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The interaction between ruptured erythrocytes and low-density lipoproteins

George Paganga[a], Catherine Rice-Evans[a], Rebecca Rule[a] and David Leake[b]

[a]*Division of Biochemistry, United Medical and Dental Schools of Guy's and St. Thomas's Hospitals, Lambeth Palace Road, London SE1 7EH, UK* and [b]*Department of Physiology and Biochemistry, University of Reading, Reading, UK*

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Low-density lipoproteins (LDL) are oxidatively modified on interaction with haem proteins. The interaction of ruptured erythrocytes with LDL induces oxidative damage as detected by alterations in electrophoretic mobility and the peroxidation of the polyunsaturated fatty acyl chains. Difference spectroscopy reveals that the amplification of the oxidative process by the haem protein is related to the transition of the oxidation state of the haemoglobin in the erythrocyte lysate from the oxy [X-Fe^{II}-O_2] to the ferryl [X-Fe^{IV}=O] form. The incorporation of the lipid-soluble antioxidant, butylated hydroxy toluene, at specific time points during the LDL–erythrocyte interaction prolongs the lag phase to oxidation and eliminates the oxy-to-ferryl conversion of the haemoglobin. The timescale of this haem conversion is related to the antioxidant status of the LDL.

Erythrocyte; Haemoglobin; Low-density lipoprotein (LDL); Ferryl radical species; Iron release

1. INTRODUCTION

Many experimental approaches are currently being applied to the study of the susceptibility of low-density lipoprotein (LDL) in vitro, to oxidative modification [1–4]. Specifically, it has been noted that all the systems applied involve at least the co-participation of transition metal ions or haem proteins. Thus, incubation in in vitro systems with transition metal ions, especially copper [1], metmyoglobin or ferryl myoglobin [5], and with cellular systems such as cultured macrophages, smooth muscle cells or endothelial cells [3,4,6] are capable of mediating LDL oxidation. Furthermore, the macrophage-induced oxidation has an absolute requirement for the presence of iron in the culture medium [2]. Since oxidation of LDL is likely to be involved in the initial stages of an atherosclerotic lesion ([7,8], reviewed in [9]), it is relevant to consider a source of oxidants which may be located, under specific circumstances, in the artery wall.

We have explored the possibility of erythrocyte disruption and subsequent oxidative changes in oxyhaemoglobin as a potential mediator of LDL oxidation. Thus we have investigated the ability of LDL to interact with haemoglobin in lysates released from ruptured erythrocytes, and the conditions under which such erythrocytes induce oxidative modification of LDL. The results show that the onset of oxidative modification of LDL, as detected by altered surface charge and LDL peroxidation, is related to the time-range during which oxyhaemoglobin [HX-Fe^{II}..O_2] becomes activated to the ferryl state [HX-Fe^{IV}=O]. The time of the haem conversion is dependent on the antioxidant status of the LDL. The incorporation of lipid-soluble antioxidants into the LDL at specific time points during the exposure to erythrocytes prolongs the lag phase ot oxidation, eliminates the oxy-to-ferryl conversion of the haemoglobin and delays the oxidative modification of LDL.

Correspondence address: C. Rice-Evans, Division of Biochemistry, UMDS St. Thomas's Hospital, Lambeth Palace Road, London SE1 7EH, UK. Fax: (44) (71) 928 3619.

2. MATERIALS AND METHODS

Fresh human blood was obtained from human volunteers (with informed consent) and the plasma was separated after centrifugation for use in the isolation of LDL. The buffy coat was removed and discarded and the packed cells were washed three times with isotonic 5 mM phosphate-buffered saline, pH 7.4, by centrifugation at 4,000 × *g*, 4°C for 20 min. Erythrocytes were lysed with hypertonic phosphate buffer, the membranes discarded and the oxyhaemoglobin concentration of the supernatant determined from the extinction coefficients [10] by measuring the absorbance in the Soret region. LDL was prepared from the fresh plasma in a Kontron 2070 ultracentrifuge fitted with a fixed-angle Kontron rotor, according to the method of Chung et al. [11] for 3 h at 100,000 × *g* and then the LDL was re-centrifuged for 14 h at 100,000 × *g* to remove minor protein contaminants. The concentration of protein was determined by a modified procedure of Markwell et al. [12] and the LDL was used at a final concentration of 0.5 mg protein/ml.

