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Haemoglobin-induced oxidative stress is associated with both endogenous peroxidase activity and H_2O_2 generation from polyunsaturated fatty acids

TSAI-MU CHENG[1,2], SIMON J.T. MAO[1], SHIAU-TING LAI[3,4], CHIA-CHING CHANG[1,5], MING-CHI YANG[6], NAI-CHI CHEN[6], SHIU-CHING CHOU[7] & JU-PIN PAN[4,7]

[1]*College of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan, ROC,* [2]*Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, ON, Canada, K1A 0R6,* [3]*Division of Cardiovascular Surgery, Department of Surgery, Taipei Veterans General Hospital, Taipei, Taiwan, ROC,* [4]*School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC,* [5]*Institute of Physics, Academia Sinica, Taipei, Taiwan, ROC,* [6]*Life Science Group, Scientific Research Division, National Synchrotron Radiation Research Center, Hsinchu, Taiwan, ROC, and* [7]*Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*

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Abstract

Patients with increased haemolytic haemoglobin (Hb) have 10–20-times greater incidence of cardiovascular mortality. The objective of this study was to evaluate the role of Hb peroxidase activity in LDL oxidation. The role of Hb in lipid peroxidation, H_2O_2 generation and intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) was assessed using NaN_3, a peroxidase inhibitor, catalase, a H_2O_2 decomposing enzyme and human umbilical vein endothelial cells (HUVECs), respectively. Hb induced H_2O_2 production by reacting with LDL, linoleate and cell membrane lipid extracts. Hb-induced LDL oxidation was inhibited by NaN_3 and catalase. Furthermore, Hb stimulated ICAM-1 and VCAM-1 expression, which was inhibited by the antioxidant, probucol. Thus, the present study suggests that the peroxidase activity of Hb produces atherogenic, oxidized LDL and oxidized polyunsaturated fatty acids (PUFAs) in the cell membrane and reactive oxygen species (ROS) formation mediated Hb-induced ICAM-1 and VCAM-1 expression.

Keywords: *Hemoglobin, peroxidase, LDL peroxidation, cell adhesion, ICAM-1, VCAM-1, probucol*

Introduction

Rupture of red blood cells (RBCs) is often observed within vessels with haemorrhagic atheromatous plaques, which are found in patients with acute coronary syndromes and those undergoing invasive thrombolytic therapy for acute myocardial infarction [1,2]. Increased haemolytic haemoglobin (Hb) resulting from RBC rupture has been observed in patients undergoing hemo- and peritoneal dialysis [3] and cardiovascular mortality in these patients is 10–20-times greater as compared to the general population. Accordingly, cell-free Hb in the circulation is a significant risk factor for cardiovascular diseases. In addition, oxidative stress can be induced by the presence of cell-free Hb, leading to the formation of reactive oxygen species (ROS) [4–6] and early *in vivo* studies indicated that oxidative stress is involved in the oxidative modification of low-density lipoproteins (LDLs), resulting in the formation of foam cells associated with the development of cardiovascular diseases [7–9].

Recent studies suggest that Hb produces oxidized LDL *in vivo* [3,10,11]. Several speculative mechanisms by which Hb might exert its high oxidative reactivity toward LDL have been proposed. For example, one study reported covalent linkage of apoB

Correspondence: Ju-Pin Pan, MD, Division of Cardiology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC. Tel: +886-2-2871-2121 ext. 2358. Fax: +886-2-2876-3336. Email: jppan@vghtpe.gov.tw

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molecules upon exposure to Hb, thus forming apoB aggregates [12–15] that are atherogenic and induce foam-cell formation [10,14,16]. Others have pointed out that globin-radicals of Hb are at least partly responsible for LDL oxidation [13,17]. Notably, these studies were carried out in the presence of an exogenous source of H_2O_2. Because Hb also possesses a weak peroxidase activity [18], relative to conventional horse-radish peroxidase (HRP), the role of Hb in LDL oxidation remains interesting and elusive. Furthermore, the contribution of Hb to vascular cell changes other than LDL oxidation and its influence on atherogenesis has yet to be fully explored.

Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) play a significant role in the process of atherosclerosis as they ensure the recruitment of inflammatory cells [19] and are involved in the development of early, mature atherosclerotic plaques [20]. Expression of ICAM-1 and VCAM-1 is induced in the presence of heme/Hb [21–23] and is associated with Hb-induced oxidative stress [21]. Because ROS can induce the expression of ICAM-1 and VCAM-1 on the endothelial surface, cell-free Hb could act as a signal for the inflammatory process that takes place within the atherosclerotic plaque [24].

The purpose of the present study was 2-fold. First, the role of endogenous Hb-peroxidase activity in Hb-induced LDL lipid peroxidation was examined. Subsequently, the hypothesis that Hb might directly produce H_2O_2 from LDL and the cell membrane was examined. Second, Hb-mediated expression of ICAM-1 and VCAM-1 in cultured human umbilical vein endothelial cells (HUVECs) was also examined. In addition to the previously proposed mechanisms by which Hb induces LDL oxidation, the combined effect of the endogenous peroxidase activity of Hb to generate H_2O_2 from lipids may also play a crucial role in forming free radicals. Furthermore, cell-free Hb may also influence the cell-adhesion properties of endothelial cells.

Materials and methods

Lipoprotein isolation

LDL (d.1.019–1.063 g/mL) was isolated from human plasma (containing 0.01% sodium azide and 0.05% EDTA) by sequential ultracentrifugation according to the method previously established [25,26]. The LDL was exhaustively dialysed against phosphate buffered saline (PBS) containing 0.12 M NaCl and 12 mM phosphate (pH 7.2) to remove the EDTA and sodium azide and then passed through a 0.45 μm sterilized filter prior to the oxidation assays.

Lipid sample preparation

Stock solutions of commercial lipids, such as linoleic acid (linoeate; 0.9 mg/mL), palmitic acid (0.9 mg/mL) and 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC; 0.9 mg/m:), were dissolved in 50% glycerol to carry out the reactions with Hb.

Preparation and purification of human Hb

Hb was purified from human RBCs according to the procedure previously described [27]; it was purchased from Sigma-Aldrich (Steinheim, Germany) and was predominantly methemoglobin, metHb(FeIII). In brief, blood was collected in 0.1% EDTA and immediately centrifuged at 3000 ×g for 15 min. The remaining RBCs were washed five times with at least six volumes of PBS and then lysed with two volumes of deionized water at 4°C. Following removal of cell debris, the solution was fractionated using 60% saturated ammonium sulphate at 4°C for 30 min followed by centrifugation at 4500 ×g for 15 min at 4°C. The supernatant containing Hb was exhaustively dialysed against a 20 mM phosphate buffer (pH 8.0) at 4°C and was mixed with DE-52 cellulose (Whatman, Maidstone, UK) for 15 min and centrifuged to remove globin-free hemin [14]. The Hb solution was further purified via a DEAE column (10 × 1.5 cm) equilibrated with 20 mM phosphate buffer (pH 8.0) and eluted with the initial buffer at a flow-rate of 1 mL/min using an HPLC system (Waters, Milford, MA), which consisted of an automatic sample injector and a photodiode array detector. The Hb purified via this method was ferrous Hb, oxyHb(FeII) [27,28]. Protein concentrations were determined by a modified Lowry assay using BSA as a standard [29].

LDL oxidation by Hb and HRP

Conjugated dienes, one of the intermediate products formed during the peroxidation of polyunsaturated fatty acids (PUFAs) of cholesterol esters, phospholipids and triglycerides, were monitored during LDL oxidation using methods described previously [30–32]. The kinetics of human LDL oxidation were measured continuously *via* the change in absorbance at 234 nm [30].

