57BL6/J background were studied, with mice bred as described previously [26]. All mice were fed a high-fat pro-atherogenic Western diet for 12 weeks (HFD; Harlan Teklad Laboratory; TD 88137). In addition, 9/13 mice in batch 1, and 5/10 mice in batch 2, were given 10 mM sodium thiocyanate (NaSCN) in their drinking water for the duration of the study. Mice were weighed approximately fortnightly, and blood was collected from the retro-orbital sinus under isoflurane anesthesia. Blood collection at sacrifice was by cardiac puncture under Avertin® anesthetic. Serum was prepared, and stored at –80 °C until analyzed.

Serum biochemical analyses

MPO protein levels were quantified by ELISA (Immundiagnostik AG, Bensheim, Germany). Total cholesterol and triglycerides were quantified by colorimetric assay (WAKO, Richmond VA, USA) [27]. Protein-bound thiols were quantified by optical absorbance at 412 nm following reaction with 5,5'-dithionitrobenzoic acid (DTNB), with concentrations determined by comparison to glutathione (0–0.5 mM) standards [28].

Quantification of serum anions

Serum anions were quantified by ion-exchange chromatography. SCN[–] was quantified at all time-points; Br[–] at both sacrifice and some intermediate time points, and NO₂[–] at sacrifice only due to limitations in serum volume. Samples were prepared by diluting serum 1:1 or 1:2 with water (in a limited number of samples where <10 µL of serum was available greater dilutions were utilized), then mixed 1:1 with acetonitrile, before centrifugation and filtration as previously [10]. Chromatography was performed at 30 °C using a Dionex ICS-2100 system (Thermo Scientific, Lane Cove, NSW, Australia) fitted with an ASRS 300 suppressor. Samples (12.5 µL) were injected using an AS-AP autosampler (at 4 °C) with peaks detected electrochemically (at 35 °C). For determination of SCN[–] alone, an IonPac AS16 column (4 × 250 mm) and AG16 guard (4 × 50 mm) were used, with peaks eluted over 12 min with 50 mM potassium hydroxide at 1.5 mL/min, and a suppressor current of 186 mA. Detection of Br[–], NO₂[–] and SCN[–] was carried out using an IonPac AS19 column (4 × 250 mm) and AG19 guard (4 × 50 mm), with peaks eluted over 48 min using a potassium hydroxide (KOH) gradient method at 1 mL/min (suppressor current 186 mA), with 10–15 mM KOH over 10 min (curve 6); 15–75 mM KOH over 25 min (curve 6); 75

mM KOH for 4 min; 75-10 mM KOH over 1 min (linear); and then re-equilibration with 10 mM KOH for 8 min. Peak areas were quantified using Chromeleon chromatography data system software (v. 7.1; Dionex/Thermo Scientific), with anion concentrations quantified by comparison to standard curves (NaBr, NaNO₂, NaSCN; 0-200 µM).

Atherosclerotic lesion analysis

The heart and proximal aorta were embedded in paraffin, and 5 µm sections analyzed at 50 µm intervals over the region spanning the aortic sinus and valve regions. Sections were stained with Masson's Trichrome stain. Digital images were acquired with ScanScope (Aperio, Vista, CA, USA), and subsequently analyzed using ImageScope (Aperio) software, for multiple sections (usually 3) in the region where the valve leaflets were visible, by drawing around the plaque perimeters. Plaque areas were normalized to the total vessel area for each section. The analyst was blinded to the identities of the samples.

Statistical analyses

All analyses were performed using GraphPad Prism v. 5 for Mac OS X (GraphPad Software, San Diego, USA) as detailed in Figure/Table captions. Significance was assumed if $P < 0.05$. One-tailed tests were employed only when increases/decreases were predicted before assay. Correlation analysis used the total exposure over the duration of the study for each analyte, determined by calculating the area under the curve (AUC) for each mouse; any mouse with missing data points for a particular analyte was excluded.