LDL was incubated at 37°C in the presence of the haemolysate at final concentration of 20 μM oxyhaemoglobin. In some experiments hydrogen peroxide was added at a final concentration of 25 μM. Oxidative modification of the LDL was assessed by measuring the altered surface charge. Samples were applied to agarose gels (Beckman paragon lipo electrophoresis system) to assess the increased relative electrophoretic mobility of the LDL. Lipoproteins were visualised by staining with Sudan black B stain. The extent of lipid peroxidation was assayed using a modified thiobarbituric acid assay [13] with the ab-

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sorbance of the chromophore measured at 532 nm, corrected for background absorbance at 580 nm due to possible contributions from haem compounds. Appropriate controls were incorporated according to Gutteridge et al. [14].

The visible spectra were recorded on a Beckman DU65 spectrophotometer fitted with Quant 1 software and linked to an IBM PC/2. Spectroscopic evaluation of the haem oxidation states in terms of the concentration of oxyhaemoglobin, methaemoglobin, ferryl haemoglobin and deoxyhaemoglobin were calculated by applying the millimolar extinction coefficients of each of the haemoglobin species at the appropriate wavelengths [15]. Observations of the effects of LDL oxidation on the state of the haemoglobin as a function of time were measured as difference spectra with prior subtraction of the LDL spectrum. After interaction between haemolysate and LDL, total haemoglobin levels were measured after conversion to cyanmethaemoglobin with potassium ferricyanide, or reduction to deoxyhaemoglobin by sodium dithionite, and calculated by applying the standard extinction coefficients of these different forms [15].

3. RESULTS

LDL (0.5 mg/ml) was exposed to oxyhaemoglobin (20 μM), in freshly prepared membrane-free erythorcyte lysate, in the presence and absence of hydrogen peroxide (25 μM) for a range of time periods up to 6 h at 37°C.

The oxidative responses were studied by measuring (i) changes in the surface charge altering the electrophoretic mobility of the LDL, (ii) the oxidation state of the haemoglobin by visible spectroscopy, (iii) the extent of lipid peroxidation.

Tabel I shows the alteration in electrophoretic mobility of LDL after treatment with membrane-free erythrocyte lysate for up to 6 h. The additional presence of hydrogen peroxide in the incubation medium induces no further alteration in the surface charge. These results are consistent with the timescale for the erythrocyte-induced lipid peroxidation (Fig. 1), as assessed by the formation of thiobarbituric acid-reactive substances. The membrane-free haemolysate in the absence and presence of hydrogen peroxide elicited the same extent of oxidation to the polyunsaturated fatty acid side-chains of the lipids.

Observations on the influence of LDL on the oxidation state of the haemoglobin in the haemolysate were undertaken by scanning the difference spectrum of LDL in the presence of haemolysate, in the presence and absence of exogenous hydrogen peroxide, with the subtraction of the spectrum of LDL. The changes in the spectra of the oxidised forms of the haemoglobin with time are illustrated in Fig. 2. As the time proceeds, the spectra reveal that the oxyhaemoglobin, characterised by peaks at 541 and 577 nm, declines slowly initially on interaction with the LDL. After the slow phase a sudden transition to deoxyhaemoglobin occurs (at t = 210 min for this LDL prep.), as shown on the spectrum, with the transient appearance of a single peak at 555 nm, characteristic of deoxyhaemoglobin replacing the 541 and 577 doublet of oxyhaemoglobin. This is followed by conversion to a spectrum, changing with time, with the appearance characteristic of mixed spectral forms of met, ferryl and oxy haemoglobin. Calculation of the proportion of oxy, met and ferryl haemoglobin by applying the extinction coefficients (Winterbourn [35]) allows the determination of the concentration of each oxidised form, expressed in Fig. 3 as the percentage of the original haem concentration. The production of ferryl haemoglobin is confirmed by the characteristic shifts of the Soret band, the red shift from 414 to 420 nm representing ferryl formation and the blue shift to 403 nm representing the met state (Fig. 2). Concomitantly the propagation of lipid peroxidation is amplified after progression through the lag phase (as seen in Fig. 1).