To study the dose effect of Hb on LDL oxidation, each well of a microtiter plate was mixed with 20 μL of Hb (0–20 μM) and 15 μg of LDL in a final volume of 200 μL PBS and read at 234 nm over time at 30°C on a Spectra MAX 190 spectrophotometer (Molecular Devices, Sunnyvale, CA). Wells containing only LDL, Hb or PBS served as blanks. A similar procedure was conducted to study the effect of HRP (Sigma-Aldrich, Steinheim, Germany) on LDL oxidation.

Inhibition of peroxidase activity

Endogenous peroxidase activity of Hb was measured according to the method previously described [33] using 2,2′-azinobis (3-ethylbenzthiazoline-6-

sulphonic acid) (ABTS; MP Biomedicals, Illkirch, France) as a chromogen (1 mM) in the presence of 0.01% H_2O_2. Briefly, enzymatic activities were determined using ABTS as the reductant substrate and measured spectrophotometrically as the rise in absorbance due to the formation of the reaction products, an ABTS radical, at 414 nm ($\varepsilon_{460\,nm}$ = 31.1 $mM^{-1}cm^{-1}$) [34]. To block Hb-peroxidase activity, NaN_3 was used as an inhibitor [35] at concentrations between 0–800 μM. Catalase isolated from Fluca (Sigma-Aldrich, Steinheim, Germany) was used as another inhibitor of LDL oxidation [36] at concentrations between 0–1000 U. The reaction was initiated in a microtiter well containing 10 μM Hb in a final volume of 200 μL PBS at 30°C. Peroxidase activity was monitored as the increasing absorption at 415 nm after 15 min.

AAPH-induced LDL oxidation

A water-soluble free radical initiator, 2, 2′-azobis (2-amidinopropane)-dihydrochloride (AAPH; Wako Co, Osaka, Japan), was added to 15 μg LDL without Hb. The LDL oxidation reaction was initiated in a microtiter well with final AAPH concentrations between 0–4 mM, which is similar to that described above.

Lipid extraction from cells

Lipids were extracted according to the procedures previously described [37]. In brief, the membrane fraction isolated from HUVECs was resuspended in 10 mL of ice-cold chloroform-methanol-H_2O (10:10:1). After constant stirring for 8 h and three washes with the same reagent, the sample was centrifuged at 100 000 ×g for 5 min. The insoluble lipid extract was dried under a flush of nitrogen and resuspended in PBS.

Measurement of H_2O_2

Production of H_2O_2 was determined utilizing xylenol orange reagent and was measured as the change in absorbance of the xylenol orange (XO) complex that forms with oxidized metals such as ferric iron [38]. In an assay mix containing XO and ferrous ion, the formation of a ferric ion–XO complex is dependent on hydroperoxides, including H_2O_2. To 150 μL XO reagent (100 μM xylenol orange, 250 μM ammonium ferrous sulphate and 25 mM H_2SO_4), 50 μL of test samples were added, vortexed and incubated at room temperature for the desired time and monitored by a scanning spectrophotometer over time at 560 nm. $CuSO_4$ (4 μM) reaction with linoleate was used as a positive control. H_2O_2 (0–50 nM) was employed as a standard and positive control.

Cell culture

HUVECs were obtained from fresh human umbilical veins according to a standard procedure previously described [39]. In general, cells were grown in M199 medium (Gibco, Carlsbad, CA) supplemented with 20% heat-inactivated foetal bovine serum (Gibco) containing penicillin (100 U/mL), streptomycin (100 μg/mL) and endothelial cell growth supplement (25 μg/mL) (Upstate Biotechnology, Lake Placid, NY) and allowed to grow in a 10-cm Petri dish in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 5–6 days until confluence. The growth medium was renewed every 2–3 days. Unless otherwise specified, all culture experiments were conducted at 37°C. Cells were then treated with 0.025% trypsin containing 0.01% EDTA and sub-cultured in gelatin-coated 24-well plates (1×10^5 cells/well). When the monolayer became confluent, cells were washed with PBS and cultured in a conditioned M199 medium containing 2% lipoprotein-deficient foetal bovine serum, incubated for 16 h, and then aspirated before proceeding with experiments.

Cell treatment with Hb, AAPH and probucol

Hb was first diluted in conditioned medium to a final concentration of 0.5–50 μM. The effect of Hb or AAPH on the expression of adhesion molecules was conducted by adding 1 mL of Hb- or AAPH-containing media to each well, followed by incubation for 0–30 h. Probucol (Sigma-Aldrich, Steinheim, Germany) was dissolved in dimethyl sulphoxide to yield a 1 mM stock solution and was slowly mixed with conditioned medium to final concentrations ranging from 0.3125–20 μM. The entire experiment was similarly conducted in the presence of 10 μM Hb and incubated for 8 h. All assays were performed in triplicate.

Immunoassay for surface expression of HUVEC adhesion molecules

Expression of ICAM-1 and VCAM-1 was monitored using a standard immunoassay procedure previously described [40]. Briefly, HUVECs cultured in 24-well plates were washed three times with PBS and then fixed with 1% paraformaldehyde in PBS for 15 min at room temperature. Following washes with a blocking buffer containing 0.3% (v/v) skim milk and 0.02% (w/v) sodium azide, 500 μL of diluted ICAM-1 (1:300) or VCAM-1 (1:400) monoclonal antibody (R & D Systems, Minneapolis, MN) in blocking buffer was added and incubated for 1 h at room temperature. HUVECs without Hb served as a baseline, while the addition of normal mouse sera or unrelated monoclonal antibodies was used to evaluate non-specific binding. After washing, cells were incubated

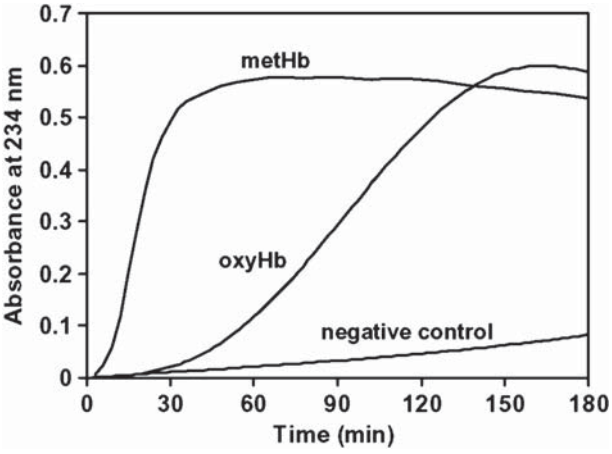

Figure 1. Effect of metHb and oxyHb on Hb-induced lipid peroxidation. Differential oxidative states of Hb (metHb and oxy Hb) induced LDL oxidation. In each well, 15 μg of LDL was mixed with 10 μM of metHb or oxy Hb in a final volume of 200 μL PBS. The negative control consisted of LDL only. All the conditions were the same as experiment, except replacing the Hb with PBS. The formation of conjugated dienes was monitored at 234 nm.

with 0.5 mL of diluted horseradish peroxidase (HRP)-labelled anti-mouse IgG (1:1000; Amersham, Buckinghamshire, UK) for 30 min and then washed with blocking buffer and PBS. Finally, 1 mL of 3,3′,5,5′-tetramethyl-benzidine developer containing 0.003% H_2O_2 was added while shaking gently for 30–60 min. The reaction mixture (200 μL) from each well was transferred to a 96-well plate and read at 630 nm. Each assay was conducted in triplicate.

Results

Hb-induced LDL oxidation

To determine if oxyHb(FeII) or metHb(FeIII) oxidizes LDL, the oxidation curve of different Hb moieties for conjugated diene oxidative curve changes was determined at 234 nm. Representative time-dependent spectral changes of oxyHb and metHb incubated with LDL are shown in Figure 1. Because native oxyHb is readily oxidized when in contact with air, the freshly-prepared Hb for experiments gradually transferred to metHb.

