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Ruptured erythrocytes inhibit the oxidation of membranes by 15-hydroperoxy-eicosatetraenoic acid

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In this study, the pro-oxidant effects of the hydroperoxide, 15-hydroperoxy-eicosatetraenoic acid (15-HPETE), on erythrocyte membranes and the modulation of the oxidation by haem proteins released from ruptured erythrocytes have been assessed. The results indicate that ruptured erythrocytes may act as an antioxidant in protecting membranes against oxidative stress induced by hydroperoxides and that it is the oxyhaemoglobin that is the active constituent of the protective mechanism. An important feature of the mechanism is the peroxidatic action of oxyhaemoglobin and its rate of reaction with 15-HPETE.

Haemoglobin; Hydroperoxide; Erythrocyte membrane; Lipid peroxidation; Thiol

1. INTRODUCTION

Oxidation of polyunsaturated fatty acids leads to the formation of hydroperoxides (LOOH), which are potential mediators in physiological and pathological processes. They may be generated by either non-enzymic mechanisms or enzymic pathways via the action of cylooxygenase or of lipoxygenase, an enzyme present in different forms in blood cells and tissues (platelets, leukocytes, neutrophils) [1]. The accumulation of hydroperoxides and their subsequent decomposition to alkoxyl ($LO^{\cdot}$) and peroxyl ($LOO^{\cdot}$) radicals accelerates the chain reaction of lipid peroxidation and is able to induce oxidative damage in the membranes. In particular, transition metals and haem proteins are effective catalysts for decomposition of hydroperoxides [2,3]:

$$LOOH + Fe^{II}\text{-complex} \rightarrow LO^{\cdot} + Fe^{III}\text{-complex} + OH^{-}$$
$$LOOH + Fe^{III}\text{-complex} \rightarrow LO^{\cdot} + [Fe^{IV} = O]\text{-complex} + H^{+}$$
$$\rightarrow LOO^{\cdot} + Fe^{II}\text{-complex} + H^{+}$$

Under conditions of oxidative stress, erythrocyte membranes are prone to the deleterious effects of hydroperoxides due to the high concentration of polyunsaturated fatty acid side chains and sulfhydryl functions. It is well-recognised that haemoglobin plays an important role in promoting oxidative stress [4]. However, haemoglobin is also able to act as an oxidant sink in protecting the membranes from exogenous free radical attack [5] via its conversion to methaemoglobin.

In the present paper, the ability of a physiological hydroperoxide to oxidise haemoglobin-free erythrocyte membranes was studied in terms of lipid peroxidation and thiol group oxidation. 15-hydroperoxy-eicosatetraenoic acid (15-HPETE), derived from the 15-lipoxygenation of arachidonic acid, was used as a naturally occurring hydroperoxide and compared to the organic peroxide, *tert*-butyl hydroperoxide (tBH), which has been extensively used as a model compound. The ability of membrane-free ruptured erythrocytes to promote or protect against membrane damage induced by these hydroperoxides was examined in comparison with that of oxyhaemoglobin and methaemoglobin. These results indicate that ruptured erythrocytes may act as an antioxidant in protecting membranes against oxidative stress induced by hydroperoxides and that it is the oxyhaemoglobin that is the active constituent of the protective mechanism.

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Abbreviations: 15-HPETE, 15-hydroperoxy-eicosatetraenoic acid; tBH, *tert*-butyl hydroperoxide; LOOH, hydroperoxide; LDL, low density lipoproteins.

2. MATERIAL AND METHODS

2.1. *Materials*

(15S)-Hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HPETE) was purchased from Cascade Biochem Ltd. (Reading, UK) and supplied as a solution in ethanol free of antioxidants (purity ≥ 98%). The volume of ethanol added with each sample was kept below 1%. All other chemicals were purchased from the Sigma Chemical Co. (Poole, UK).