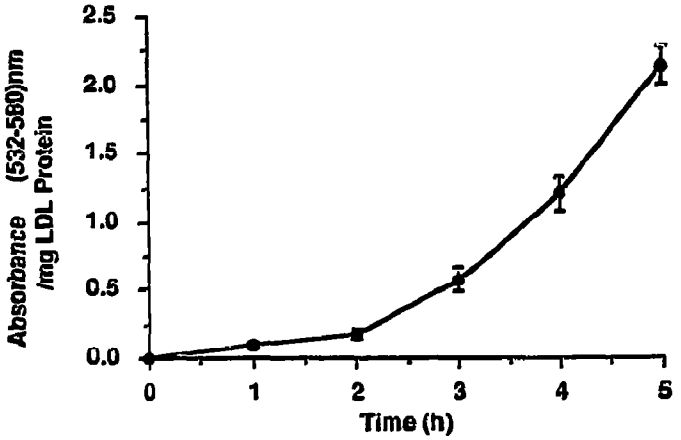

Fig. 1. The time dependency of the extent of lipid peroxidation of LDL (0.5 mg/ml protein) induced by erythrocyte lysate (20 μM) ± hydrogen peroxide (2.5 μM), as thiobarbituric acid-reactive compounds.

Assessment of the effects of adding a lipid-soluble antioxidant to the LDL on the time to oxidative transition of the oxyhaemoglobin was undertaken (Fig. 4). Incorporation of butylated hydroxytoluene (BHT) into the LDL/erythrocyte incubations 30 and 90 min after the initiation of the interaction suppresses the oxidising effects of the haemoglobin system on the LDL (with suppression of lipid peroxidation) and this is reflected in the oxidation state of the haem protein, as shown in the spectra (Fig. 4). Addition of BHT attenuates the loss of oxyhaemoglobin as shown in panel B compared to

Table I

The effects of erythrocyte lysate (20 μM) ± hydrogen peroxide (25 μM) on the relative electrophoretic mobility (REM) of LDL (0.5 mg/ml protein) as a function of time

Time (h)	REM
0	1
0.5	1
1	1.06
2	1.20
3	1.40
4	1.50
5	1.60
6	1.68

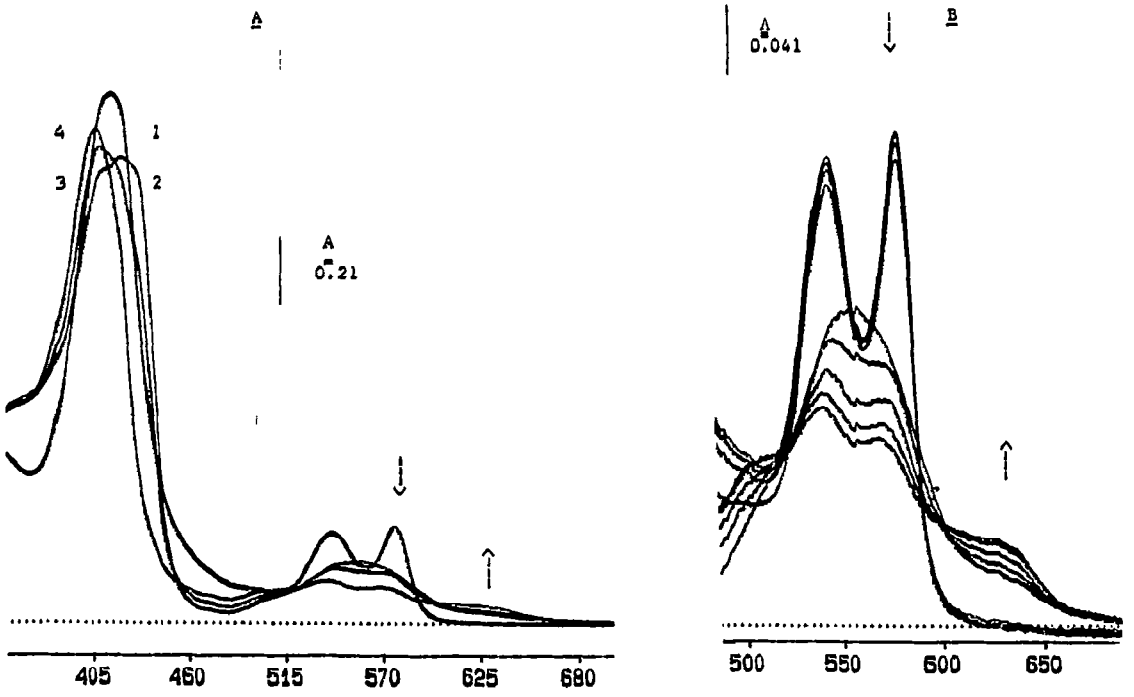

Fig. 2. Difference spectra as a function of time of oxyhaemoglobin in erythrocyte lysate ± hydrogen peroxide (25 μM) as it oxidises LDL, with the subtraction of the LDL. (Panel A) The Soret region: (1) 50 min, 100 min, (2) 250 min, (3) 300 min, (4) 450 min. The arrows indicate the direction of the absorbance changes. (Panel B) Visible longwave spectral region: the arrows indicate the direction of the absorbance changes at time points of 50, 100, 150, 200, 250, 300, 350, 400 and 450 min.