Peroxidase activity is required for Hb-induced LDL oxidation

Hb possesses peroxidase activity; the role of this endogenous enzymatic activity in LDL oxidation was assessed. As shown in Figure 2A, NaN_3 (0–800 μM), a peroxidase inhibitor [35], inhibited Hb-dependent peroxidase activity (10 μM Hb) in a dose-dependent manner. Furthermore, the addition of NaN_3 blocked Hb-induced LDL oxidation (Figure 2B). These data

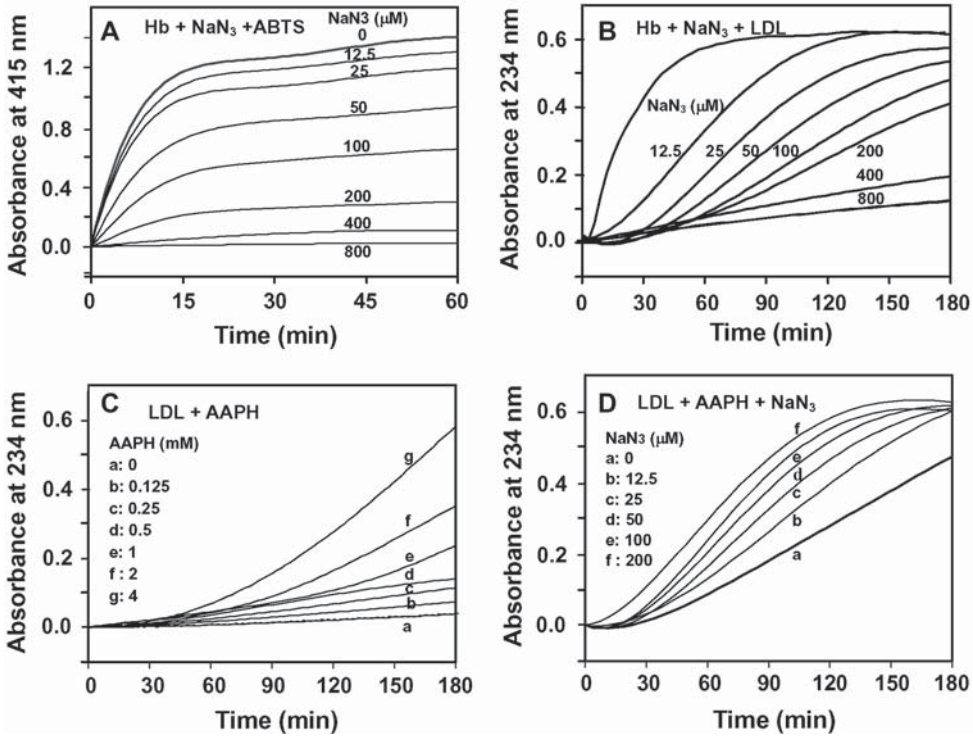

Figure 2. Effect of NaN_3 on Hb peroxidase activity and Hb-induced lipid peroxidation. (A) Effect of NaN_3 on the endogenous peroxidase activity of Hb. Hb (600 μM) was incubated with various amounts of NaN_3 prior to the addition of ABTS (1 mM) and 0.01% of H_2O_2. The enzyme activity was monitored at 415 nm. (B) Effect of NaN_3 on Hb-induced LDL oxidation. LDL in the presence of Hb (10 μM) was incubated with various amounts of NaN_3 and lipid peroxidation was monitored at 234 nm. (C) The water-soluble free radical initiator, AAPH (0 and 4 mM), was used to perform LDL oxidation. (D) Effect of NaN_3 (0–200 μM) on AAPH-induced LDL lipid peroxidation. Reactions were performed at 30°C in a microtiter plate in a final volume of 200 μL.

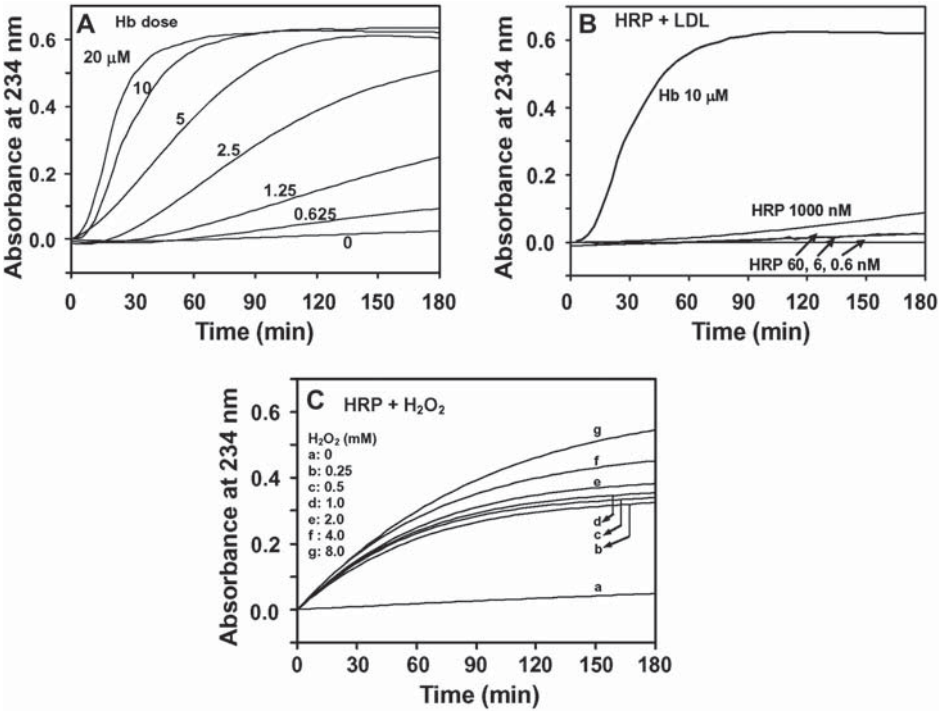

Figure 3. Hb-induced LDL oxidation in the absence of exogenous H_2O_2. (A) Freshly prepared LDL (10 μg) was reacted with Hb (0.625–20 μM). Formation of conjugated dienes, an index for Hb-induced lipid peroxidation, was monitored continuously using a scanning Spectra MAX 190 at 234 nm over time. The baseline absorption of LDL or Hb alone was subtracted prior to generating the plot. (B) HRP (0.6–600 nM) was incubated with LDL in the absence of both H_2O_2 and Hb, while Hb (10 μM) incubated with LDL in the absence of H_2O_2 and HRP (upper curve) served as a positive control. (C) HRP (60 nM) was incubated with LDL in the presence of H_2O_2. Reactions were performed at 30°C in a microtiter plate in a final volume of 200 μL.

indicated that Hb-induced LDL oxidation required the peroxidase activity of Hb.

To rule out the possibility that NaN_3 may non-specifically suppress the free-radical-induced formation of conjugated dienes, AAPH, which is known to generate carbon-centred akyl or peroxyl radicals, was used to initiate LDL oxidation. Figure 2C demonstrates that AAPH was capable of forming conjugated dienes, which was not inhibited by NaN_3 (Figure 2D). On the contrary, slightly enhanced oxidation via an unknown mechanism was observed upon the addition of NaN_3.

Hb-induced LDL oxidation in the absence of exogenous H_2O_2

As shown in Figure 3A, a typical Hb-induced LDL oxidation reaction as monitored by the formation of conjugated dienes over time was observed. Unlike a typical procedure, no exogenous H_2O_2 was added as a peroxide producer. The extent of lipid peroxidation was dependent on the Hb concentrations used (0–20 μM).

Because HRP, a heme-containing redox enzyme, possesses much higher peroxidase activity than Hb [41], its ability to oxidize LDL in the absence of exogenous H_2O_2 was also evaluated. HRP (0.6–1000 nM) produced little or no LDL oxidation in the absence of exogenous H_2O_2 (Figure 3B); however, in the presence of H_2O_2, HRP (at concentrations as low as 60 nM) initiated oxidation in a dose-dependent manner (Figure 3C). H_2O_2 alone (0.25–8 mM) did not yield any conjugated dienes (data not shown). These results indicated that Hb, but not HRP, initiated lipid oxidation in the absence of exogenous H_2O_2.

