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# Hydrogen sulfide destroys lipid hydroperoxides in oxidized LDL

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Markus K. MUELLNER <sup>\*</sup>, Sabine M. SCHREIER <sup>\*</sup>, Hilde LAGGNER <sup>\*</sup>, Marcela HERMANN <sup>†</sup>, Harald ESTERBAUER <sup>‡</sup>, Markus EXNER <sup>§</sup>, Bernhard M.K. GMEINER <sup>\* 1</sup>  
and Stylianos KAPIOTIS <sup>‡, □</sup>

<sup>\*</sup> *Department of Medical Chemistry, CPP, Medical University Vienna, Vienna, Austria*

<sup>†</sup> *Department of Medical Biochemistry, MFPL, Medical University Vienna, Vienna, Austria*

<sup>‡</sup> *Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University Vienna, Vienna, Austria*

<sup>§</sup> *Labor Gruppenpraxis Bauer, Spitzauer & Partner, Vienna, Austria*

<sup>□</sup> *The Central Laboratory, Hospital of the Divine Redeemer, Vienna, Austria*

<sup>1</sup> Correspondence: Bernhard M.K. Gmeiner MD, PhD, CPP, Department of Medical Chemistry, Medical University Vienna, Waehringerstr. 10, A-1090 Vienna, Austria.  
Tel.: +43-1-4277-60825, e-mail: [bernhard.gmeiner@meduniwien.ac.at](mailto:bernhard.gmeiner@meduniwien.ac.at)

**ABSTRACT**

Lipid hydroperoxides (LOOHs) in oxidized LDL (oxLDL) are potentially atherogenic compounds. Recently, H<sub>2</sub>S was identified as the third endogenous gasotransmitter in the vasculature. Hydrogen peroxide is known to be destroyed by H<sub>2</sub>S. Assuming that H<sub>2</sub>S may also react with LOOHs, the results show that H<sub>2</sub>S can destroy LOOHs in oxLDL. The ability of LOOH-enriched LDL to induce HO-1 in endothelial cells was abolished by H<sub>2</sub>S pretreatment. HPLC analysis showed that 9-HPODE (9-hydroperoxy-octadecadienoic acid), a compound found in oxLDL, was reduced to 9-HODE (9-hydroxy-octadecadienoic acid) in presence of H<sub>2</sub>S. Thus, H<sub>2</sub>S may act as an antiatherogenic agent by reducing LOOHs to the less reactive LOHs and could abrogate the pathobiological activity of oxLDL.

Key words: LDL oxidation; lipid hydroperoxides; hydrogen sulfide; atherosclerosis; 9-HODE; 9-HPODE

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## INTRODUCTION

The oxidative modification of LDL may play a role in atherogenesis [1-4]. The formation of the potentially atherogenic lipid hydroperoxides (LOOHs) in LDL is an early event in LDL lipid oxidation. Thus, destroying LOOHs in LDL can limit its pathobiological potential.

Recently, H<sub>2</sub>S has been identified as a third endogenous gasotransmitter in the vasculature (beside CO and NO) [5]. As H<sub>2</sub>S is known to destroy hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [6-8] one may speculate that H<sub>2</sub>S may also have the potential to destroy LOOHs in LDL, abrogating the pathobiological activity of oxLDL.

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## EXPERIMENTAL

### Materials

Sodium hydrogen sulfide (NaHS), triphenylphosphine (TPP) and xylenol orange were from Sigma-Aldrich. H<sub>2</sub>O<sub>2</sub> (30 % solution) was supplied by Merck.

9-HPODE (9S-hydroperoxy-10E, 12Z-octadecadienoic acid) and 9-HODE (9S-hydroxy-10E, 12Z-octadecadienoic acid) were delivered by Cayman Chemicals, Ann Arbor, USA as ethanolic solutions (1 mg/mL).

### Methods

#### H<sub>2</sub>S generation

NaHS was used to generate H<sub>2</sub>S in solution. H<sub>2</sub>S concentration was taken as 30 % of the NaHS concentration according to Beauchamp et al. [9].