2.2. *Preparation of red blood cell membranes*

Fresh venous blood was obtained from healthy adult volunteers, using acid-citrate-dextrose as anticoagulant. After centrifugation at 800 × *g* for 20 min at 4°C, the plasma and the buffy coat were removed

and the erythrocytes were washed three times with 5 mM isotonic phosphate buffer (pH 7.4). Haemoglobin-free erythrocyte membranes were prepared by hypotonic haemolysis according to the method of Dodge et al. [6]. The membrane protein concentration was determined by the Lowry assay [7], using bovine serum albumin as a standard, and the membranes were used at final concentrations of 0.5 mg/ml.

2.3. *Preparation of haemoglobins*

Methaemoglobin (bovine) was purified by oxidation with potassium ferricyanide and subsequent separation on a Sephadex G15-column. Oxyhaemoglobin was prepared from bovine methaemoglobin by reduction with sodium dithionite and chromatographically purified on a Sephadex G15-column using phosphate buffer (pH 7.4) as an eluant.

2.4. *Haemoglobin oxidation*

The visible spectra of haem species were recorded on a Beckman DU65 spectrophotometer fitted with Quant 1 software. Spectroscopic evaluation of the haem oxidation states, expressed as percentages of oxyhaemoglobin, methaemoglobin and ferrylhaemoglobin, were calculated from the extinction coefficients of each of the haemoglobin species at the appropriate wavelengths [8] applying the following equations:

$$[\text{Oxyhaemoglobin}] = 119\ A_{577} - 39\ A_{630} - 89\ A_{560}$$
$$[\text{Methaemoglobin}] = 28\ A_{577} + 307\ A_{630} - 55\ A_{560}$$
$$[\text{Haemichrome}] = -133\ A_{577} - 114\ A_{630} + 233\ A_{560}$$

2.5. *Measurement of lipid peroxidation*

The extent of lipid peroxidation was assessed by application of the thiobarbituric assay [9]. 0.5 ml of 10% trichloroacetic acid was added to 0.5 ml of sample followed by 0.5 ml of 0.75% thiobarbituric acid in 0.1 N HCl. The mixture was heated for 20 min at 95°C and, after cooling, centrifuged at $1000 \times g$ for 10 min. The absorbance of the supernatant was measured at 532 nm and the background absorbance at 580 nm, due to possible contributions from haem proteins, was subtracted. Appropriate controls were incorporated according to Gutteridge [10].

2.6. *Membrane thiol levels*

Thiol levels of membrane proteins were determined spectrophotometrically at 412 nm after derivatization with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) [11]. Standards were run simultaneously using reduced glutathione. Appropriate controls including those containing the hydroperoxides were run and showed no interference with the DTNB assay.

3. RESULTS

Firstly, haemoglobin-free membranes (0.5 mg/ml) were incubated with different concentrations of 15-HPETE (from 50 μM to 200 μM) for 3 h at 37°C and the membrane alterations were investigated by measuring the membrane lipid peroxidation as well as sulfhydryl group oxidation. The effects of 15-HPETE were compared with those of tBH, a model organic peroxide. Increasing relative concentrations of 15-HPETE to membranes resulted in an enhanced lipid peroxidation, as assessed by the formation of breakdown products of lipid peroxidation (Fig. 1). Interestingly, for each of the concentrations used, the extent of lipid peroxidation was about twice higher in membranes treated with 15-HPETE than in membranes treated with tBH, while lipid peroxidation was minimal in control membranes. Membrane thiol levels, used as a marker of oxidative