panel A; the later addition of the BHT, within the time period during which the pronounced loss of oxyhaemoglobin would have occurred in its absence, sustains the oxyhaemoglobin level even further and inhibits the oxidation of LDL by the haemoglobin.

In the absence of BHT (Fig. 5) the oxyhaemoglobin level slowly declines as oxidation occurs, the slow phase being considerably shorter for this LDL preparation compared to that in Fig. 3 due to the well-documented variation in the susceptibility of LDL isolated from different donors to oxidation [16,17]. At 100 min there is a sudden rapid transition to the deoxy form, as described above for the LDL preparations shown in Fig. 3. In the presence of BHT, the deoxyhaemoglobin conversion is prevented and the formation of ferrylhaemoglobin is inhibited, with the oxyhaemoglobin level slowly and progressively declining with time (Fig. 5). The maintenance of the oxyhaemoglobin state is very pronounced with the later addition of the antioxidant, within the timescale prior to the induction of the pronounced oxidative activation of the haemoglobin. The data show no loss of haem and no release of iron from the haemoglobin during the period of the reaction. Up to 100 min (Fig. 5) there is no distinction between the slow rates of oxidation of the oxyhaemoglobin whether the BHT is present or not. As the duration of the interaction between the LDL and the haemolysate is prolonged, the BHT prevents the oxidative transition of oxy, via deoxy, to ferryl. The addition of the antioxidant during the lag phase (prior to the time at which the rapid decrease in the oxyhaemoglobin levels occurs) suppresses lipid peroxidation as assessed by measurements of electrophoretic mobility and inhibition of the production of thiobarbituric acid-reactive substances.

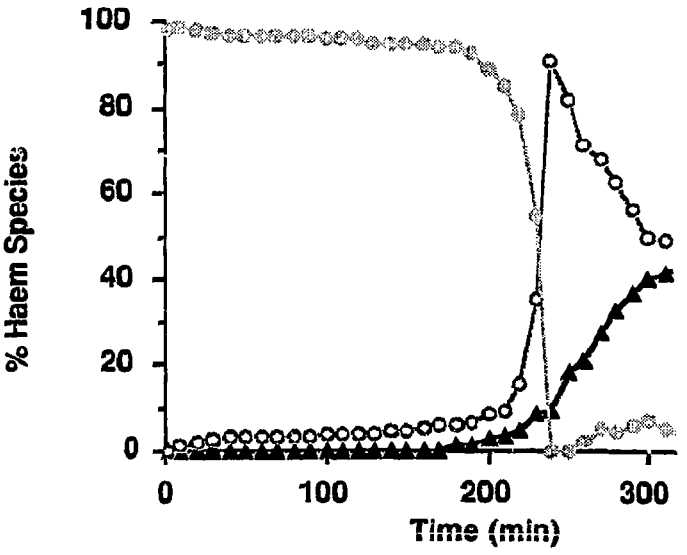

Fig. 3. The proportions of oxy-, met- and ferryl haemoglobin generated during the interaction of erythrocyte lysate (20 μM haemoglobin) with LDL (0.5 mg/ml protein).