#### Lipoprotein isolation

LDL preparations were isolated by ultracentrifugation as reported previously [10]. The final preparations were subjected to gel chromatography to get rid of KBr and filter sterilized. Protein was estimated by a modified Lowry method [11]. All LDL concentrations are given as mg protein/mL.

#### LDL oxidation

Prior to LDL oxidation, the lipoprotein was passed over a gel column equilibrated in PBS pH 7.4. Routinely, LDL (0.2 mg/mL PBS) was incubated for 2 hours in the presence of 10 μmol/L Cu<sup>++</sup> at 37 °C and the reaction was stopped by the addition of EDTA (100 μmol/L). Alternatively LDL (1 mg/mL, 10 mmol/L MOPS, 150 mmol/L NaCl pH 7.4) was dialysed for 24 hours at 4 °C against MOPS buffer containing 5 μmol/L Cu<sup>++</sup> and

oxidation was stopped by dialysis against PBS containing 100  $\mu\text{mol/L}$  EDTA for 24 hours at 4 °C according to Gerry et al. [12].

#### H<sub>2</sub>S treatment of oxidized LDL samples

Cu<sup>++</sup> oxidized LDL was incubated at 37° C in the absence or presence of NaHS for up to 1 hour. For cell experiments, samples of Cu<sup>++</sup> oxidized LDL (0.2 mg/mL) were brought to 0.4 mg/mL by the addition of native LDL and after incubation with or without NaHS for 30 min at 37 °C the preparations were subjected to gel chromatography to get rid of NaHS.

#### Estimation of LDL oxidation

##### *Estimation of lipid peroxides*

LOOHs were estimated using the ferrous oxidation in xylenol orange assay (FOX assay) [13]. 50  $\mu\text{L}$  of sample was mixed with 450  $\mu\text{L}$  of FOX reagent and after 30 min at room temperature absorbance was estimated at 560 nm. Hydrogen peroxide was used to calculate concentrations. NaHS at the highest concentrations used did not interfere with the assay as no inhibition of color development was observed when NaHS was added to FOX-reagent/LOOH mixtures.

##### *Diene formation*

LDL oxidation was monitored as the increase in conjugated diene formation by measuring  $A_{234\text{ nm}}$  [14].

##### *Measurement of malondialdehyde*

TBARS formation in LDL was measured as reported [15]. NaHS did not interfere with TBARS formation as tested using pure substance malondialdehyde.

## Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as reported previously [16].

## Real time PCR

Isolation of total RNA, cDNA synthesis and quantitative PCR were performed as described [17]. Primers for real-time PCR were designed using the Primer3 software at [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and exon junction primers were selected to avoid amplification of residual DNA contaminations (heme oxygenase-1: 5'-AGACGGCTTCAAGCTGGTGA-3' and 5'-TCTCCTTGTTGCGCTCAATCTC-3'; acidic ribosomal phosphoprotein P0 5'-CTCGCCAGGCGTCCTCGT-3' and 5'-TTGCCCATCAGCACCACAG-3'). For normalization mean threshold cycles ( $C_t$ -values) of all replicate analyses were normalized to RPLP0 within each sample to obtain sample-specific  $\Delta C_t$  values ( $= C_t \text{ HMOX1} - C_t \text{ RPLP0}$ ). To compare the effect of various treatments with untreated controls  $2^{-\Delta\Delta C_t}$  values were calculated to obtain fold expression levels, where  $\Delta\Delta C_t = (\Delta C_t \text{ treatment} - \Delta C_t \text{ control})$ . Real-time analysis using primers specific for cyclophilin b (GenBank NM\_000942) as reference standard, and normalization for equal amounts of total RNA input (1  $\mu\text{g}$  for each sample) gave comparable results (data not shown).

