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ABSTRACT

Lipid hydroperoxides (LOOHs) in oxidized LDL (oxLDL) are potentially atherogenic compounds. Recently, H₂S was identified as the third endogenous gasotransmitter in the vasculature. Hydrogen peroxide is known to be destroyed by H₂S. Assuming that H₂S may also react with LOOHs, the results show that H₂S can destroy LOOHs in oxLDL. The ability of LOOH-enriched LDL to induce HO-1 in endothelial cells was abolished by H₂S 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 H₂S. Thus, H₂S 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, H₂S has been identified as a third endogenous gasotransmitter in the vasculature (beside CO and NO) [5]. As H₂S is known to destroy hydrogen peroxide (H₂O₂) [6-8] one may speculate that H₂S 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. H₂O₂ (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

H₂S generation

NaHS was used to generate H₂S in solution. H₂S 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⁺⁺ 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⁺⁺ 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].

H₂S treatment of oxidized LDL samples

Cu⁺⁺ 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⁺⁺ 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 μL of sample was mixed with 450 μL 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, 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 ΔC_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 μg for each sample) gave comparable results (data not shown).

HPLC separation of standard 9-HODE and 9-HPODE

Separations were performed by injecting 5 μ L of 64 μ mol/L HPODE for time-effect or 15 μ L of 21.3 μ mol/L HPODE for concentration-effect experiments into the HPLC (Waters 2695 Alliance system, Nova-Pak C18 3.9 x 150 mm column, 4 μ m) with a mobile phase of 0.3 % H_3PO_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 μ 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 $^\circ$ 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

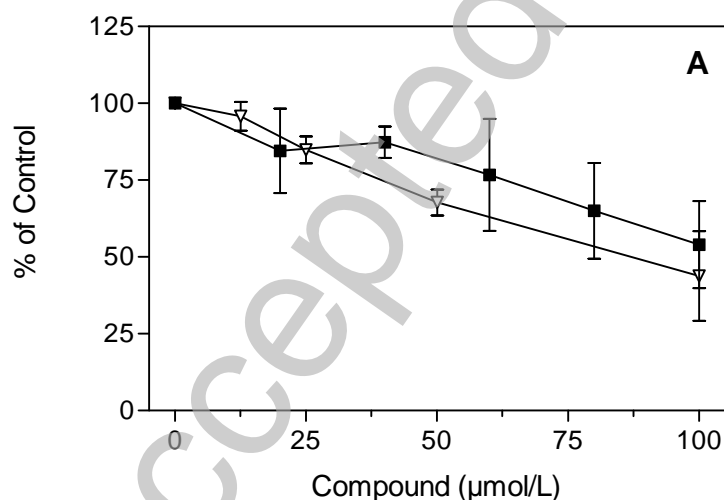
The results are presented as mean values of two to five experiments. As appropriate specific effects were evaluated by one-way analysis of variance (ANOVA) with *post hoc* testing using Newman-Keuls' test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Influence of H₂S 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 $\mu\text{mol/L}$ H₂S 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 H₂S on LOOHs. Under the conditions employed after 30 min of H₂S 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, H₂S 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).



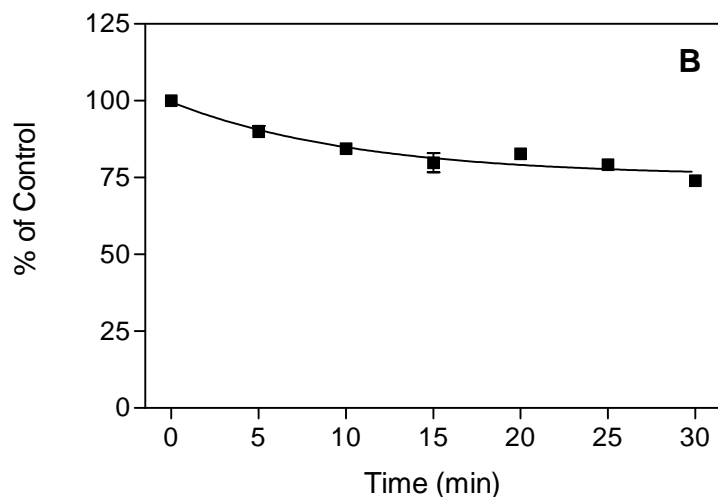


Figure 1: Influence of H₂S treatment on LOOHs in oxLDL (A).

LDL was oxidized with Cu⁺⁺ and subsequently treated with H₂S (■) 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 H₂S on LOOHs in LDL (B).

Cu⁺⁺ oxidized LDL (0.2 mg/mL PBS) was further incubated in the absence or presence of H₂S (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).

H₂S 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 H₂S for 30 min at 37 °C, which completely destroyed LOOHs (data not shown), abolished the ability of the LDL preparation to induce HO-1.

