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Hydrogen sulphide: A novel physiological inhibitor of LDL atherogenic modification by HOCl

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Abstract

Hypochlorite (HOCl), the product of the activated myeloperoxidase/H_2O_2/chloride (MPO/H_2O_2/Cl^-) system is favored as a trigger of LDL modifications, which may play a pivotal role in early atherogenesis. As HOCl has been shown to react with thiol-containing compounds like glutathione and *N*-acetylcysteine protecting LDL from HOCl modification, we have tested the ability of hydrogen sulfide (H_2S)—which has recently been identified as an endogenous vasorelaxant—to counteract the action of HOCl on LDL. The results show that H_2S could inhibit the atherogenic modification of LDL induced by HOCl, as measured by apolipoprotein alterations. Beside its HOCl scavenging potential, H_2S was found to inhibit MPO (one may speculate that this occurs via H_2S/heme interaction) and destroy H_2O_2. Thus, H_2S may interfere with the reactants and reaction products of the activated MPO/H_2O_2/Cl^- system. Our data add to the evidence of an anti-atherosclerotic action of this gasotransmitter taking the role of HOCl in the atherogenic modification of LDL into account.

Keywords: *LDL, hypochlorite, hydrogen sulphide, atherosclerosis*

Introduction

The oxidative modification of low density lipoprotein (LDL) may play a role in early atherogenesis [1–4]. However, the reactions initiating LDL modification *in vivo* are still a matter of debate. In recent years, the activated myeloperoxidase/H_2O_2/Cl^-—system of neutrophils has been favored as a trigger of LDL atherogenic modifications *in vivo* [5,6]. HOCl, the reaction product of the MPO/H_2O_2/Cl^--system [7,8] has been found to modify LDL and HOCl-modified LDL has been detected in atherosclerotic plaques [9–12] supporting the possible *in vivo* relevance of the system. HOCl, a strong bio-reagent has been shown to be inactivated by vitamin C and some thiol-compounds like *N*-acetylcysteine and GSH which can inhibit LDL modification by HOCl [13,14].

Recently, hydrogen sulfide (H_2S) has been identified as a third endogenous gasotransmitter (beside CO and NO) [15]. Cystathionine ß-synthase (CBS, EC 4.2.1.22.) and cystathionine gamma-lyase (CSE, EC 4.4.1.1.) are responsible for the endogenous

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production of H_2S. Both enzymes use L-cysteine as substrate. Up to 100 μmol/l H_2S can be found in human serum [16]. One main physiological function of H_2S within the cardiovascular system is its vasorelaxant effect [17]. Results from recent work done by Li et al. [18] and Bhatia et al. [19,20] strongly suggest that H_2S plays an important role as endogenous pro-inflammatory agent. Mok et al. [21] pointed to a role of H_2S in hemorrhagic shock (induced in rats) as treatment with CSE inhibitors before or after shock induction produced a rapid, partial restoration in arterial blood pressure and heart rate. Yusuf et al. [22] reported on enhanced tissue hydrogen sulfide biosynthesis in streptozotocin-induced diabetes in the rat and the authors propose that this may have vasculoprotective effects due to vasodilatation [17], effect on NO [23], smooth muscle cell proliferation inhibition [24] and scavenging peroxynitrite [25].

H_2S which can reach relatively high concentrations in the brain, has been found to counteract toxic effects of HOCl on neuronal cells [26]. Thus, one may speculate that the novel gasotransmitter H_2S may have the potential to inhibit the atherogenic modification of LDL induced by HOCl. In the present paper we report that H_2S can interfere with all the reactants and the reaction product of the activated ($MPO/H_2O_2/Cl^-$) system.

Materials and methods

Materials

Sodium hydrogen sulfide (NaHS) was from Aldrich. Sodium hypochlorite was from Merck. Cysteine, homocysteine, methionine, taurine, guaiacol, DNPH (2,4-dinitrophenylhydrazine) and TMB (3,3′,5,5′-tetramethylbenzidine) was obtained from Sigma. Myeloperoxidase (MPO, EC 1.11.1.7) was supplied by Calbiochem.

H_2S generation

NaHS was used to generate H_2S in solution. H_2S concentration was taken as 30% of the NaHS concentration according to R. Beauchamp et al. [27].