Results

Mouse plaque areas and physiology

Aortic root plaque areas (normalized to vessel area) were determined for 11/13 of the SCN⁻ supplemented mice, and 8/9 of the non-supplemented controls. SCN⁻ supplementation decreased plaque areas by 26% compared to controls (Figure 1A; $P=0.0417$; 2-tailed t -test). Plaque morphology was as previously reported in this model [22], with a combination of lesions, ranging from aggregation of macrophages in early fatty streaks (Figure 1B, left panel), through to large complex lesions with large cholesterol clefts (Figure 1B, right panel). Numerous plaques in both SCN⁻ supplemented and non-supplemented mice were multi-layered with several lipid cores, suggesting no beneficial effects of SCN⁻ supplementation on plaque morphology once advanced lesions were present. However, plaques in the SCN⁻ supplemented mice were statistically smaller, due to either less advanced lesions, and/or smaller lipid cores. The use of normalized plaque areas controlled for any differences in vessel size. The SCN⁻ supplemented mice did not show any significant difference, when compared to the non-supplemented mice, with regard to gain in mass over the complete study (Figure 1C). This suggests that elevated levels of SCN⁻ do not have significant effects on overall metabolism in these animals.

Quantification of MPO protein levels and MPO substrates (SCN⁻, Br⁻, NO₂⁻)

Serum MPO protein levels steadily increased during the study, in both SCN⁻ supplemented and non-supplemented groups (Figure 2A). There were no significant differences in MPO protein levels between the two groups at any of the time-points examined.

Mean serum SCN⁻ concentrations in both groups of mice were initially not significantly different ($124 \pm 20 \mu\text{M}$ in mice to be supplemented vs. $107 \pm 34 \mu\text{M}$ in controls, $P=0.1469$), but SCN⁻ supplementation resulted in a rapid and significant increase in serum SCN⁻ levels (Figure 2B), resulting in mean concentrations ~2-3 times that of the non-supplemented controls (supplemented ~200-320 μM ; non-supplemented < 120 μM). These concentrations remained significantly elevated for the rest of the study except for the 12-week (sacrifice) time-point (Figure 2B).

Serum Br⁻ concentrations measured at sacrifice were unexpectedly high, but not significantly different between the two groups (mean Br⁻ in supplemented mice $1.004 \pm 0.265 \text{ mM}$; non-supplemented $1.108 \pm 0.273 \text{ mM}$, $P=0.3873$; not shown). This high level is believed to arise from post-mortem degradation or impurities in the anesthetic used at sacrifice (Avertin®, tribromoethanol). Measurement of serum Br⁻ levels in 8 randomly-selected mice (4 from each experimental cohort; insufficient serum was available to assay all mice) at both the initial time-point, and the sample obtained prior to sacrifice (where isoflurane was used as anesthetic), indicated that average serum Br⁻ levels were much lower (<80 μM) for the duration of the study (data not shown).

Serum NO₂⁻ levels at sacrifice (Figure 2C) were not significantly different between the supplemented and non-supplemented groups (supplemented group, $7.6 \pm 3.5 \mu\text{M}$; non-supplemented, $6.6 \pm 3.2 \mu\text{M}$, $P=0.4928$). Cl⁻ was not measured, as it is present endogenously at very high levels (~100 mM) [2].

Serum thiols and lipoproteins

Serum protein thiols were measured as a marker of oxidative stress. Thiol levels were significantly lower in the supplemented mice only at a single time-point compared to the non-supplemented mice (Figure 3A). Serum cholesterol (Figure 3B) and triglycerides (Figure 3C) were also quantified throughout the study period; although the values were slightly lower in the supplemented mice this did not reach statistical significance at any time-point.

Correlation analysis

The relationship between normalized aortic root plaque areas and serum biochemical parameters was examined by correlation analysis (Table 1A). A significant positive correlation was observed between plaque area and total cholesterol levels as expected on the basis of previous studies [29]. A strong inverse correlation was observed between plaque area and total SCN⁻ exposure, but no significant correlations were detected between plaque area at sacrifice and the AUC values for serum thiols, MPO, or triglycerides.

The interplay between total SCN⁻ exposure and other parameters measured for the duration of the study was also examined (Table 1B). With the exception of the correlation between total SCN⁻ exposure and plaque area (Table 1A), there were no significant correlations for any of the parameters measured.