### HPLC separation of standard 9-HODE and 9-HPODE

Separations were performed by injecting 5  $\mu\text{L}$  of 64  $\mu\text{mol/L}$  HPODE for time-effect or 15  $\mu\text{L}$  of 21.3  $\mu\text{mol/L}$  HPODE for concentration-effect experiments into the HPLC (Waters 2695 Alliance system, Nova-Pak C18 3.9 x 150 mm column, 4  $\mu\text{m}$ ) with a mobile phase of 0.3 %  $\text{H}_3\text{PO}_4$ /acetonitrile (40:60 v/v) for 30 min with a flow of 1 mL/min. UV absorbance was monitored at 210 nm and 234 nm in dual wavelength mode. HPODE and HODE were prepared in PBS (chelexed). Substances were quantified via peak area.

### HPLC analysis of oxidized LDL

Lipid oxidation products were determined by HPLC analysis of saponified lipids extracted from LDL according to Thomas and Jackson [18]. In brief, 200  $\mu\text{g}$  of LDL oxidized by the dialysis method (see above) was extracted with 2 mL ether-ethanol (3:1) and protein precipitated by centrifugation. Decanted extracts were dried under nitrogen and subsequently lipid dissolved in 0.5 mL ethanol/0.05 mL 10 N NaOH. After saponification (20 min at 60  $^\circ\text{C}$ ) samples were neutralised and dried under nitrogen. Water was added and lipids extracted into heptane. Dried heptane phases were dissolved in ethanol and subjected to HPLC analyses as outlined above.

### Statistics

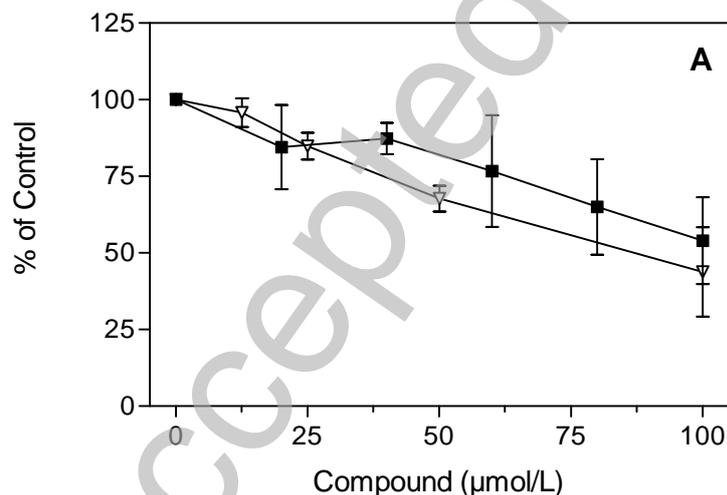
The results are presented as mean values of two to five experiments. As appropriate specific effects were evaluated by one-way analysis of variance (ANOVA) with *post hoc* testing using Newman-Keuls' test. A value of  $p < 0.05$  was considered statistically significant.

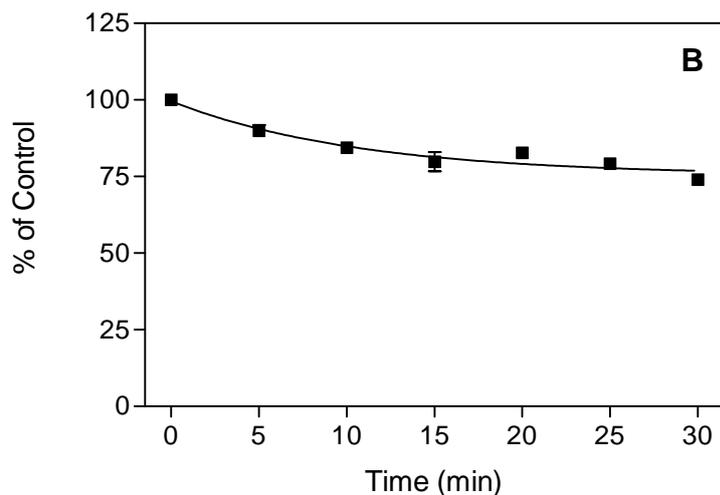
## RESULTS

### Influence of H<sub>2</sub>S on lipid hydroperoxides in oxLDL

As can be seen in figure 1 when samples of oxLDL were incubated with NaHS for 60 min at 37 °C a concentration dependent decrease in LOOHs was observed. About 50 % ( $p < 0.01$ ) of LOOHs were destroyed by 100  $\mu\text{mol/L}$  H<sub>2</sub>S treatment (figure 1A). Triphenylphosphine (100  $\mu\text{mol/L}$ ) which is known to reduce LOOHs [19, 20] were run as a control and showed comparable results ( $p < 0.001$ ). Figure 1B depicts the time dependent influence of H<sub>2</sub>S on LOOHs. Under the conditions employed after 30 min of H<sub>2</sub>S treatment about 25 % of the LOOHs in oxLDL were destroyed ( $p < 0.001$  for all time points versus control).