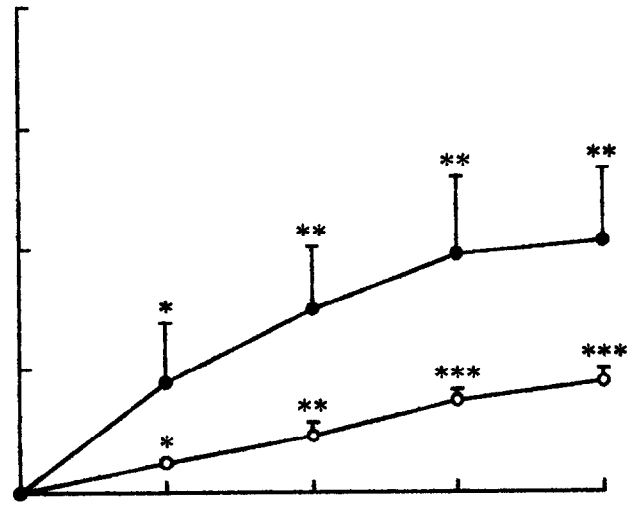

Fig. 1. The effects of different concentrations of hydroperoxides on membrane lipid peroxidation. Results are the means ± S.D. of 3 experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared to control. (○) tBH; (●) 15-HPETE. The contribution of 15-HPETE in the TBARS assay ($A_{532} = 0.018$, 50 μM 15-HPETE) was subtracted from the samples incubated with 15-HPETE.

protein damage, progressively decreased on incubation with 15-HPETE and a plateau was reached at 150 μM (Fig. 2). At comparable hydroperoxide concentrations, thiol groups were oxidised to approximately the same extent in 15-HPETE-treated membranes as in tBH-treated membranes.

In order to investigate the effects of haem species on membrane hydroperoxide-induced stress, the concentration corresponding to 50 μM hydroperoxide was selected for further experiments. Membranes (0.5 mg/ml) were incubated with fresh lysate, purified oxyhaemoglobin or methaemoglobin (final concentration 50 μM), with or without addition of 15-HPETE, for 3 h at 37°C.

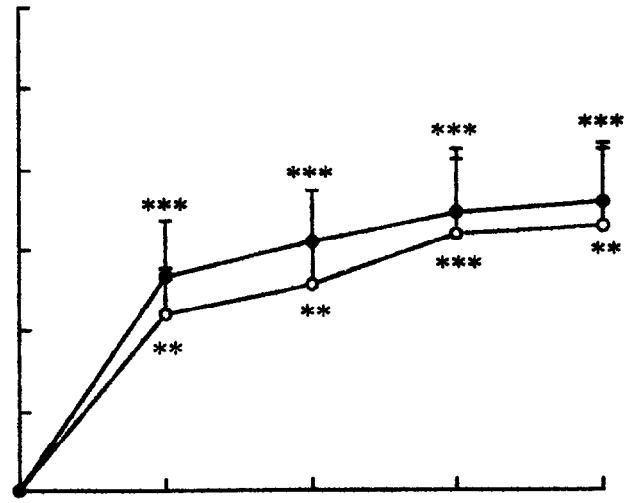

Fig. 2. The effects of different concentrations of hydroperoxides on membrane thiol levels. Results are the means ± S.D. of 3 experiments. $**P < 0.01$, $***P < 0.001$, compared to control. (○) tBH; (●) 15-HPETE.

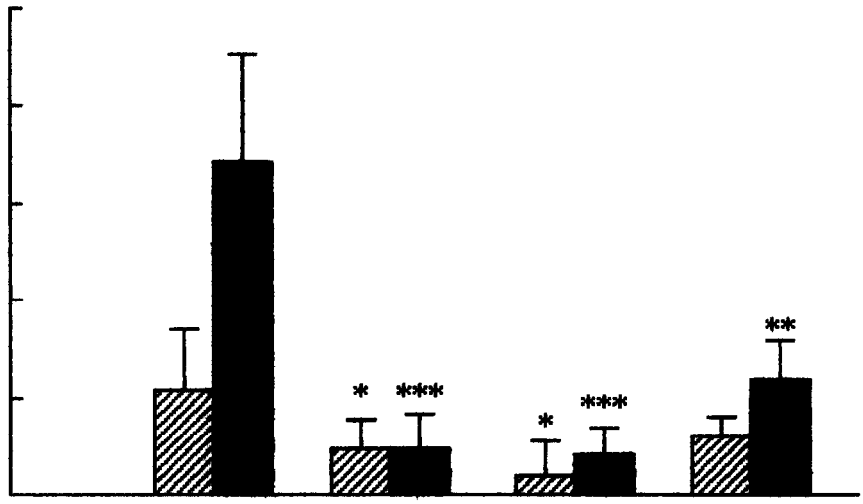

Fig. 3. The effects of erythrocyte lysate (50 μM) oxyhaemoglobin (50 μM) and methaemoglobin (50 μM) on the lipid peroxidation of membranes (0.5 mg/ml) incubated with either tBH (50 μM) or 15-HPETE (50 μM) for 3 h at 37°C. Results are the means ± S.D. of 3–5 experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared to control membranes. (□) + tBH; (■) + 15-HPETE.