Discussion

Previous studies have provided compelling evidence for an association between elevated levels of MPO and increased atherosclerosis and cardiovascular disease [30,31]. However, the nature of the oxidants that gives rise to this elevated risk remains to be fully elucidated. This study has examined atherosclerotic plaque development, and various biochemical parameters, upon supplementation of an atherosclerosis-prone LDLR^{-/-} mouse model transgenic for human MPO with a major alternative MPO substrate. The data show that atherosclerotic plaque growth was markedly decreased by 12 weeks of dietary SCN⁻ supplementation that increased serum levels of this anion by approximately 3 fold. This supplementation did not however affect the levels of other anion substrates of MPO, or MPO protein levels. The significant inverse correlation between serum SCN⁻ concentrations and normalized plaque areas suggests that elevated SCN⁻ levels are associated with the decreased atherosclerosis. To our knowledge, this is the first study to show a positive effect of SCN⁻ supplementation in an atherosclerosis model, and supports the reported association between elevated plasma SCN⁻ and a decreased incidence of all-cause mortality in humans [15].

The use of a mouse model transgenic for human MPO avoids some of the issues associated with other mouse models. Murine neutrophils have <20% of the MPO activity of human neutrophils [32], resulting in suggestions that mice are not appropriate models of human disease. Furthermore, some studies using MPO-knockout mice have reported increased, rather than decreased, atherosclerosis [33], though other studies suggest differently (e.g. [34]). The human transgenic MPO model used here circumvents these potential problems, with levels of MPO and its associated analytes comparing favorably with those reported in humans [10]. Thus the MPO levels detected in the mice at the commencement of this study are similar to those in “healthy” humans [10]. These levels increased during the experiments, with levels at sacrifice within the range reported for patients with acute coronary syndromes [31]. Likewise, the mouse serum SCN⁻ levels detected throughout the study are comparable to those reported for human smokers and non-smokers [2,10]. Although the serum Br⁻ levels at sacrifice were approximately 10-fold higher than those detected in humans [10], these high levels were only detected post-mortem, and the mean Br⁻ levels detected at intermediate time-points were within the range observed in humans [10,35]. The high levels of Br⁻ detected post-mortem should not have influenced lesion formation or MPO-derived oxidation, as these appear to be an artifact of anesthetic decomposition. NO₂⁻ levels were also comparable with human data [2,10].

The marked increase in serum SCN⁻ concentrations in the supplemented mice would be expected to modify the mix of oxidants produced by MPO, with increased production of the thiol- specific oxidant HOSCN, and decreased formation of the more reactive, and less specific, HOCl [2]. Unlike HOSCN-mediated damage, which is largely reversible, HOCl generates higher yields of non-repairable oxidation [11]. Use of a previously developed kinetic model [10,11], with a representative MPO concentration from this study (3 nM, i.e. 400 ng/mL), suggests that as plasma SCN⁻ concentrations increase, the HOSCN yield increases and that of HOCl decreases. Thus, at 100 μM SCN⁻, ~21% of the H₂O₂ utilized by MPO gives HOSCN-derived products; this doubles to ~44% at 300 μM SCN⁻, with

concomitant decreases in HOCl-derived products (Pattison *et al*, unpublished data). This is in accord with previous experimental evidence from human studies showing high plasma SCN⁻ can increase thiol oxidation [10], and decrease levels of the HOCl-dependent biomarker 3-chlorotyrosine on human proteins [11]. Quantification of protein 3-chlorotyrosine levels, and correlation of these values with SCN⁻ concentrations, would have been a valuable addition to the current study, but this was not possible due to the low levels of available material; this is a limitation of the current study. The postulated increase in HOSCN, and decrease in HOCl, is believed to give rise to altered levels and types of damage within the artery wall, and result in smaller atherosclerotic lesions. This may be due to decreased cholesterol accumulation in the artery wall as a result of a lower extent of modification of positively charged (Lys, His and Arg) residues on low-density lipoproteins (LDL). His and Lys residues are well-established targets of HOCl (but not HOSCN) and alteration at these sites is known to result in the formation of modified high-uptake forms of LDL that are recognized by the scavenger receptors of macrophage cells [36,37]. Internalization of such modified LDL gives rise to lipid-laden (“foam”) cells, a well-established hallmark, and key driver, of atherosclerosis. Alternatively the decreased levels of HOCl may result in less damage to high-density lipoproteins (HDL), which are atheroprotective in their unmodified (but not oxidized / damaged) forms [38].