Lipoprotein isolation

LDL preparations were isolated by ultracentrifugation as reported previously [28]. The final preparations were dialyzed against 150 mmol/l NaCl containing 0.1 mmol/l EDTA or subjected to gel chromatography to get rid of KBr and filter sterilized. Protein was estimated by a modified Lowry method [29] using bovine serum albumin as a standard. All LDL concentrations are given as milligram protein/milliliter.

LDL modification by HOCl

LDL 1 mg/ml PBS containing 100 μmol/l DTPA was incubated for 1 h at 37°C in the absence or presence of 1 mmol/l HOCl (molar ratio LDL/HOCL 1:500). Reagent HOCl concentration was estimated spectrophotometrically as reported [30].

Preparation of taurine chloramine

Taurine 1 mmol/1 PBS pH 7.4 was reacted with 50 μmol/l HOCl for 30 min at 37°C.

Estimation of LDL modification

Apolipoprotein modification was estimated by altered relative electrophoretic mobility (REM) of the LDL particle [31] and determination of protein carbonyls [32].

Electrophoresis

At the end of incubations samples (≈2 μg protein) of untreated or treated LDL were applied to cellulose acetate stripes (Cellogel-E, Biolab) and electrophoresed at 200 V for 15 min. Protein was stained with Ponceau Red S.

ApoB carbonyl assay

LDL 1 mg/ml PBS containing 100 μmol/l DTPA was incubated for 1 h at 37°C in the absence or presence of 1 mmol/l HOCl in the absence or presence of the respective thio-compound. LDL protein carbonyl concentration was estimated with 2,4-dinitrophenylhydrazine (DNPH) according to Yan et al. [32].

Myeloperoxidase activity

MPO activity was estimated in 50 mmol/l phosphate buffer pH 7.0 containing 0.56 mmol/l H_2O_2 and guaiacol (100 mmol/l) as the substrate according to Desser et al. [33]. Five microliter of the respective enzyme solution (0.1 μmol/l MPO in PBS) was added to 500 μl of substrate/H_2O_2 and after incubation for 1 min at 25°C the absorbance was read at 470 nm and enzyme activity calculated. NaHS at the highest concentration used (300 μmol/l) in the MPO-inhibition experiments (preincubation) did not interfere with the assay when present at 3 μmol/l.

Estimation of H_2O_2

H_2O_2 was estimated using the ferrous oxidation in xylenol orange assay (FOX assay) [34].

Fifty microliter of sample was mixed with 450 μl of FOX reagent and after 30 min at room temperature absorbance was estimated at 560 nm.

Estimation of HOCl and chloramine

HOCl and taurine chloramine were estimated by the TMB (3,3′,5,5′-tetramethylbenzidine) assay reported by Dypbukt [35]. Two hundred microliter of the respective sample was mixed with 50 μl of 2 mmol/l TMB in 400 mmol/l acetate buffer, pH 5.4, containing 10% dimethylformamide and 100 μmol/l sodium iodide. After 10 min the absorbance was measured at 650 nm in a plate reader. As higher concentrations of residual NaHS can reduce the TMB oxidation product, the NaHS concentration in the HOCl and chloramine scavenging experiments did not exceed the respective HOCl or chloramine concentration (both 50 μmol/l).

Statistics

The results are presented as means ± standard deviation (SD) of three experiments. Specific effects were evaluated by one-way analysis of variance (ANOVA) with *post hoc* testing using Newman–Keuls' test. A value of $p < 0.05$ was considered statistically significant. If no error bars are evident these are smaller than the symbols used.

Results

Effect of H_2S or thio-amino acids on LDL modification induced by HOCl

Figure 1 shows the effect of H_2S, cysteine, homocysteine and methionine on LDL modification by HOCl as analyzed by REM. Increased REM was completely reduced in presence of 3 and 1.5 mmol/l NaHS. At a molar ratio of HOCl/NaHS of 1/0.75 (mmol/l) REM was reduced to 50%. Cysteine, homocysteine and methionine at 1 and 0.5 mmo/l were very effective in inhibiting HOCl induced LDL protein modification.

Figure 2 depicts the effect of H_2S and thio-amino acids on HOCl induced carbonyl formation in LDL.