The effects of catalase on LDL oxidation were also assessed. Catalase decreased conjugated diene formation (Figure 4) in the presence of either metHb (Figure 4A) or oxyHb (Figure 4B). However, addition of up to 1000 U catalase did not fully inhibit Hb-induced LDL oxidation.

Hb produces H_2O_2 in the presence of unsaturated fatty acids

There are at least two possible sources of H_2O_2 available for Hb-induced LDL oxidation: one is from Hb or LDL alone and the other is from the reaction of Hb with LDL. To address these possibilities, the chromogenic reagent, ABTS, which has been widely used to measure peroxidase activity in the presence of an H_2O_2 substrate, was employed. As shown in Figure 5A, Hb alone, in the absence of exogenous H_2O_2, was not able to oxidize ABTS. However, oxidized ABTS was detected when Hb was mixed with LDL in the absence exogenous H_2O_2. Further spiking

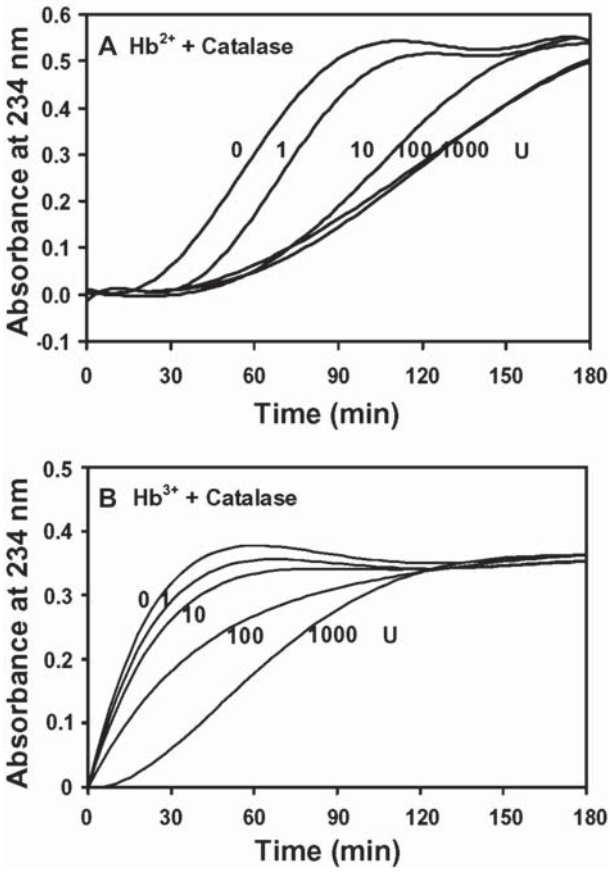

Figure 4. Catalase inhibits Hb-induced LDL oxidation in a dose-dependent manner. Catalase (0~1000 U), a H_2O_2 consuming enzyme, was pre-incubated with LDL and then metHb (A) or oxyHb (B) was added to the reaction mixture. Lipid peroxidation was monitored at 234 nm.

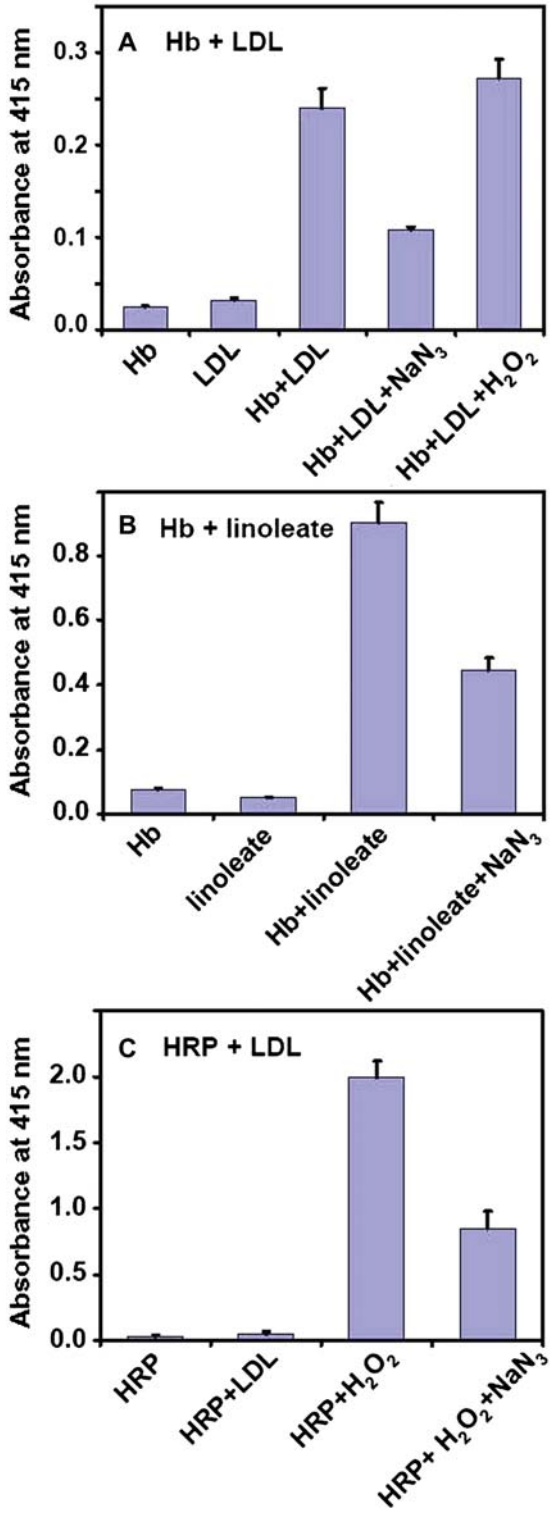

Figure 5. Hb generates H_2O_2 when reacted with unsaturated fatty acids. ABTS was used as a chromogen to estimate the free radical production from H_2O_2 peroxidation. H_2O_2 generation by Hb from (A) LDL and (B) the unsaturated fatty acid, linoleate was measured. (C) H_2O_2 generation by HRP from LDL was determined. The effect of NaN_3 on Hb and HPR peroxidase activity was also assessed. H_2O_2 was monitored at 415 nm.

of H_2O_2 did not increase ABTS oxidation in the presence of Hb, suggesting that the mixture of Hb and LDL already produced sufficient levels of H_2O_2. A similar result was generated when mixing Hb with linoleate (Figure 5B). Pure linoleate is easily oxidized by metal ions, resulting in linoleate-induced oxidized ABTS [42]. This reaction reflected why inhibition of Hb peroxidase could not fully suppress ABTS oxidation in a mixture containing Hb, linoleate and NaN_3 (Figure 5B). On the other hand, the mixture of HRP and LDL did not yield H_2O_2, as measured in the same assay (Figure 5C). Thus, the data indicated that Hb, but not HRP, was uniquely capable of producing H_2O_2 in the presence of LDL. Furthermore, in all three reactions, addition of NaN_3 partially reduced ABTS oxidation. In addition to possible Hb peroxidase activity, another pathway may induce ROS formation. Specifically, PUFA often contains lipid hydroperoxide (LOOH) [43], which may react with Hb, producing ROS that will initiate the radical reaction.