In addition to the LOOH destroying effect, H<sub>2</sub>S could directly inhibit copper ion induced LDL oxidation as monitored by diminished TBARS formation and increased lag-phase during conjugated diene kinetics (results not depicted).





**Figure 1: Influence of H<sub>2</sub>S treatment on LOOHs in oxLDL (A).**

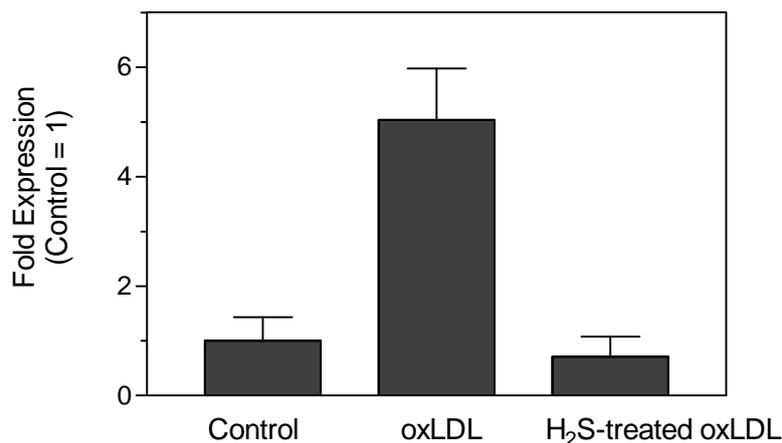
LDL was oxidized with Cu<sup>++</sup> and subsequently treated with H<sub>2</sub>S (■) or triphenylphosphine (▽) for 60 min at 37 °C and residual lipid hydroperoxides were estimated as given in Methods. 100 % = 70.6 ± 4.5 μmol/L LOOH (n=3).

**Time course of the effect of H<sub>2</sub>S on LOOHs in LDL (B).**

Cu<sup>++</sup> oxidized LDL (0.2 mg/mL PBS) was further incubated in the absence or presence of H<sub>2</sub>S (100 μmol/L) for the indicated time at 37°C and residual LOOHs were estimated. 100 % = 67.5 ± 0.5 μmol/L LOOH (n=3).

**H<sub>2</sub>S treatment of oxLDL reduces its ability to induce HO-1**

LOOHs in oxLDL can induce heme oxygenase 1 (HO-1) in vascular cells [21-23]. As seen in figure 2 oxLDL (100 μg/mL for 18 hours) resulted in a 5 fold induction of HO-1 in HUVEC. Pretreatment of oxLDL with 1 mmol/L H<sub>2</sub>S for 30 min at 37 °C, which completely destroyed LOOHs (data not shown), abolished the ability of the LDL preparation to induce HO-1.