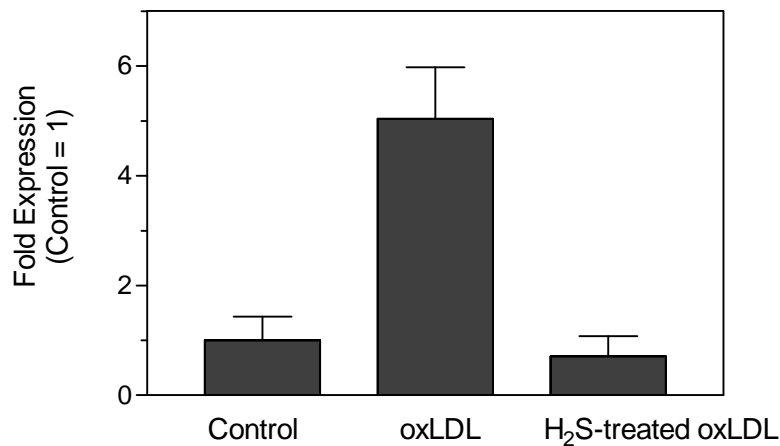


Figure 2: H₂S treatment reduces oxLDL induced heme oxygenase 1 in HUVEC.

Cu⁺⁺ oxidized LDL was treated with or without H₂S (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 H₂S

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 H₂S with 9-HPODE by HPLC. When 9-HPODE was incubated for 30 min at 37 °C with H₂S this treatment resulted in the formation of 9-HODE. The H₂S 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 H₂S. About 50 % of 9-HPODE were reduced after 30 min.

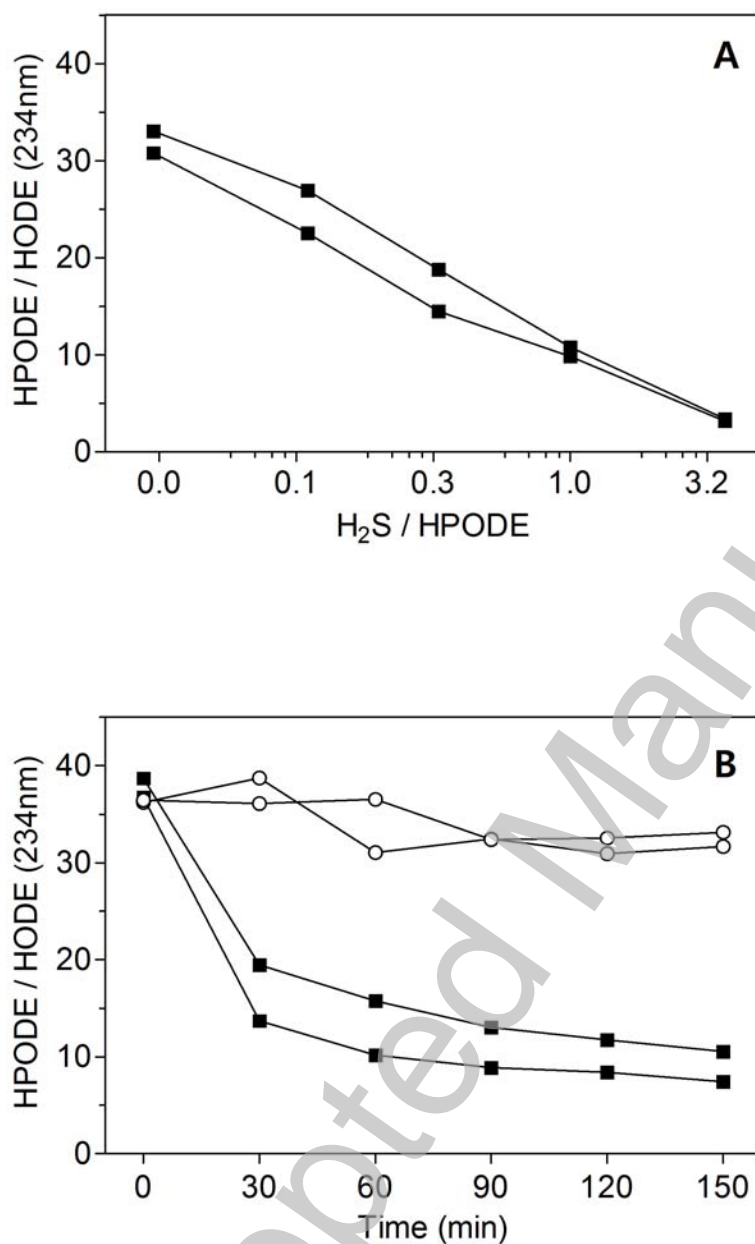


Figure 3: Concentration dependent reduction of 9-HPODE to 9-HODE by H₂S (A).

9-HPODE (21.3 $\mu\text{mol/L}$ PBS) was incubated with the respective concentration of H₂S 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 H₂S (B).