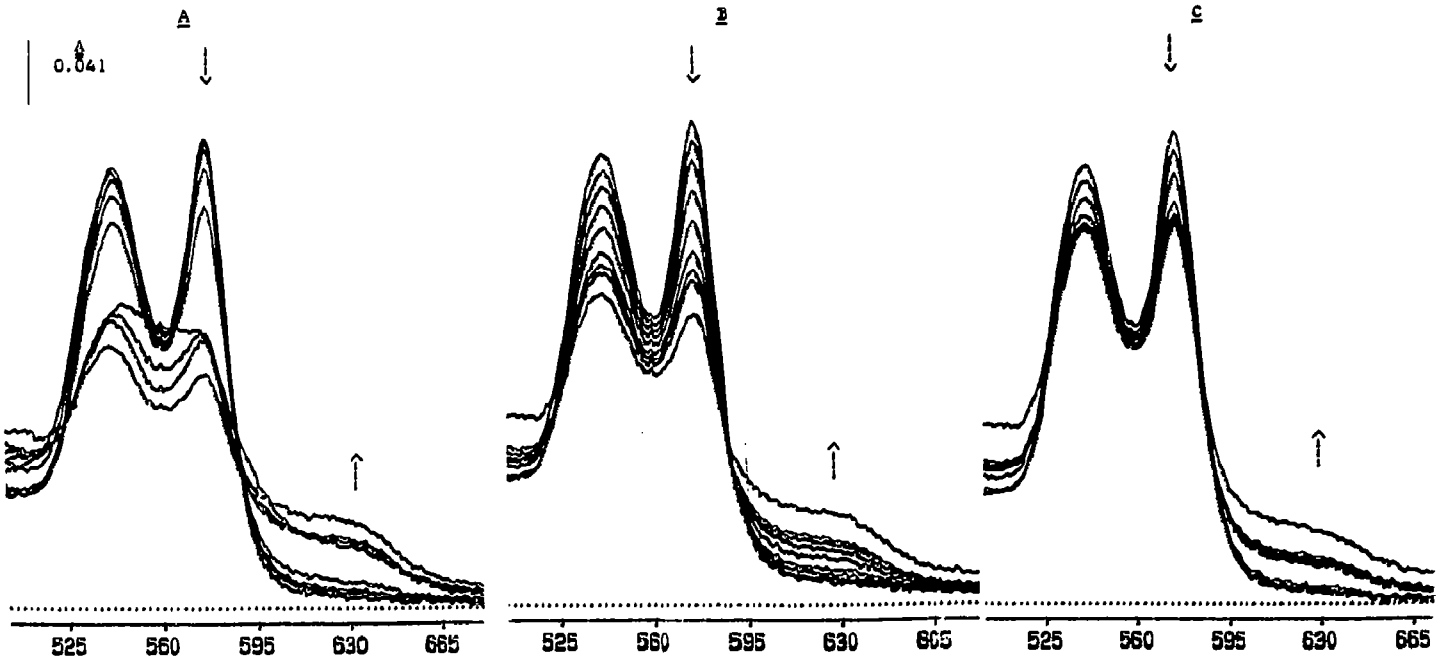

Fig. 4. Difference spectra of the effects of the antioxidant, butylated hydroxytoluene, on the spectral transition of oxyhaemoglobin in erythrocyte lysate as it reacts with LDL, with the subtraction of the LDL. (Panel A) LDL (0.5 mg/ml protein), erythrocyte lysate (20 μM haemoglobin). (Panel B) Addition of BHT (100 μM final concentration) 30 min after the initiation of the reaction. (Panel C) Addition of BHT 90 min after the initiation of the interaction. The arrows show the direction of the absorbance changes at time points of 3, 30, 60, 120, 150, 180, 210 and 300 min.

4. DISCUSSION

It is well known that haemoglobin possesses peroxidase activity [18] and that the protein in both its oxy- and met forms (as well as free haemin) catalyses the oxidation of unsaturated fatty acids [19–21]. Additionally, the haem proteins, haemoglobin and myoglobin, activated by hydrogen peroxide can initiate the oxidation of cell membranes and of LDL [5,22,23]. Elevated levels of hydrogen peroxide or organic hydroperoxides induce destabilisation of the haem ring and release iron from haemoglobin [24,25]. Similar effects of excess hydrogen peroxide on myoglobin have been observed [23,26]. Previous work from this group has shown that ruptured cardiac myocytes under conditions of oxidative stress [27] generate free radicals, the identity of which can be assigned to the ferryl myoglobin radical species. Giulivi and Davies [28] have demonstrated that continual exposure of intact erythrocytes to a flow of hydrogen peroxide leads to the formation of ferryl haemoglobin; their proposal is that the synproportionation reaction rapidly reduces the ferryl haemoglobin to methaemoglobin, a mechanism which has also been described in myoglobin/hydrogen peroxide systems in which the mole ratio of the haem protein concentration was in excess of that of the oxidising component [29].