The detection of identical levels of circulating MPO protein and lipid levels in both the supplemented and control groups shows that SCN⁻ supplementation does not directly modulate either serum MPO, cholesterol or triglyceride levels. This is consistent with local effects of increased SCN⁻ concentrations on the nature and extent of damage within the artery wall, rather than changes in systemic parameters, such as the level of chronic systemic inflammation induced by high-fat / high-cholesterol diets. In addition, it should be noted that neither cholesterol nor triglycerides react rapidly with MPO-derived oxidants and hence are unlikely to be major targets of oxidation [39,40]. Furthermore, SCN⁻ supplementation did not result in chronic depletion of serum thiols, a change previously associated with increased atherosclerosis risk [41]. The absence of any correlation between these parameters may again reflect localized changes rather than systemic alterations, though it may also result from altered levels and activities of repair or catabolic enzymes between the artery wall and serum. Thus, any increase in endogenous steady-state thiol oxidation in serum, arising from increased HOSCN formation, may be compensated for by repair mechanisms, such as the glutaredoxin and thioredoxin systems [42]; a similar phenomenon has also been observed in smokers (P.E. Nedoboy, unpublished results).

Overall, this study provides evidence that increasing circulating SCN⁻ levels, to those detected in some human subjects, results in significantly decreased atherosclerotic plaque areas in high-fat-fed mice transgenic for human MPO. This is ascribed to a change in the type of oxidants formed by the key inflammation-associated enzyme MPO, with lower levels of damaging HOCl formed. This is postulated to result in lower levels of modified (high-uptake) forms of LDL and / or decreased damage to athero-protective HDL, within the artery wall, and hence a decreased accumulation of lipids. The current study should be followed up with more in-depth studies on the effects of SCN⁻ supplementation on lesion morphology and the extent of plaque rupture (e.g. in the brachiocephalic artery), and the

levels of modified LDL. The effects of SCN^- supplementation on thiol repair systems (e.g. the thioredoxin and glutaredoxin systems) and 3-chlorotyrosine levels should also be examined. These data suggest a potentially promising role for SCN^- supplementation as an alternative therapy to the use of MPO inhibitors in the prevention of MPO-derived cardiovascular injury, as such changes can be readily achieved by dietary means.

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Abbreviations

AUC	area under the curve
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
GSH	glutathione in its reduced form
H₂O₂	hydrogen peroxide
HDL	high-density lipoprotein
HOBr	hypobromous acid
HOCl	hypochlorous acid
HOSCN	hypothiocyanous acid
KOH	potassium hydroxide
LDL	low-density lipoprotein
LDLR^{-/-}	low-density lipoprotein receptor deficient
MPO	myeloperoxidase

References

- [1]. Nussbaum C, Klinke A, Adam M, Baldus S, Sperandio M. Myeloperoxidase: a leukocyte-derived protagonist of inflammation and cardiovascular disease. *Antioxid Redox Signal*. 2013; 18:692–713. [PubMed: 22823200]
- [2]. van Dalen CJ, Whitehouse MW, Winterbourn CC, Kettle AJ. Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem J*. 1997; 327:487–492. [PubMed: 9359420]
- [3]. van Dalen CJ, Winterbourn CC, Senthilmohan R, Kettle AJ. Nitrite as a substrate and inhibitor of myeloperoxidase. Implications for nitration and hypochlorous acid production at sites of inflammation. *J Biol Chem*. 2000; 275:11638–11644. [PubMed: 10766781]
- [4]. Choi DK, Koppula S, Choi M, Suk K. Recent developments in the inhibitors of neuroinflammation and neurodegeneration: inflammatory oxidative enzymes as a drug target. *Exp Opin Therapeut Patents*. 2010; 20:1531–1546.
- [5]. Davies MJ, Hawkins CL, Pattison DI, Rees MD. Mammalian heme peroxidases: from molecular mechanisms to health implications. *Antioxid Redox Signal*. 2008; 10:1199–1234. [PubMed: 18331199]
- [6]. Malle E, Furtmuller PG, Sattler W, Obinger C. Myeloperoxidase: a target for new drug development? *Br J Pharmacol*. 2007; 152:838–854. [PubMed: 17592500]