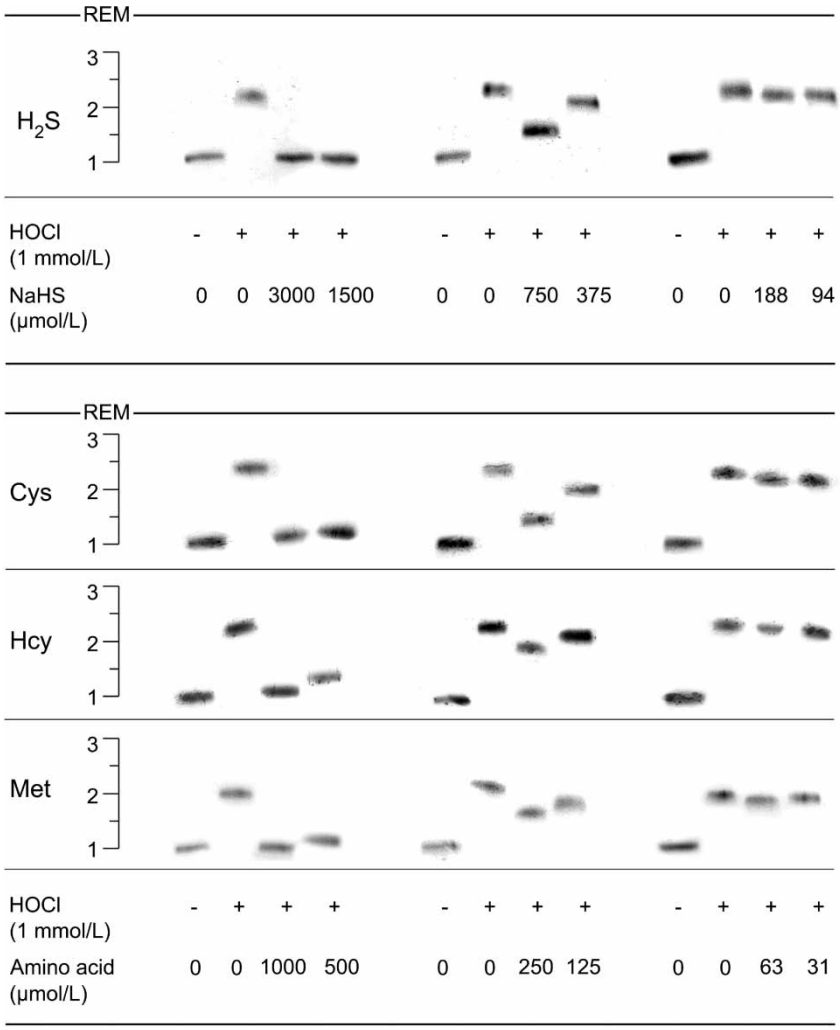

Figure 1. Effect of H_2S and thio-amino acids on HOCl induced apolipoprotein modification estimated by REM. LDL (1 mg/ml PBS containing 100 μmol/L DTPA) was incubated for 1 h at 37°C in the absence or presence of 1000 μmol/L HOCl and the respective concentration of NaHS, cysteine (Cys), homocysteine (Hcy) or methionine (Met). At the end of incubation samples were subjected to electrophoresis as given in Methods.

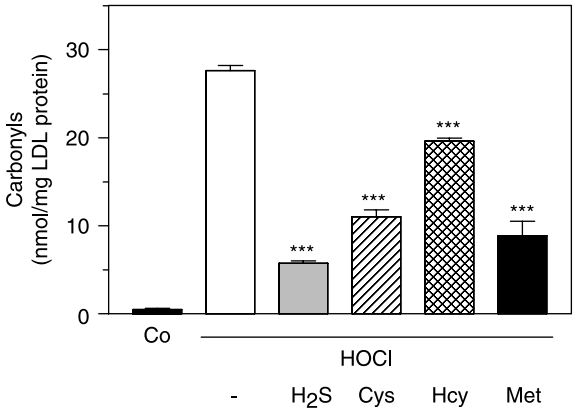

Figure 2. Effect of H_2S and thio-amino acids on HOCl induced carbonyl formation in LDL. LDL (1 mg/ml) was treated with 1 mmol/L HOCl in the absence or presence of 750 μmol/L NaHS or 250 μmol/L cysteine (Cys), homocysteine (Hcy) or methionine (Met) for 60 min at 37°C. Carbonyls were then measured as described in Methods ($n = 3$). *** $p < 0.001$ vs. LDL treated with HOCl.

When LDL (1 mg/ml) was treated with 1 mmol/l HOCl for 60 min at 37°C carbonyl concentration increased from 0.51 ± 0.13 to 27.6 ± 0.56 nmol/mg protein. NaHS like all other thio-componds when present during HOCl treatment was effective in protecting LDL from HOCl induced carbonyl formation.