These results were further confirmed using a different reporter assay; the generation of H_2O_2 in the following experiments was determined employing an XO reporter to monitor ferrous oxidation [15]. As shown in Figure 6A, Hb or delipidated LDL (apoB) alone did not generate H_2O_2. Furthermore, the combination of apoB and Hb were not able to produce

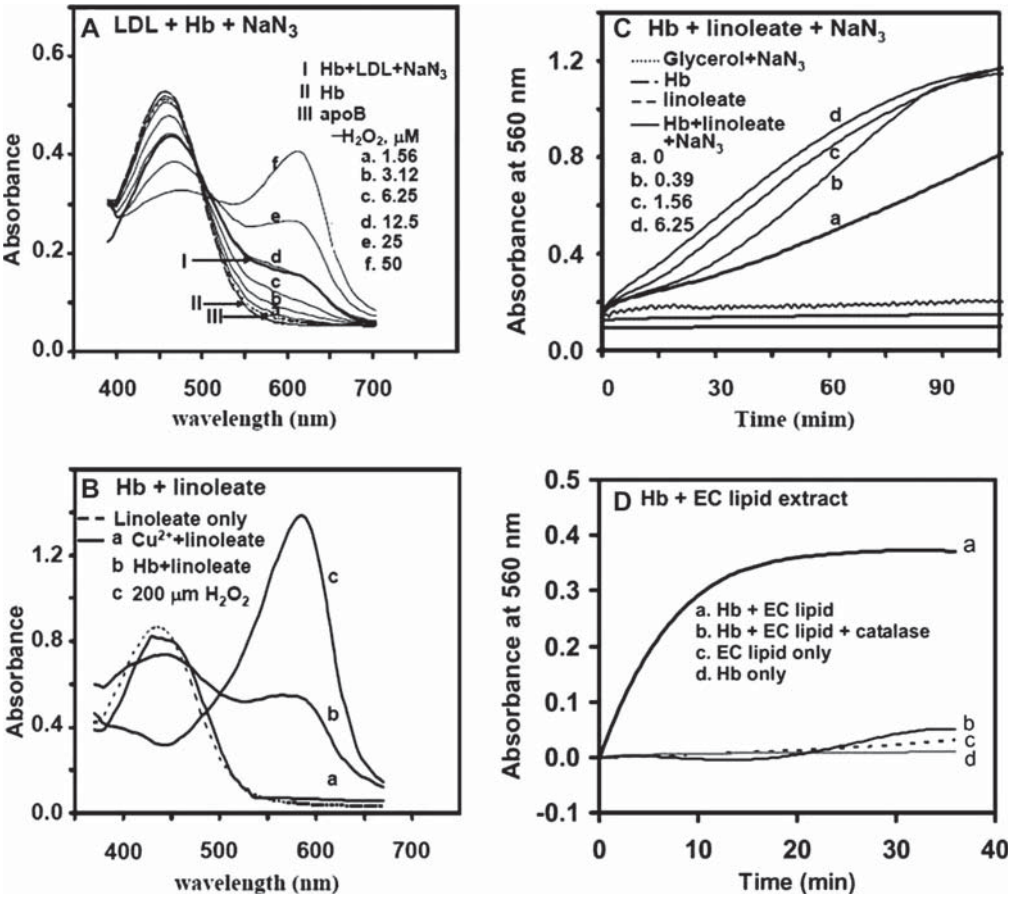

Figure 6. Generation of H_2O_2 induced by Hb. H_2O_2 generation was detected using the XO chromogenic reagent and scanning spectral analysis. (A) Hb (5 μM)-treated LDL (0.3 mg/mL) in 50 μL was immediately added to 150 μL of XO reagent without addition of H_2O_2. Standard curves were made by addition of various concentrations of H_2O_2 (0–50 μM). Hb alone and delipidated LDL (apoB) served as negative controls. (B) Linoleate (0.9 mg/mL) was pre-mixed with Hb (5 μM) or Cu^{2+} (4 μM) in a volume of 50 μL, followed by the addition of 150 μL XO reagent without H_2O_2. H_2O_2 was used as a positive control. (C) The effect of NaN_3 on Hb-induced H_2O_2 degradation. H_2O_2 was produced from the mixture of Hb and linoleate as described in (B). (D) Generation of H_2O_2 from the mixture of Hb and HUVEC lipid extracts. In addition, catalase (1000 U) consumed Hb-induced H_2O_2.

H_2O_2. However, the mixture of Hb and LDL (with peroxidase activity blocked) was able to generate H_2O_2. Based on these results, we hypothesized that H_2O_2 is likely derived from the lipid component of LDL upon reacting with Hb.

To test this hypothesis, an unsaturated fatty acid, linoleate (enriched in LDL lipids), as a substrate for Hb in the XO reporter assay was next used. The reaction mixture containing linoleate and Hb produced H_2O_2 to levels similar to that of LDL and Hb (Figure 6B). Cu^{2+}, which also induces LDL oxidation [25,41], did not induce H_2O_2 production in the presence of linoleate. Copper (4 μM) may induce lipids to form hydroxyl radicals directly [32], which will initiate linoleate peroxidation. Thus, no H_2O_2 was produced or accumulated during copper-induced lipid peroxidation. Notably, the addition of NaN_3 was necessary in order to inhibit the consumption of H_2O_2 by Hb-peroxidase, as shown in Figure 6C. Furthermore, the lipid fraction isolated from endothelial cells (ECs) produced H_2O_2 upon reacting with Hb (Figure 6D). The addition of catalase (1000 U) consumed the Hb produced H_2O_2 from ECs (Figures 6B and D). Together, these data indicated that Hb is capable of producing H_2O_2 in the presence of unsaturated fatty acids.

Saturated fatty acids are not a substrate for Hb-induced H_2O_2 generation

The ability of Hb to induce H_2O_2 formation in the presence of saturated fatty acids was next examined. As shown in Figure 7, little or no H_2O_2 was produced when Hb was reacted with either palmitic acid or dimyristoylphosphatidylcholine (DMPC), which are both saturated fatty acids. Addition of catalase (100 U) did not affect H_2O_2 generation. Thus, Hb is only capable of producing H_2O_2 in the presence of unsaturated fatty acids (e.g. linoleate). However, the mixture of Hb, linolate and catalase was able to decrease H_2O_2 generation in a dose-dependent trend, although not completely (Figures 4 and 7).

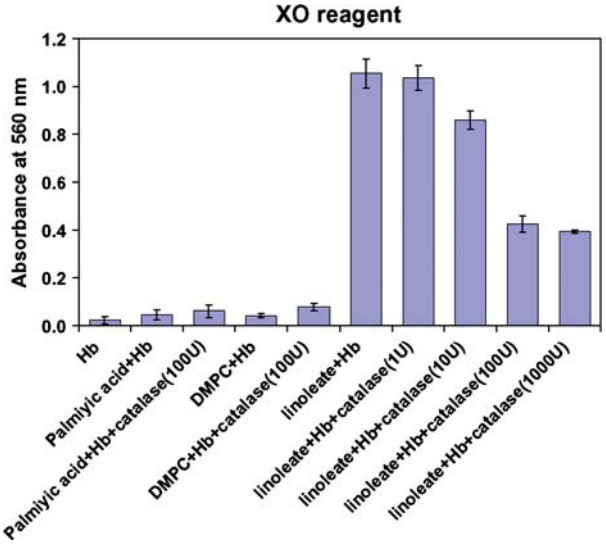

Figure 7. The effect of saturated fatty acids on H_2O_2 production by Hb. Determination of H_2O_2 was conducted using the XO chromogenic reagent and scanning spectral analysis. Incubation of Hb (5 μM) with linoleate (0.9 mg/mL) was used as a positive control. H_2O_2 generation by Hb (5 μM) with palmitic acid (0.9 mg/mL) or DMPC (0.9 mg/mL), both saturated fatty acids, was also assessed. Catalase (1–1000 U) was used to consume H_2O_2 in the Hb/linoleate mixture.