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

Reduction of 9-HPODE in oxidized LDL by H₂S

Figure 4 shows the influence of H₂S treatment on 9-HPODE present in oxidized LDL. H₂S treatment caused a significant decrease (as quantified via peak area) in 9-HPODE from 842 ± 118 (ox-LDL) to 544 ± 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 ± 18).

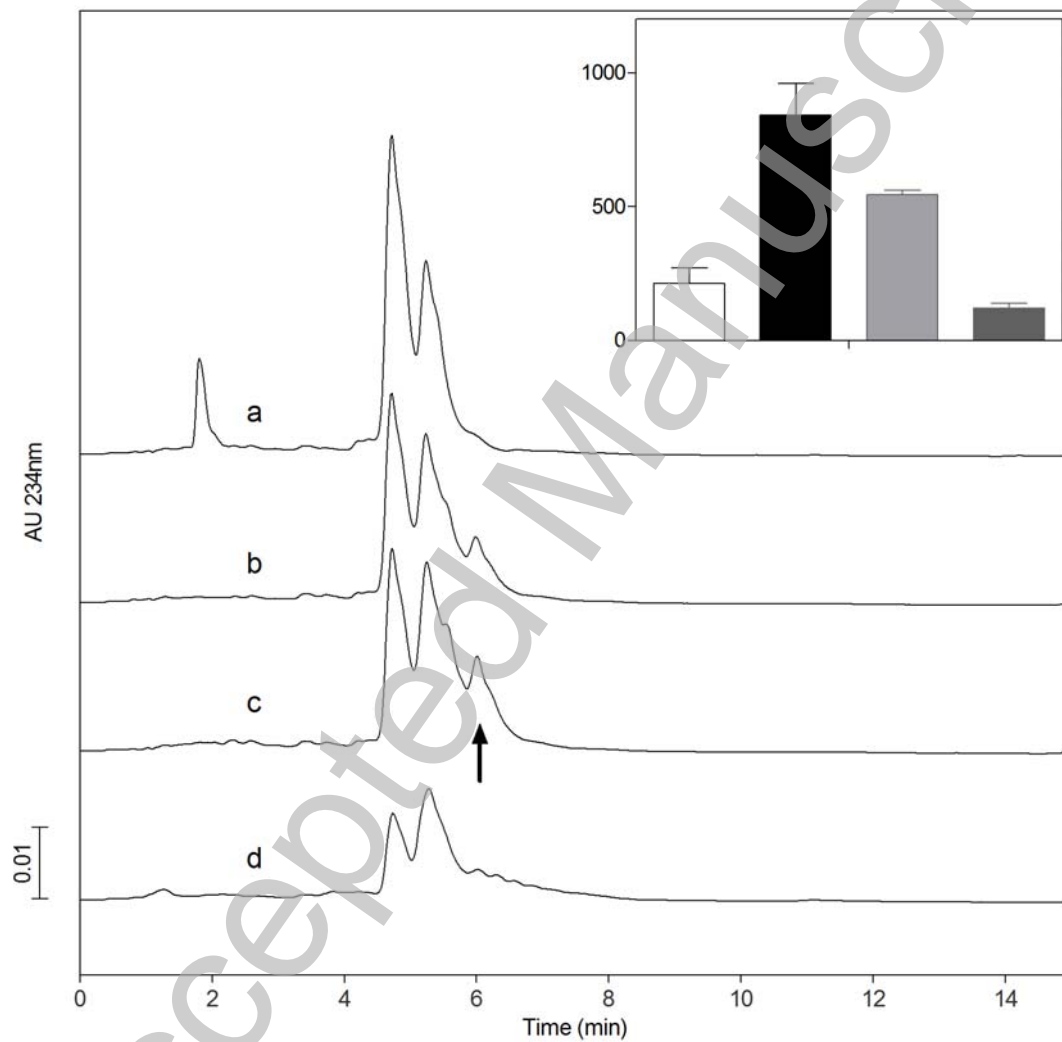


Figure 4: Reduction of 9-HPODE in oxidized LDL by H₂S or ebselen treatment analysed by HPLC.

Oxidized LDL (1 mg/mL) was incubated with H₂S (1 mmol/L) or ebselen/GSH (100 μ 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), H₂S treatment (b) and ebselen/GSH (a). The arrow indicates 9-HPODE. Inset: LDL (open bar), oxidized LDL (black bar), H₂S treatment (light gray bar), ebselen treatment (dark gray bar). Substances were quantified via peak area (n=3).

DISCUSSION

The third gaseous transmitter H₂S has been shown to protect cells and proteins from oxidative modifications by peroxynitrite and HOCl [7, 26]. H₂O₂ is also known to be destroyed by H₂S [6-8]. Thus one may assume that H₂S 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 H₂S. This resulted in the inability of oxLDL to induce HO-1 in endothelial cells. Both results indicate that H₂S 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[•]) as well as peroxy radicals (LOO[•]) 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 H₂S 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 H₂S *in vivo* are much lower than previously thought [34]. On the other hand, as H₂S 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 H₂S 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 H₂S - 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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