- [7]. Skaff O, Pattison DI, Davies MJ. Hypothiocyanous acid reactivity with low-molecular-mass and protein thiols: Absolute rate constants and assessment of biological relevance. *Biochem J.* 2009; 422:111–117. [PubMed: 19492988]
- [8]. Nagy P, Jameson GN, Winterbourn CC. Kinetics and mechanisms of the reaction of hypothiocyanous acid with 5-thio-2-nitrobenzoic acid and reduced glutathione. *Chem Res Toxicol.* 2009; 22:1833–1840. [PubMed: 19821602]
- [9]. Pattison DI, Hawkins CL, Davies MJ. What are the plasma targets of the oxidant hypochlorous acid? A kinetic modeling approach. *Chem Res Toxicol.* 2009; 22:807–817. [PubMed: 19326902]
- [10]. Morgan PE, Pattison DI, Talib J, Summers FA, Harmer JA, Celermajer DS, Hawkins CL, Davies MJ. High plasma thiocyanate levels in smokers are a key determinant of thiol oxidation induced by myeloperoxidase. *Free Radic Biol Med.* 2011; 51:1815–1822. [PubMed: 21884783]
- [11]. Talib J, Pattison DI, Harmer JA, Celermajer DS, Davies MJ. High plasma thiocyanate levels modulate protein damage induced by myeloperoxidase and perturb measurement of 3-chlorotyrosine. *Free Radic Biol Med.* 2012; 53:20–29. [PubMed: 22609005]
- [12]. Ashby MT, Kreth J, Soundarajan M, Sivuilu LS. Influence of a model human defensive peroxidase system on oral streptococcal antagonism. *Microbiology.* 2009; 155:3691–3700. [PubMed: 19684069]
- [13]. Chandler JD, Min E, Huang J, Nichols DP, Day BJ. Nebulized thiocyanate improves lung infection outcomes in mice. *Br J Pharmacol.* 2013; 169:1166–1177. [PubMed: 23586967]
- [14]. Chandler JD, Day BJ. Thiocyanate: a potentially useful therapeutic agent with host defense and antioxidant properties. *Biochem Pharmacol.* 2012; 84:1381–1387. [PubMed: 22968041]
- [15]. Nedoboy PE, Morgan PE, Mocatta TJ, Richards AM, Winterbourn CC, Davies MJ. High plasma thiocyanate levels are associated with enhanced myeloperoxidase-induced thiol oxidation and long-term survival in subjects following a first myocardial infarction. *Free Radic Res.* 2014; 48:1256–1266. [PubMed: 25050609]
- [16]. Hadfield KA, Pattison DI, Brown BE, Hou L, Rye KA, Davies MJ, Hawkins CL. Myeloperoxidase-derived oxidants modify apolipoprotein A-I and generate dysfunctional high-density lipoproteins: comparison of hypothiocyanous acid (HOSCN) with hypochlorous acid (HOCl). *Biochem J.* 2013; 449:531–542. [PubMed: 23088652]
- [17]. Exner M, Hermann M, Hofbauer R, Hartmann B, Kapiotis S, Gmeiner B. Thiocyanate catalyzes myeloperoxidase-initiated lipid oxidation in LDL. *Free Radic Biol Med.* 2004; 37:146–155. [PubMed: 15203186]
- [18]. Barrett TJ, Pattison DI, Leonard SE, Carroll KS, Davies MJ, Hawkins CL. Inactivation of thiol-dependent enzymes by hypothiocyanous acid: role of sulfenyl thiocyanate and sulfenic acid intermediates. *Free Radic Biol Med.* 2012; 52:1075–1085. [PubMed: 22248862]
- [19]. Cook NL, Pattison DI, Davies MJ. Myeloperoxidase-derived oxidants rapidly oxidize and disrupt zinc-cysteine/histidine clusters in proteins. *Free Radic Biol Med.* 2012; 53:2072–2080. [PubMed: 23032100]
- [20]. Talib J, Kwan J, Suryo Rahmanto A, Witting PK, Davies MJ. The smoking-associated oxidant hypothiocyanous acid induces endothelial nitric oxide synthase dysfunction. *Biochem J.* 2014; 457:89–97. [PubMed: 24112082]
- [21]. Hawkins CL. The role of hypothiocyanous acid (HOSCN) in biological systems. *Free Radic Res.* 2009; 43:1147–1158. [PubMed: 19905977]
- [22]. Castellani LW, Chang JJ, Wang X, Lusic AJ, Reynolds WF. Transgenic mice express human MPO –463G/A alleles at atherosclerotic lesions, developing hyperlipidemia and obesity in –463G males. *J Lipid Res.* 2006; 47:1366–1377. [PubMed: 16639078]
- [23]. McMillen TS, Heinecke JW, LeBoeuf RC. Expression of human myeloperoxidase by macrophages promotes atherosclerosis in mice. *Circulation.* 2005; 111:2798–2804. [PubMed: 15911707]
- [24]. Asselbergs FW, Tervaert JW, Tio RA. Prognostic value of myeloperoxidase in patients with chest pain. *New Eng J Med.* 2004; 350:516–518. author reply 516–518. [PubMed: 14752891]
- [25]. Tang N, Wang Y, Mei Q. Myeloperoxidase G-463A polymorphism and susceptibility to coronary artery disease: a meta-analysis. *Gene.* 2013; 523:152–157. [PubMed: 23583798]