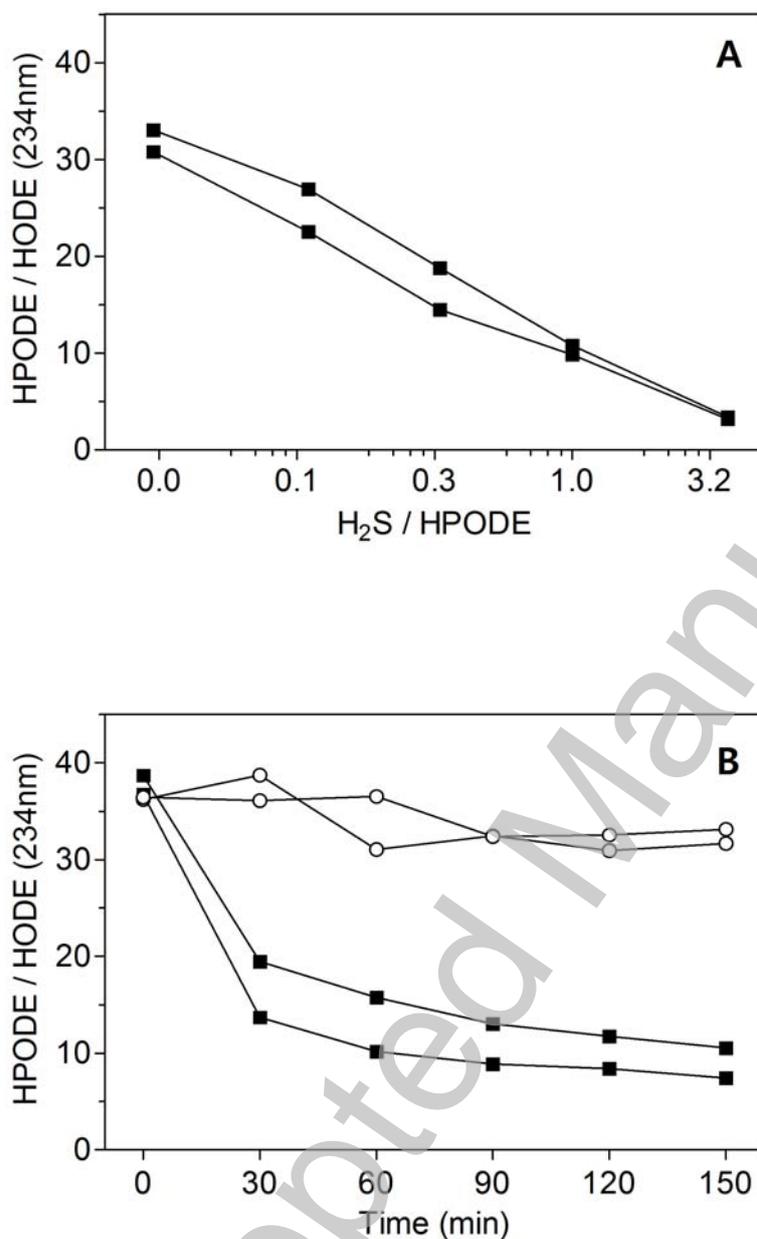


**Figure 2: H<sub>2</sub>S treatment reduces oxLDL induced heme oxygenase 1 in HUVEC.**

Cu<sup>++</sup> oxidized LDL was treated with or without H<sub>2</sub>S (1 mmol/L) as given in Methods and LDL preparations (100 µg/mL) were added to cell monolayers. After 18 hours of incubation RNA was extracted and used for PCR analysis (n=3).

### Reduction of 9-HPODE to 9-HODE by H<sub>2</sub>S

9-HPODE is a fatty acid peroxidation product found in oxLDL [24]. Reduction of LOOHs to LOHs by sulfhydryl residues on paraoxonase have been reported by Aviram et al [25]. Thus, we analyzed the reaction product(s) of H<sub>2</sub>S with 9-HPODE by HPLC. When 9-HPODE was incubated for 30 min at 37 °C with H<sub>2</sub>S this treatment resulted in the formation of 9-HODE. The H<sub>2</sub>S concentration dependent reduction (expressed as ratio HPODE/HODE) is shown in figure 3A. Figure 3B depicts the time dependent conversion of 9-HPODE to 9-HODE in presence of H<sub>2</sub>S. About 50 % of 9-HPODE were reduced after 30 min.