Similar experiments were performed using tBH as an organic hydroperoxide. Results relative to membrane lipid peroxidation are reported in Fig. 3 and those relative to thiol levels are shown in Fig. 4. As previously described above, lipid peroxidation was induced extensively and thiol level was decreased by 47% in the membranes treated with 15-HPETE alone. Relatively little peroxidation but a similar thiol oxidation (−41%) occurred in the membranes treated with tBH alone. On the contrary, red cell membranes incubated with lysate were less susceptible to hydroperoxide-induced damage than control membranes. Lysate significantly counteracted the increased lipid peroxidation and the decreased thiol content induced by 15-HPETE. In order to ascertain which components of the lysate contributed to the protective effects, similar experiments were carried out using purified oxyhaemoglobin. Co-incubation of membranes with oxyhaemoglobin protected them against 15-HPETE-induced lipid peroxidation as well as thiol oxidation, reflecting the lysate response. The ability of methaemoglobin to protect membranes against hydroperoxide-induced damage was also investigated. Addition of methaemoglobin protected the membranes from the increase of lipid peroxidation induced by 15-HPETE and partly counteracted the decrease of thiol content.

The effects of haem species against tBH-induced membrane oxidative stress were also investigated. Lysate (50 μM) provided partial protection against the small amount of lipid peroxidation induced by tBH. It

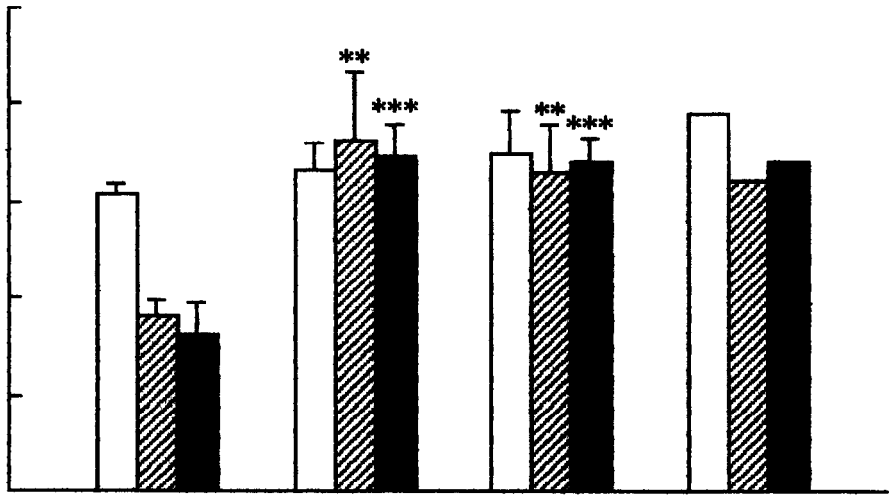

Fig. 4. The effects of erythrocyte lysate (50 μM) oxyhaemoglobin (50 μM) and methaemoglobin (50 μM) on the thiol level of membranes (0.5 mg/ml) incubated with either tBH (50 μM) or 15-HPETE (50 μM) for 3 h at 37°C. Results are the means ± S.D. of 4 experiments or the mean of 2 experiments. **$P < 0.01$; ***$P < 0.001$, compared to control membranes. (□) control; (□) +tBH; + (■) + 15-HPETE.

should be noted that lower concentrations of lysate (10 μM) protected to a lesser extent against hydroperoxide-induced damage whereas higher concentrations of lysate (100 μM) provided a higher protection (data not shown). In comparison with the lysate, addition of oxyhaemoglobin prevented the tBH-induced lipid peroxidation even more whereas methaemoglobin did not. On the other hand, co-incubation of membranes with fresh lysate or purified oxyhaemoglobin counteracted the oxidation of thiol groups induced by tBH to the same extent.