The results described in this paper demonstrate that membrane-free red cell haemolysates mediate the oxidative modification of LDL without addition of exogenous hydrogen peroxide; transition of the oxidation state of the oxyhaemoglobin in the haemolysate signals the enhancement of lipid peroxidation and the altered surface charge on the LDL.

The mode of action of transition metals or haem proteins, such as haemoglobin and myoglobin, in the

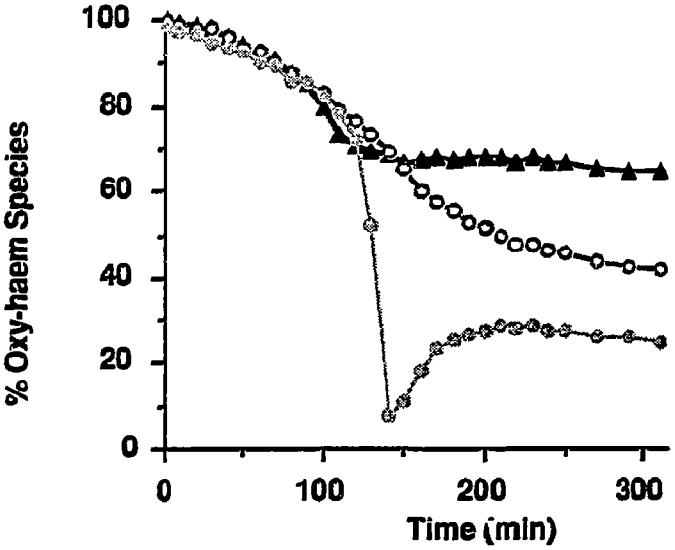

Fig. 5. The decline in the oxyhaemoglobin levels as a function of time in erythrocyte lysate during interaction with LDL, in the presence and absence of BHT. (●) No BHT addition; (○) BHT added 30 min after the initiation of the interaction; (▲) BHT added 90 min after the initiation of the interaction.

oxidation of LDL is through the catalysis of the propagation of the oxidation of preformed hydroperoxides [30,31]:

$$LOOH + M^{(n+1)+} \longrightarrow LOO^{\cdot} + M^{n+}$$
$$LOOH + M^{n+} \longrightarrow LO^{\cdot} + M^{(n+1)+}$$
$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$$
$$LO^{\cdot} + LH \longrightarrow LOH + L^{\cdot}$$
$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$$
$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$$

The data here suggest that after an initial slow phase corresponding to the antioxidant capacity of the LDL, hydroperoxides can interact with haemoglobin in a similar manner to hydrogen peroxide, forming ferryl haemoglobin which is then rapidly reduced to mixtures consisting mainly of oxy- and met forms, possibly by the synproportionation reaction [28].

Incorporation of the chain-breaking antioxidant arrests the rapid transition from the oxy to the ferryl form and inhibits LDL oxidation, supporting the idea that it is the interaction between lipid hydroperoxides and oxyhaemoglobin which is essential for the haemoglobin-mediated modification to LDL to take place. Enhancement of the antioxidant status of the LDL increases the resistance of LDL to oxidation and to oxidative damage induced by erythrocyte lysate; thus the antioxidant capacity of the LDL is a controlling factor in the oxidation of oxyhaemoglobin to more reactive, damaging forms.

Balla et al. [32] have recently reported the destruction of haem and the release of iron on interaction of LDL with haemin/hydrogen peroxide mixtures (ten- or twenty-fold molar excess of peroxide). Our studies clearly show that during interaction between LDL and erythrocyte lysate, LDL oxidation and oxidative activation of the oxyhaemoglobin occur with no requirement for exogenous oxidants, such as hydrogen peroxide, and involving no haem destruction nor iron release during the timescale studied. Any added hydrogen peroxide would clearly be removed by catalase action in the haemolysate.

It has been shown that probucol or BHT lower the frequency of occurrence of atherosclerotic lesions in animals [33,34]. Our examination of the incorporation of the lipid-soluble antioxidant, butylated hydroxytoluene, into the LDL/haem protein systems reveals a delay in the onset of the oxidative conversion of the oxyhaemoglobin, the abolition of the transition to the ferryl- and met state and the inhibition of LDL oxidation. Thus haem proteins leaking from ruptured cells may be capable of enhancing the oxidation of LDL which has penetrated the endothelium of the coronary vessels. This may occur by haem protein-mediated decomposition of preformed peroxides in LDL which has already been minimally oxidised by contact with neighbouring cells or the enzymatic activity of lipoxygenases.

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