**Figure 3: Concentration dependent reduction of 9-HPODE to 9-HODE by H<sub>2</sub>S (A).**

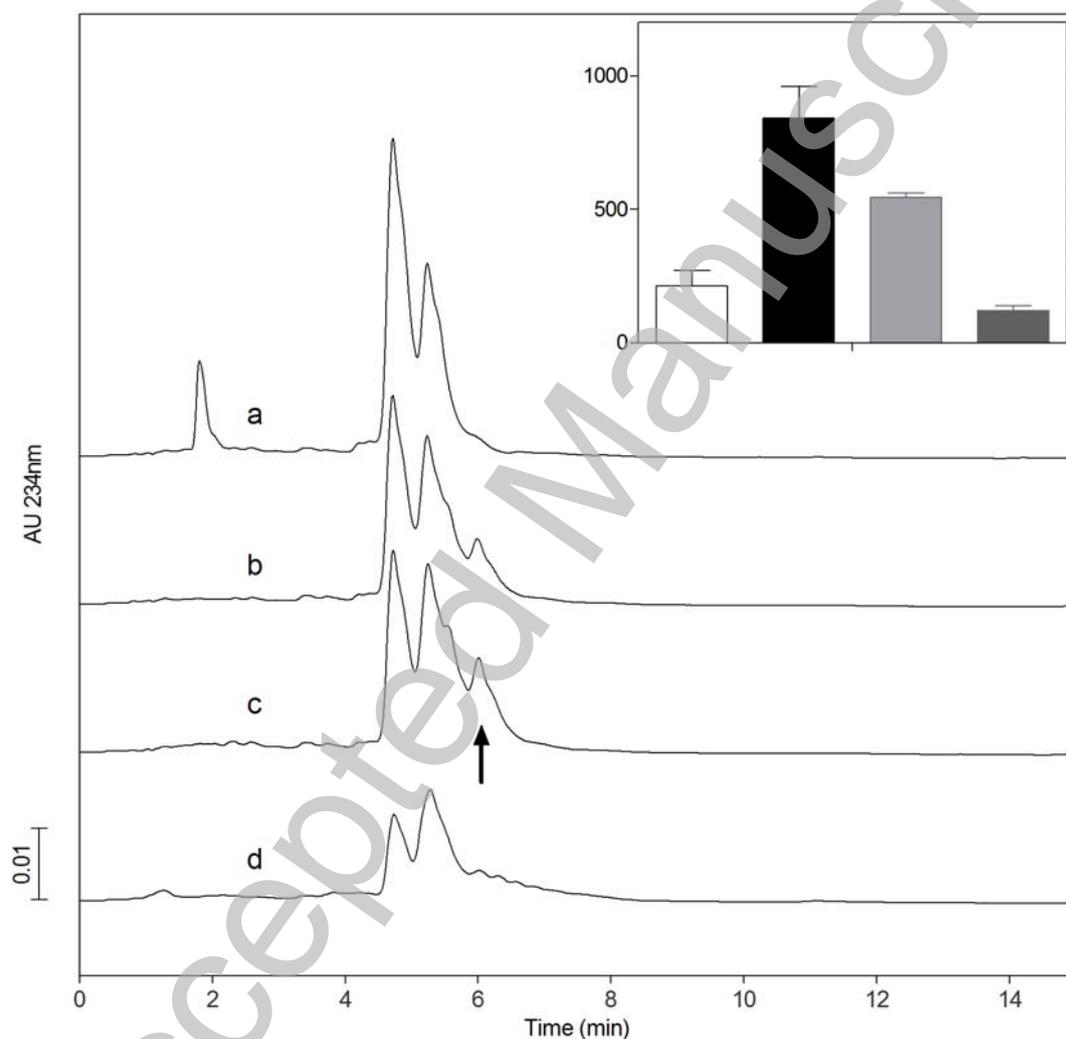
9-HPODE (21.3  $\mu\text{mol/L}$  PBS) was incubated with the respective concentration of H<sub>2</sub>S relative to 9-HPODE for 30 min at 37 °C and the samples were analysed by HPLC as given in Methods (n=2).

**Time dependent reduction of 9-HPODE to 9-HODE by H<sub>2</sub>S (B).**

9-HPODE (64  $\mu\text{mol/L}$  PBS) was incubated at 37°C in the absence ( $\circ$ ) or presence of equimolar amounts of NaHS ( $\blacksquare$ ). At the indicated time samples were withdrawn and analysed by HPLC (n=2).