- [26]. Kumar AP, Piedrafita FJ, Reynolds WF. Peroxisome proliferator-activated receptor gamma ligands regulate myeloperoxidase expression in macrophages by an estrogen-dependent mechanism involving the -463GA promoter polymorphism. *J Biol Chem.* 2004; 279:8300–8315. [PubMed: 14668325]
- [27]. Mehrabian M, Qiao JH, Hyman R, Ruddle D, Laughton C, Lusis AJ. Influence of the apoA- II gene locus on HDL levels and fatty streak development in mice. *Arterioscler Thromb.* 1993; 13:1–10. [PubMed: 8422330]
- [28]. Hawkins CL, Morgan PE, Davies MJ. Quantification of protein modification by oxidants. *Free Radic Biol Med.* 2009; 46:965–988. [PubMed: 19439229]
- [29]. Verschuren WM, Jacobs DR, Bloemberg BP, Kromhout D, Menotti A, Aravanis C, Blackburn H, Buzina R, Dontas AS, Fidanza F. Serum total cholesterol and long-term coronary heart disease mortality in different cultures. Twenty-five-year follow-up of the seven countries study. *JAMA.* 1995; 274:131–136. others. [PubMed: 7596000]
- [30]. Nicholls SJ, Wilson Tang WH, Brennan D, Brennan ML, Mann S, Nissen SE, Hazen SL. Risk prediction with serial myeloperoxidase monitoring in patients with acute chest pain. *Clin Chem.* 2011; 57:1762–1770. [PubMed: 21940659]
- [31]. Baldus S, Heeschen C, Meinertz T, Zeiher AM, Eiserich JP, Munzel T, Simoons ML, Hamm CW. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. *Circulation.* 2003; 108:1440–1445. [PubMed: 12952835]
- [32]. Rausch PG, Moore TG. Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. *Blood.* 1975; 46(6):913–919. [PubMed: 173439]
- [33]. Brennan ML, Anderson MM, Shih DM, Qu XD, Wang X, Mehta AC, Lim LL, Shi W, Hazen SL, Jacob JS. Increased atherosclerosis in myeloperoxidase-deficient mice. *J Clin Invest.* 2001; 107:419–430. others. [PubMed: 11181641]
- [34]. van Leeuwen M, Gijbels MJ, Duijvestijn A, Smook M, van de Gaar MJ, Heeringa P, de Winther MP, Tervaert JW. Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR^{-/-} mice. *Arterioscler, Thromb Vasc Biol.* 2008; 28:84–89. [PubMed: 17991873]
- [35]. Olszowy HA, Rossiter J, Hegarty J, Geoghegan P. Background levels of bromide in human blood. *J Anal Toxicol.* 1998; 22:225–30. [PubMed: 9602940]
- [36]. Haberland ME, Olch CL, Fogelman AM. Role of lysines in mediating interaction of modified low density lipoproteins with the scavenger receptor of human monocyte macrophages. *J Biol Chem.* 1984; 259:11305–11311. [PubMed: 6088540]
- [37]. Hazell LJ, van den Berg JJ, Stocker R. Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. *Biochem J.* 1994; 302:421–428.
- [38]. Shao B, Oda MN, Oram JF, Heinecke JW. Myeloperoxidase: an oxidative pathway for generating dysfunctional high-density lipoprotein. *Chem Res Toxicol.* 2010; 23:447–454. [PubMed: 20043647]
- [39]. Pattison DI, Davies MJ. Reactions of myeloperoxidase-derived oxidants with biological substrates: gaining insight into human inflammatory diseases. *Curr Med Chem.* 2006; 13:3271–3290. [PubMed: 17168851]
- [40]. Pattison DI, Davies MJ, Hawkins CL. Reactions and reactivity of myeloperoxidase-derived oxidants: differential biological effects of hypochlorous and hypothiocyanous acids. *Free Radic Res.* 2012; 46:975–995. [PubMed: 22348603]
- [41]. Ashfaq S, Abramson JL, Jones DP, Rhodes SD, Weintraub WS, Hooper WC, Vaccarino V, Harrison DG, Quyyumi AA. The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults. *J Am Coll Cardiol.* 2006; 47:1005–1111. [PubMed: 16516085]
- [42]. Berndt C, Lillig CH, Holmgren A. Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system. *Am J Physiol Heart Circ Physiol.* 2007; 292:H1227–1236. [PubMed: 17172268]