In order to elucidate the mechanism of action of the haem proteins, the oxidation state of haemoglobin was determined by difference spectroscopy after incubation of membranes with hydroperoxides and either fresh lysate, oxyhaemoglobin or methaemoglobin and calculation of the percentages of haemoglobin as oxyhaemoglobin, methaemoglobin or ferrylhaemoglobin (Table I). The production of ferrylhaemoglobin was confirmed by addition of sodium sulfide which resulted in the appearance of the sulfhaemoglobin peak at 617 nm [12]. Incubation of the lysate with 15-HPETE led mainly to the formation of a small amount of methaemoglobin (9%) and the presence of membranes in the incubation mixture did not affect the reaction between the lysate and the 15-HPETE; the addition of purified oxyhaemoglobin to 15-HPETE-treated membranes gave similar results. No spectroscopic alteration in the oxidation state of methaemoglobin was observed in membranes treated with both methaemoglobin and 15-HPETE suggesting that the reaction between methaemoglobin and 15-HPETE is extremely rapid and dependent on the reduction of the lipid hydroperoxide by trace levels of the met-haem protein.

Oxidation of haemoglobin was much more pronounced when the haem proteins were incubated with tBH. In the presence of membranes, oxyhaemoglobin was decreased by 47% in tBH-treated lysates with concomitant formation of methaemoglobin (37%) and ferrylhaemoglobin (10%). Interestingly, in the absence of membranes, incubation of the lysate with tBH resulted in a lower total oxidation of oxyhaemoglobin because of a lower accumulation of methaemoglobin (23%) while the same amount of ferryl was formed as in the presence of membranes. The addition of the purified oxyhaemoglobin in place of the erythrocyte lysate in membranes treated with tBH resulted in a similar oxidative response of the oxyhaemoglobin in protecting the membranes from oxidation. The response from methaemoglobin in place of the oxyhaem proteins was a less extensive alteration in the oxidation state of the methaem proteins in protecting the membranes from the tBH-induced oxidation, suggesting that methaemoglobin is better able to mediate the scavenging of hydroperoxides than the oxy forms of the haemoglobin.

Table I

Proportions of methaemoglobin and ferrylhaemoglobin generated during the interaction of erythrocyte lysate (50 μM), oxyhaemoglobin (50 μM) and methaemoglobin (50 μM) with 15-HPETE (50 μM) or tBH (50 μM) in the presence and absence of membranes (0.5 mg/ml) after 3 h incubation at 37°C.

	+ Membranes		− Membranes	
	% MetHb	% FerrylHb	% MetHb	% FerrylHb
Lysate +15-HPETE	8.8 ± 2.9	0.2 ± 0.6	9.6 ± 5.0	3.3 ± 2.1
OxyHb +15-HPETE	9.2 ± 1.4	0.6 ± 0.9	5.1 ± 0.1	4.5 ± 1.3
MetHb +15-HPETE	99.6 ± 0.7	0.4 ± 1.2	96.6 ± 1.4	3.4 ± 1.4
Lysate +tBH	37.3 ± 1.8	10.3 ± 2.5	23.1 ± 4.2	12.0 ± 1.1
OxyHb +tBH	40.9 ± 0.4	12.1 ± 1.8	22.3 ± 1.7	19.9 ± 0.7
MetHb +tBH	84.4 ± 1.03	15.6 ± 1.4	87.0 ± 3.9	13.0 ± 3.9

Results are the means ± S.D. of 3–5 experiments.