Hb-induced expression of ICAM-1 and VCAM-1

Hb is able to independently interact with the lipid extract of cultured endothelial cells to form H_2O_2 and, presumably, lipid radicals (Figure 6D), and because free radicals induce the expression of endothelial cell adhesion molecules, we tested the hypothesis that Hb alone might alter the expression of adhesion molecules in HUVECs. Changes in expression of ICAM-1 and VCAM-1 following an 8-h incubation with various concentrations of Hb were measured by direct immunoassay. As shown in Figures 8A and B, Hb induced both ICAM-1 and VCAM-1 expression in a concentration-dependent manner (0.5–50 μM) to levels observed with the water-soluble free-radical initiator, AAPH (Figure 8C). The kinetics of ICAM-1 and VCAM-1 induction upon the addition of 10 μM Hb was also determined (Figures 8D and E).

To test the hypothesis that free radicals are involved in the stimulation of ICAM-1 and VCAM-1 expression, probucol, a potent radical scavenger [9,44], was used. Probucol, at concentrations between 0.05–10 μM, effectively suppressed Hb-induced expression of both adhesion molecules with an IC_{50} (50% inhibitory concentration) of ~ 1.25 μM (Figure 9), suggesting that, in addition to the oxidative activity of Hb toward LDL, Hb also exerted a direct influence on atheromatous due to induction of adhesion molecules through free radical production.

Discussion

There is no doubt that production of globin-radicals by Hb is one of the mechanisms involved in the initiation of LDL oxidation. Globin-radicals are capable of withdrawing an electron from unsaturated fatty acids or fatty-acid hydroperoxides, resulting in the formation of fatty-acid radicals and leading to extensive lipid peroxidation via an amplification cycle [45]. The equations depicting the formation of ferryl-Hb from oxy-Hb (equation 1) and subsequent generation of met-Hb and superoxide ions (equation 2) in the presence of H_2O_2 are given below:

$$Hb(Fe^{2+})O_2 + H_2O_2 \rightarrow Hb(Fe^{4+}{=}O) + O_2 + H_2O \quad (1)$$
$$Hb(Fe^{4+}{=}O) + H_2O_2 \rightarrow Hb(Fe^{3+}) + O_2^{\bullet -} + H_2O \quad (2)$$

The coupling of the free radicals generated above to specific amino-acid residues of Hb (e.g. tyrosine, histidine or cysteine) to form globin-radicals are illustrated in equations (3) and (4):

$$Hb(Fe^{3+}) + H_2O_2 \rightarrow {}^{\bullet}Hb(Fe^{4+}{=}O) + H_2O \quad (3)$$
$$Hb(Fe^{4+}{=}O) + 2H^+ \rightarrow {}^{\bullet}Hb(Fe^{3+}) + H_2O \quad (4)$$

There are a number of previous studies that have demonstrated the formation of these globin-radicals and have shown their oxidative reactivity towards LDL [13,46]. However, because these experiments were carried out with the addition of exogenous H_2O_2, the ability of Hb to produce H_2O_2 in the presence of LDL has remained unexplored.

In the present study, we demonstrated that Hb directly induced LDL oxidation without the addition of H_2O_2 and showed that the source of H_2O_2 originated from the reaction with unsaturated fatty acids associated with LDL. There are several lines of evidence to support this conclusion. First, ABTS is a known chromogenic reagent that requires H_2O_2 as a substrate for developing chromogeneity [47]. In the present study, Hb was capable of catalysing ABTS in the presence of LDL or linoleate without the addition of H_2O_2 (Figure 5). Second, H_2O_2 was generated when Hb was incubated with LDL, but not with delipidated LDL (Figure 6A). Finally, incubating pure polyunsaturated linoleate enriched in LDL lipids with Hb also generated H_2O_2 (Figure 6B) and catalase consumed the H_2O_2 that was generated (Figure 7). In contrast, DMPC, containing two identical saturated fatty acyl chains, was not able to produce H_2O_2 when reacted with Hb (Figure 7), indicating that the presence of unsaturated fatty acids is a fundamental pre-requisite for the production of H_2O_2 in this reaction.

Based on the above results, we propose equation (1) in Figure 10, which is consistent with an early study using RH2 + H, where R represents a reducing agent (e.g. PUFA) [48]. Because unsaturated fatty

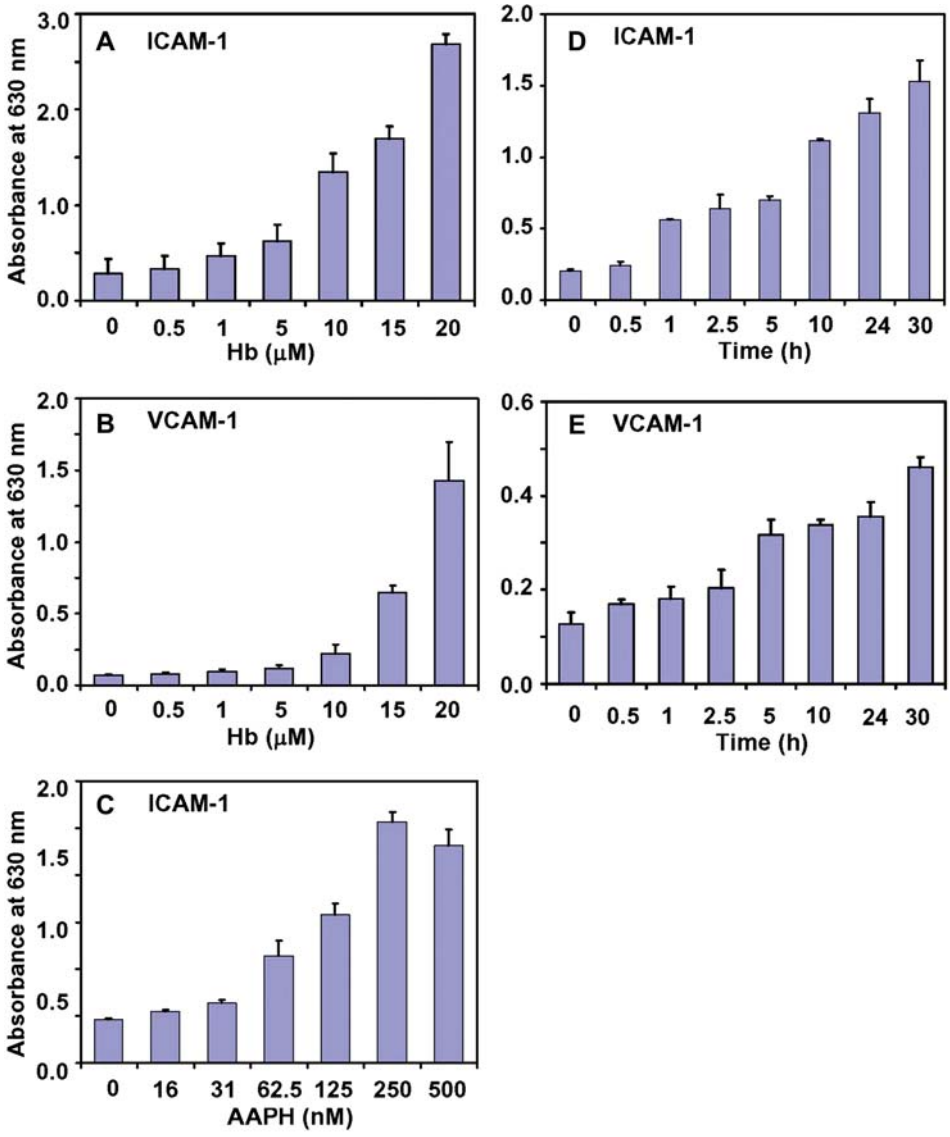

Figure 8. Effect of Hb on ICAM-1 and VCAM-1 expression in HUVECs. Cells were grown in 24-well plates and incubated with or without Hb for 8 h at 37°C. Expression of ICAM-1 (A) and VCAM-1 (B) was determined using a standard immunoassay. (C) AAPH induction of ICAM-1 expression was also assessed. ICAM-1 (D) and VCAM-1 (E) expression over time was also determined in HUVECs with 10 μM of Hb. All experiments were conducted in triplicate.

acids are particularly susceptible to peroxidation, due to the increased number of double bonds in the fatty acid side chain, it is easier to remove the hydrogen atom [49]. When Hb is incubated with LDL, the free hydrogen atom can react with the oxygen within oxy-Hb (Figure 10, equation 1a) to form H_2O_2. The oxidation of ferrous (Fe^{2+}) Hb occurs in ruptured, advanced atheromatous lesions, generating MetHb (Fe^{3+}) and resulting in more extensive oxidation to ferryl Hb ($Fe^{3+}/Fe^{4+}=O$) (Figure 10, equation 1b) [50]. Both MetHb and ferryl Hb are toxic for endothelial cells. Interestingly, the reaction is unique for Hb, as HRP has the electronic structure of compound III (bound dioxygen species) similar to that of oxy-Hb [41,51]. However, HRP was not able to generate H_2O_2 from the lipids under the same experimental conditions (Figure 5). This was further confirmed by the formation of conjugated dienes in the HRP-induced LDL oxidation reaction, in which an exogenous source of H_2O_2 was pre-requisite (Figure 3).