Effect of H_2S on reactants and reaction products of the MPO/H_2O_2/Cl^- system

Influence on MPO. Figure 3 depicts the influence of H_2S on MPO enzyme activity. When MPO (0.1 μmol/l) was preincubated with NaHS for 60 min at 37°C and subsequently the enzyme activity was estimated the results showed that MPO is inactivated by hydrogen sulfide.

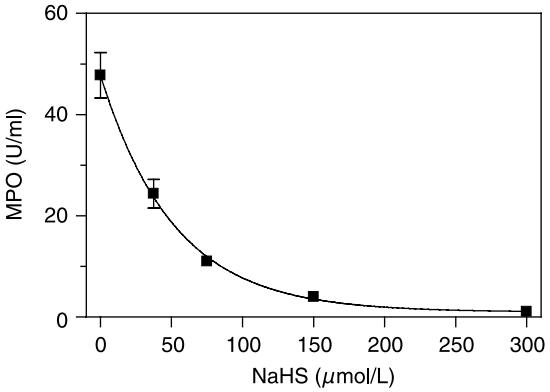

Figure 3. Effect of H_2S on MPO activity. MPO (0.1 μmol/L) was preincubated with NaHS (0–300 μmol/L) for 60 min at 37°C and subsequently enzyme activity was estimated as described in Methods ($n = 3$).

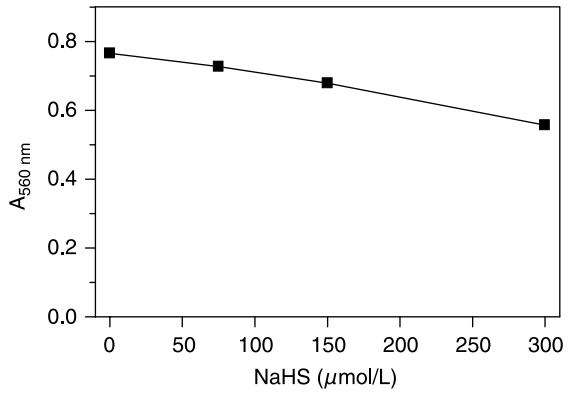

Figure 4. Influence of H_2S on decomposition of H_2O_2. H_2O_2 (100 μmol/l PBS) was incubated with 0–300 μmol/l NaHS for 30 min at 37°C and residual H_2O_2 was estimated with the FOX assay as described in Methods ($n = 3$).

Influence of H_2S on decomposition of H_2O_2. H_2O_2 is known to be destroyed by H_2S [36]. When H_2O_2 (100 μmol/l PBS) was incubated with increasing concentrations of NaHS for 30 min at 37°C about 30% of H_2O_2 was destroyed by the highest concentration of NaHS used (see Figure 4).

Scavenging of HOCl by H_2S. Fifty micromolar per liter HOCl (50 μmol/l) was incubated NaHS for 30 min at 37°C and the residual HOCl estimated. As seen in Figure 5 HOCl was completely destroyed in presence of equimolar concentrations of NaHS.

Scavenging of chloramine by H_2S. Taurine chloramine (50μmol/1) was reacted with increasing concentrations of NaHS for 30 min at 37°C and residual chloramine estimated. Figure 6 shows that NaHS could also scavenge chloramines.

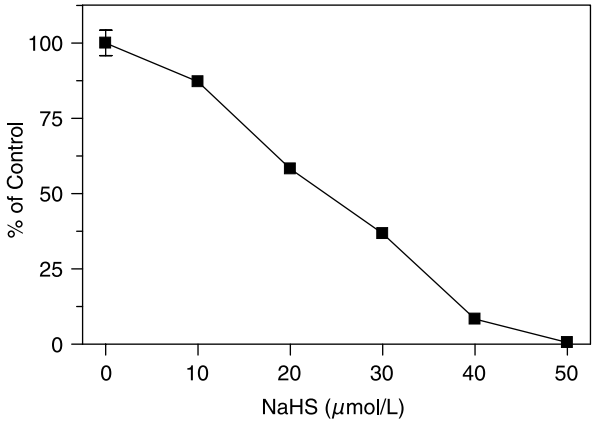

Figure 5. Scavenging of HOCl by H_2S. HOCl (50 μmol/l PBS) was incubated with 0–50 μmol/l NaHS for 30 min at 37°C and residual HOCl was estimated with the TMB assay as described in Methods ($n = 3$).