### Reduction of 9-HPODE in oxidized LDL by H<sub>2</sub>S

Figure 4 shows the influence of H<sub>2</sub>S treatment on 9-HPODE present in oxidized LDL. H<sub>2</sub>S treatment caused a significant decrease (as quantified via peak area) in 9-HPODE from  $842 \pm 118$  (ox-LDL) to  $544 \pm 18$  ( $p < 0.001$ ). Ebselen/GSH treatment run as a positive control of lipid hydroperoxide reduction [18] resulted in a decrease of about 85 % ( $121 \pm 18$ ).



**Figure 4: Reduction of 9-HPODE in oxidized LDL by H<sub>2</sub>S or ebselen treatment analysed by HPLC.**

Oxidized LDL (1 mg/mL) was incubated with H<sub>2</sub>S (1 mmol/L) or ebselen/GSH (100  $\mu$ mol/3 mmol/L) for 45 min at 37 °C and subsequently oxidized lipids were analysed after saponification as given in Methods. LDL (d), oxidized LDL (c), H<sub>2</sub>S treatment (b) and ebselen/GSH (a). The arrow indicates 9-HPODE. Inset: LDL (open bar), oxidized LDL (black bar), H<sub>2</sub>S treatment (light gray bar), ebselen treatment (dark gray bar). Substances were quantified via peak area (n=3).

## DISCUSSION

The third gaseous transmitter H<sub>2</sub>S has been shown to protect cells and proteins from oxidative modifications by peroxynitrite and HOCl [7, 26]. H<sub>2</sub>O<sub>2</sub> is also known to be destroyed by H<sub>2</sub>S [6-8]. Thus one may assume that H<sub>2</sub>S may also destroy organic hydroperoxides of pathobiological importance, like fatty acid hydroperoxides (LOOHs). Focusing on LOOHs in oxLDL the present data show that LOOHs were destroyed in presence of H<sub>2</sub>S. This resulted in the inability of oxLDL to induce HO-1 in endothelial cells. Both results indicate that H<sub>2</sub>S could abrogate the pathophysiological activity of oxLDL. Destroying LOOHs in oxLDL could limit its atherogenic potential as hydroperoxides can i) break down into lipid alkoxy (LO<sup>•</sup>) as well as peroxy radicals (LOO<sup>•</sup>) which both can attack and modify other biological molecules [27] are ii) cytotoxic and iii) break down to protein modifying aldehydes like malondialdehyde (MDA), 4-hydroxynonenal and 4-hydroxyhexenal [28, 29].

Paraoxonase (PON) associated with HDL has been implicated as an important factor in destroying LOOHs on LDL and may contribute to the beneficial effect of HDL in atherogenesis and cytoprotection of vascular cells [30]. The ability of PON to protect against LDL oxidation involves its free sulfhydryl group and not its arylesterase/paraoxonase activity [25] and the results revealed that the reduction of the levels of CE-LOOHs (cholesteryl ester hydroperoxides) by PON was due to the formation of CE-LOHs and the authors suggested a peroxidase-like action of PON on LOOHs [31]. Using 9-HPODE, an LOOH compound found in oxLDL [24], our results showed that in presence of H<sub>2</sub>S at concentrations that have been reported for *in vivo* (50–150 μmol/L) [32, 33], 9-HPODE was reduced to 9-HODE assuming the reaction:  $\text{LOOH} + \text{H}_2\text{S} + \text{O}_2 \gg \text{LOH} + \text{SO}_2 + \text{H}_2\text{O}$ .

However, recent work has suggested that levels of free H<sub>2</sub>S *in vivo* are much lower than previously thought [34]. On the other hand, as H<sub>2</sub>S can be stored as bound sulfur and released [35] one may speculate that the overall amount could be adequate to modulate

pathophysiological reactions.

In summary our results indicate that H<sub>2</sub>S beside PON may be an additional endogenous factor in reducing lipid hydroperoxides in LDL. An early event in atherogenesis is the trapping and oxidative modification of LDL in the subendothelial space [36] and H<sub>2</sub>S - which is nearby produced and released from smooth muscle cells [37] - may prevent or diminish the further atherogenic modifications of the lipoprotein.

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