H_2O_2 is subsequently catalysed by the endogenous peroxidase activity of Hb, forming ROS, such as superoxide ions [52] and hydroxyl radicals [1] (Figure 10, equation 2a). Catalase, an enzyme specific for hydrogen peroxide degradation, reduced the rates of Hb-induced LDL oxidation (H_2O_2 H_2O) [53]. Furthermore, because catalase is a major enzymatic scavenger of ROS, the inhibition of Hb-induced LDL oxidation activity by catalase was examined. Catalase significantly inhibited Hb-induced LDL oxidation in a dose-dependent manner through inhibiting peroxidase activity; however, complete inhibition by catalase was not observed (Figure 4). ROS, including hydroxyl

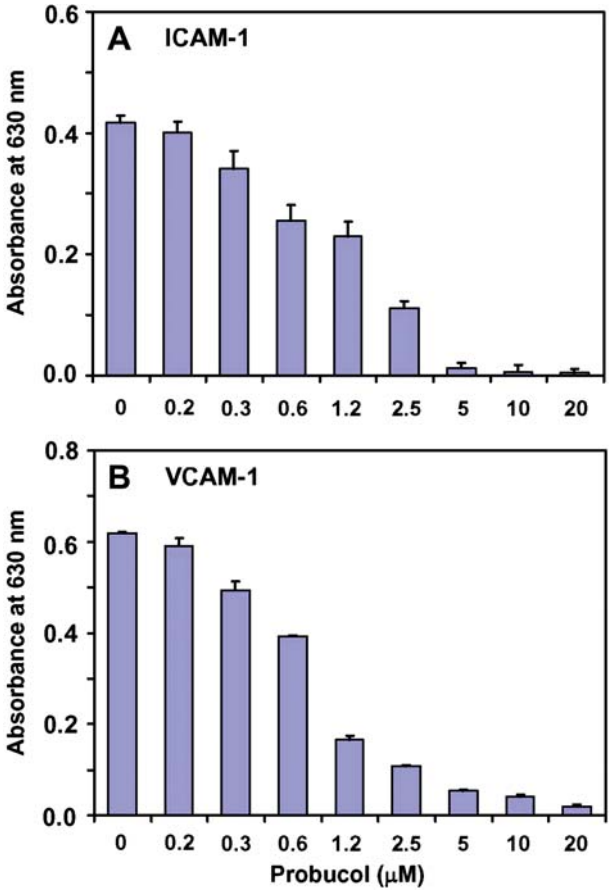

Figure 9. Effect of probucol on Hb-induced expression of ICAM-1 and VCAM-1 in HUVECs. Cells were incubated with 10 μM of Hb at 37°C for 8 h in the presence of various concentrations of probucol. The effect of probucol on Hb-induced ICAM-1 (A) and VCAM-1 (B) expression was determined. All experiments were conducted in triplicate.

radicals, superoxide and hydrogen peroxides, are a group of highly reactive molecular forms of oxygen containing unpaired electrons. Catalase can scavenge ROS, but not the highly reactive globin-radicals of Hb (equations 3 and 4), which are capable of LDL oxidation [13,46] through attracting hydrogen from LDL, thereby propagating lipid peroxidation. This might explain why catalase did not fully inhibit Hb-induced LDL oxidation. In addition, inhibition of Hb peroxidase activity did not completely inhibit Hb-induced ROS formation. These results suggest that PUFA-derived LOOH may react with Hb, contributing to radical generation in the absence of H_2O_2 (Figure 10, equations 2b and c). Oxidation of LDL by Cu^{2+} was initiated by radical generation from the reaction between LOOH and Cu^{2+} (equations 5 and 6).

$$LOOH + Cu^{2+} \rightarrow LOO^{\cdot} + Cu^{+} + H^{+} \quad (5)$$
$$LOOH + Cu^{+} \rightarrow LO^{\cdot} + Cu^{2+} + OH^{-} \quad (6)$$

The reduction of Cu^{2+} by LOOH is the rate-limiting step and Cu^{+} can be generated with reducing agent in lipid [54].

In the present study, catalase consumed the H_2O_2 produced by Hb. The given equations are rationalized using the established mechanisms proposed previously [1,17,55]. The formation of ROS is dependent on the peroxidase activity of Hb; LDL oxidation was limited in the presence of the peroxidase inhibitor, NaN_3 (Figure 2B). Therefore, Hb-induced LDL oxidation may occur through the following two steps: (i) generation of H_2O_2 from lipids by Hb (Figure 10, equations 1a and b) and (ii) ROS formation by endogenous peroxidase activity of Hb and by LOOH peroxidation (Figure 10, equations 2a–c). These ROS react with the lipid component of LDL. The resulting carbon-centred lipid radicals react with O_2 to generate peroxyl radicals, which can combine with each other or attract hydrogen from adjacent fatty acids, including LDL and membrane lipids, thereby propagating lipid peroxidation (Figure 10, equation 3). Furthermore, the lipid peroxide radicals can withdraw an electron from a chromogen (i.e. ABTS) to generate a change in absorbance under visible light (Figure 10, equation 4). In summary, these results demonstrated that Hb-induced oxidation requires both endogenous H_2O_2 production and peroxidase activity catalysed by Hb.

Hb reacts with unsaturated fatty acids to generate H_2O_2 (Figures 6 and 7). Enrichment of unsaturated fatty acids [56] and lipid peroxidation of cell membranes has been previously investigated [57]. Lipids extracted from endothelial cell membranes can generate H_2O_2 in the presence of Hb (Figure 6D) that can be further catalysed to form ROS (Figure 3A), which would likely result in membrane disruption. An *ex vivo* study observed that haemolysis-induced haemoglobin oxidation resulted in vascular dysfunction and was associated with endothelial injury [58]. The data in the present study provide a possible mechanism to explain the observance of high cardiovascular mortality in patients undergoing hemo- and peritoneal dialysis [3]. Because endothelial dysfunction is a risk factor for atherosclerosis [59], cell-free Hb circulating in the plasma represents a potent inflammatory atherogenic factor [24].