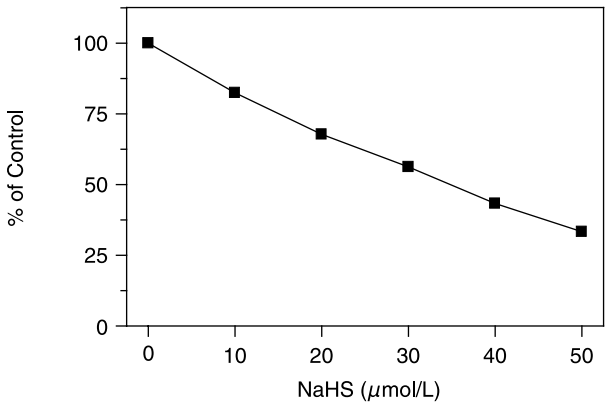

Figure 6. Scavenging of chloramine by H_2S. Taurine chlormanine (50 μmol/l PBS) was incubated with 0–50 μmol/l NaHS for 30 min at 37°C and residual chloramine was estimated with the TMB assay as described in Methods ($n = 3$).

Discussion

HOCl is the reaction product of the activated MPO/H_2O_2/Cl^--system [7,8] and has been suggested as a relevant *in vivo* agent inducing the atherogenic modification of LDL [9]. Indeed, HOCl modified LDL has been found in atherosclerotic plaques [11,12,37] and increased MPO activity has been correlated to cardiovascular disease [38]. HOCl induces the oxidation (modification) of amino acids, peptides, proteins and other biomolecules like nucleotides and DNA [39,40]. In proteins, the thio-amino acids have been found to be the most reactive structures [41]. Beside MPO-inhibitors, HOCl traps like glycine, taurine and methionine have been suggested as therapeutic agents [42]. Thiol-compounds like GSH, *N*-acetylcysteine and captopril have been found to inactivate HOCl and inhibit LDL modification by HOCl [13]. Recently, H_2S has been shown to inhibit HOCl induced brain injury [26]. Thus, one may speculate that H_2S may protect LDL from atherogenic modification by HOCl.

In almost all *in vitro and in vivo* studies investigating the biochemical/biological effects of H_2S, NaHS is used to generate H_2S. NaHS in solution forms Na^+ and HS^- which associates to H_2S. In solution one-third of NaHS exists as H_2S and the remaining two-thirds as HS^- at equilibrium with H_2S [27].

The results show that Apo B modification (measured by REM and carbonyl formation) in LDL induced by HOCl (1 mmol/l) was effectively counteracted in presence of H_2S. The HOCl scavenging property of H_2S was also verified in the TMB assay. It should be noted that *in vivo* activated neutrophils can produce 20–400 μmol/l HOCl [43] or even higher [44].

In general, modulation of the activated MPO/H_2O_2/Cl^--system may—beside HOCl trapping—involve inhibition of MPO activity, inhibition of H_2O_2 production and H_2O_2 inactivation/decomposition. Therefore, the influence of H_2S on MPO activity has been tested. The results showed that MPO was inactivated in presence of H_2S. Claesson et al. have shown that H_2S did inhibit PMN cell extracted MPO, but did not inhibit MPO activity in intact cells [45]. MPO is a heme containing enzyme. As H_2S has been shown to react with heme proteins like hemoglobin modulating oxygen binding by forming sulfhemoglobin [46]—although not experimentally proven at present—one may speculate that MPO inactivation may be mediated via H_2S/heme interactions. In presence of H_2S increased decomposition of H_2O_2 was observed over the time period and the molar ratios tested. This is in accordance with Geng and coworkers [36] who reported on the H_2O_2 and superoxide anion scavenging property of H_2S in the regulation of isoproterenol induced myocardial injury.

In contrast to the thio-amino acid/HOCl reaction products [39] at present the product(s) of the H_2S/HOCl reaction is (are) not known [26]. In this respect it is of interest that supernatants of activated neutrophils have been found to convert H_2S into sulfite [47]. If this is attributed to HOCl it warrants further investigations.

In summary the present data indicate, that H_2S which reaches levels of about 50 and 100 μmol/l in the vasculature in rats [17] and in humans [16] can act as a potent inhibitor of LDL atherogenic modification by the activated MPO/H_2O_2/Cl^- -system as H_2S can interfere with both, the reactants (MPO and H_2O_2) and the reaction product (HOCl) of the system. In this respect it is noteworthy that plasma H_2S levels were significantly lowered in patients with coronary heart disease as compared to angiographically normal controls [48].

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