4. DISCUSSION

The peroxidatic action of haemoglobin is well known [13]. In both its oxy- and met- forms, the haem protein catalyses the oxidation of unsaturated fatty acids [4,14–16]. The studies described in this paper demonstrate the role of ruptured erythrocyte lysates in protecting membranes from peroxidation induced by the physiological hydroperoxide, 15-HPETE. With increasing relative concentrations of hydroperoxides to membranes, in the absence of erythrocyte lysate, the lipid peroxidation is increased, the HPETE being much more effective than equimolar concentrations of tBH. A progressive oxidation of the membrane thiol groups is also observed to approximately the same extent with both the hydroperoxides. The greater pro-oxidant effect of the lipophilic 15-HPETE on the polyunsaturated fatty acide side-chains of the membranes may be linked to the greater accessibility of the polyunsaturated fatty acid hydroperoxide within the membranes compared to the short rigid *tert*-butyl hydroperoxide molecule. This observation is consistent with the results of Vatassery [17] which showed that the presence of the lipoxygenase-derived linoleic acid hydroperoxide induced a more extensive oxidation of α-tocopherol in the membranes than the tBH.

Incorporation of ruptured erythrocytes into the incubation system, at equimolar concentrations of oxyhaemoglobin to 15-HPETE, extensively protected the membrane polyunsaturated fatty acids from lipid peroxidation and the membrane protein thiol groups from oxidation induced by 15-HPETE. Replacing the ruptured erythrocytes with oxyhaemoglobin showed equivalent effects; thus showing that the component of the lysate which is exerting the antioxidant effects is the oxyhaemoglobin. Similar protective effects by the lysate and the oxyhaemoglobin were exerted against the tBH-induced oxidation of protein thiols. The protection against the small amount of lipid peroxidation was mar-

ginal. Observing the oxidation state of the haem protein of the lysate or the oxyhaemoglobin as they protect the membranes from the hydroperoxide-induced oxidation, formation of methaemoglobin to the extent of 9% was observed with 15-HPETE, suggesting minimal cycling of the haem protein. In the case of tBH, a decline in 50% of the oxyhaemoglobin was observed with formation of met- and ferryl haemoglobin. Comparing the reactivity of the two hydroperoxides with membranes, the oxidative damage to the membrane protein thiol groups is the same but the lipid peroxidation is more extensive for 15-HPETE; however, surprisingly the haemoglobin modification in the lysate and the oxyhaemoglobin while protecting the membranes against the 15-HPETE induced oxidation is less whereas for tBH, for the same extent of protein thiol oxidation as with HPETE, there is a relatively low oxidation of the polyunsaturated fatty acids but the extent of haemoglobin modification required to prevent the oxidative stress is greater.

These observations suggest that the mode of action of oxyhaemoglobin as an antioxidant in protecting membranes from oxidation depends on the locality of the pro-oxidant and its reactivity with oxyhaemoglobin. The membrane-located 15-HPETE is able to induce greater levels of oxidative damage to the polyunsaturated fatty acid side-chains. In the presence of the lysate in the aqueous compartment, oxidation was inhibited perhaps due to the ability of haemoglobin to decompose catalytically hydroperoxy lipids [18]. Thus it seems that a hydroperoxidatic activity of haemoglobin enables it to act as an antioxidant in protecting membranes from hydroperoxide-mediated oxidation and that oxyhaemoglobin is a more effective peroxidase against HPETE than tBH. Interestingly, it has also been shown that glutathione-peroxidase acts faster with the increasing hydrophobicity of the hydroperoxide substrate [19] and this might, in principle, be the case with haem proteins with peroxidatic activity, contributing to the rapid metabolism of the 15-HPETE in the presence of the erythrocyte lysate. In the case of tBH, the protective action of the oxyhaemoglobin against the tBH-induced membrane thiol oxidation may be by diverting its action to the cycling of the haemoglobin to its higher oxidation state with the consumption of the hydroperoxide, forming the inactive tert-butyl hydroxide.

Previous studies have shown the ability of the iron-containing proteins to redox cycle and so to serve as additional oxidant sinks protecting the membranes from the deleterious effects of hydrogen peroxide and other hydroperoxides [20–22]. These studies demonstrate that haemoglobin released from ruptured erythrocytes can act as an oxidant sink through its hydroperoxidatic action, protecting membranes against oxidative damage induced by physiological hydroperoxides such as 15-HPETE.

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