Hb might also play a role in atherogenesis by influencing the adhesion properties of vascular endothelial cells. Because RBCs contain as much as 20 mM Hb (~ 150 mg/mL RBC), a minimal amount of vascular haemolysis could result in a micromolar increase in plasma Hb; 1 mL of lysed RBCs is sufficient to produce a final circulatory concentration of ~ 3–4 μM Hb. In an oxidative environment, endothelial cells mediate LDL oxidation and upregulate adhesion molecule expression in the presence of oxidative stress [60,61]. Furthermore, in the pro-oxidative environment of atheromatous lesions in which Hb is oxidized to ferrous- and ferryl Hb, lipid oxidation is further promoted. These events will directly amplify endothelial cell injury [60]. In addition, H_2O_2 can induce up-regulation of adhesion molecule expression in

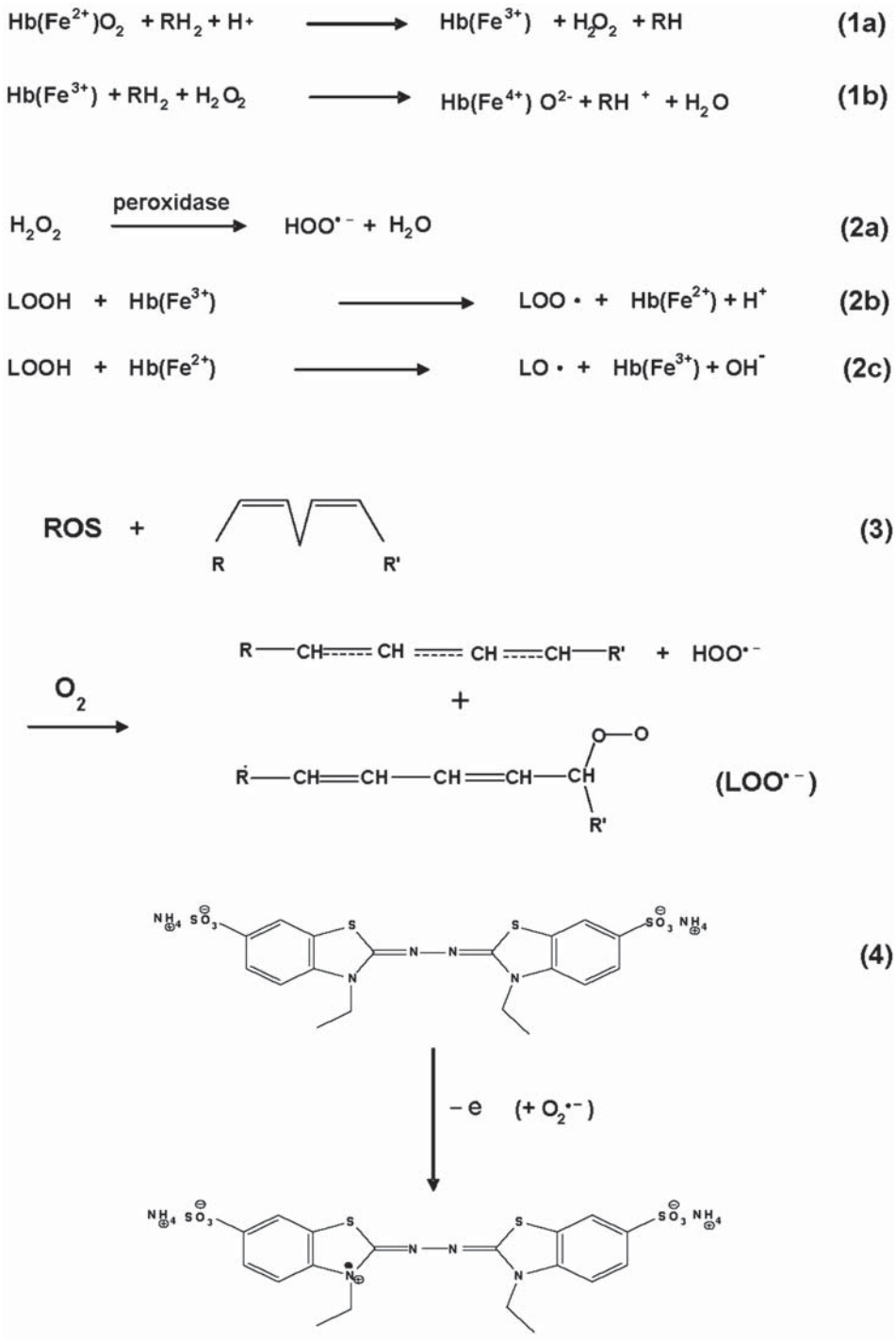

Figure 10. The proposed reaction of Hb-induced lipid peroxidation. Two species of Hb generate H_2O_2 via the lipid alkyl chain and does not require Hb-peroxidase activity (equation 1a). The extensive oxidation to ferryl Hb $(Fe^{4+})O_2$ (equation 1b). Endogenous Hb-derived reactive oxygen species (ROS) is produced from H_2O_2 (equation 2a) and/or lipid peroxide (LOOH) (equations 2b and c). ROS reacts with the unsaturated fatty acyl chains of LDLs, producing conjugated dienes (equation 3) and subsequently forming lipid peroxide radicals that further propagate the reaction. In addition, the lipid peroxide radicals withdraw an electron from ABTS (equation 4), generating a green product that is detected at 415 nm.

endothelial cells [60]. In the present study, Hb catalysed H_2O_2 formation by attracting hydrogen molecules from the endothelial cell membrane (Figure 6D) as well as unsaturated fatty acids (Figure 7). In addition, 5–10 μM Hb may be of pathogenic significance as it produces a sustained over-expression of ICAM-1 and VCAM-1. To our knowledge this is the first report showing that Hb can dramatically stimulate expression of adhesion molecules in cultured HUVECs. We propose that Hb generated H_2O_2 through PUFA oxidation, which further created free radicals through Hb peroxidase activity. Thus, Hb-mediated of lipid oxidation may increase atheromatous lesions and endothelial cytotoxicity.

To address the possibility that Hb stimulates adhesion molecule expression via radical formation, the effect of probucol, a potent antioxidant [9,41], on Hb-mediated expression of adhesion molecules was determined. At concentrations as low as 5 μM, probucol completely inhibited the Hb-induced expression of ICAM-1 and VCAM-1 (Figure 8). Therefore, the inhibitory activity of probucol is relatively potent, with an IC_{50} of ~ 1.25 μM; this concentration can be easily achieved in the circulation upon oral administration of a single dose, which has a sustaining half-life in plasma of up to 6 months [41]. In humans, probucol significantly reduced the rate of restenosis after percutaneous transluminal coronary angioplasty (PTCA) [62] and a low daily dose of probucol effectively decreased the incidence and severity of restenosis [63]. Furthermore, this potent antioxidant compound was protective against atherosclerosis [64,65] based on its ability to up-regulate heme oxygenase-1 (HO-1) expression [66,67].

Because local haemolysis may take place at the site of balloon injury or during balloon angioplasty, the release of Hb could induce the over-expression of adhesion molecules, which in turn may trap monocytes and enhance the inflammatory response, thereby triggering smooth muscle proliferation [68]. Immediate treatment with probucol during PTCA can substantially reduce the proliferation of smooth muscle cells that occurs during restenosis. After trapping the radicals, probucol metabolizes them into diphenolquinone, spiroroquinone and bisphenol, which are found in human plasma following oral ingestion. Interestingly, bisphenol itself is a potent antioxidant [9,32]. Thus, probucol inhibits Hb-induced adhesion molecular expression in endothelial cells by scavenging Hb-produced ROS, which may explain, in part, the effect of probucol in reducing the incidence of restenosis that normally occurs in ~ 40% of the patients undergoing PTCA [69].

In summary, Hb reacts with polyunsaturated fatty acyl chains to generate H_2O_2 independent of Hb-peroxidase activity. However, the formation of conjugated dienes of LDL by Hb required the endogenous peroxidase activity of Hb to react with H_2O_2, forming ROS and promoting the amplification cycle of lipid peroxidation. Therefore, in addition to the previously proposed mechanisms by which Hb induces LDL oxidation via globin-radicals and haemoglobin-derived iron (i.e. heme transfer to LDL), the combined endogenous peroxidase activity of Hb and its ability to generate H_2O_2 from lipids plays a crucial role in free radical formation. Moreover, Hb alone induces ICAM-1 and VCAM-1 expression in HUVECs via interaction with the endothelial membrane, which is completely inhibited by scavenging of free radicals.

Declaration of interest

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