Figure 1.

LDLR^{-/-} mice transgenic for human MPO were fed a high-fat Western diet with and without SCN⁻ supplementation. (A) Aortic root plaque areas, normalized to total vessel area, for SCN⁻-supplemented mice (11/13) and non-supplemented controls (8/9). (B) Representative images of the aortic root stained with Masson's Trichrome demonstrating early lesions (left panel), and complex multilayered lesions with large cholesterol clefts (right panel), typical of those from both SCN⁻-supplemented and control mice. Scale line for both panels 200 μm. (C) Mean values for gain in mouse weight (grams per day) for the SCN⁻-supplemented (black bar) and control mice (white bar) over the course of the study. Statistical analysis was carried out in (A) by two-tailed *t*-test, and (C) two-way ANOVA with Bonferroni post-test; * *P* < 0.05.

Figure 2.

Serum MPO substrates in SCN⁻-supplemented (○) and control (■) mice. (A) MPO protein over study time course; (B) Thiocyanate (SCN⁻) over study time course; (C) Nitrite (NO₂⁻) at sacrifice. Data in (A-B) are means ± SEM, with statistical analysis by two-way ANOVA with Bonferroni post-test; statistical analysis in (C) by two-tailed *t*-test; ** *P*<0.01; *** *P*<0.001.

Figure 3.

Serum protein thiol and lipid levels in MPO-expressing LDLR^{-/-} mice fed a high-fat diet over the study time course. (A) Thiols; (B) Cholesterol; (C) Triglycerides. (○) SCN⁻ supplemented; (■) control mice. Data are means ± SEM; statistical analyses by two-way ANOVA with Bonferroni post-test; ** $P < 0.01$.

Table 1

Correlation analyses comparing (A) normalized aortic root plaque areas to the serum parameters measured over the 12-week duration of the study, and (B) SCN⁻ to other parameters. AUC, area under curve. Any mouse with missing data points was excluded. Statistically-significant correlations are shown in bold (two-tailed Pearson correlations, except plaque area vs. SCN⁻ and cholesterol, which were one-tailed).

Parameter	Pearson r (P value)
(A) Normalized plaque areas vs.:	
Thiocyanate AUC (n=15)	-0.5693 (0.0134)
Thiols AUC (n=16)	0.1875 (0.5034)
MPO AUC (n=19)	-0.1857 (0.4755)
Cholesterol AUC (n=18)	0.4742 (0.0468)
Triglycerides AUC (n=18)	0.2393 (0.3388)
(B) Thiocyanate AUC vs.:	
Thiols AUC (n=15)	-0.2647 (0.3405)
MPO AUC (n=16)	0.0261 (0.9236)
Cholesterol AUC (n=16)	-0.2526 (0.3451)
Triglycerides AUC (n=16)	-0.3668 (0.1623)