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Peroxide-induced membrane damage in human erythrocytes

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Erythrocytes exposed to H_2O_2 or *t*-butyl hydroperoxide (tBHP) exhibited lipid peroxidation and increased passive cation permeability. In the case of tBHP a virtually complete inhibition of both processes was caused by butylated hydroxytoluene (BHT), whereas pretreatment of the cells with CO increased both lipid peroxidation and K^+ leakage. In the experiments with H_2O_2, on the other hand, both BHT and CO strongly inhibited lipid peroxidation, without affecting the increased passive cation permeability. These observations indicate different mechanisms of oxidative damage, induced by H_2O_2 and tBHP, respectively. The SH-reagent diamide strongly inhibited H_2O_2-induced K^+ leakage, indicating the involvement of SH oxidation in this process. With tBHP, on the contrary, K^+ leakage was not significantly influenced by diamide. Thiourea inhibited tBHP-induced K^+ leakage, without affecting lipid peroxidation. Together with other experimental evidence this contradicts a rigorous interdependence of tBHP-induced lipid peroxidation and K^+ leakage.

Introduction

The important role of oxidative stress in the development of different pathological processes is emphasized increasingly in recent literature [1–3]. Experimental evidence demonstrates that peroxides frequently play an important, direct or intermediate role in oxidative cell damage. Despite many studies the exact mechanism of peroxide-induced injury is still unknown and, moreover, may be dissimilar in different cell types. For instance, killing of mouse 3T3 cells by H_2O_2 has been attributed to DNA damage [4]. Moreover, it was suggested that this DNA damage was not caused by H_2O_2 itself, but by iron-mediated generation of hydroxyl radicals via a Fenton type reaction [4,5]. In other cases of oxidative damage membrane deterioration is involved. Rubin and Farber [6], e.g. presented evidence for at least two mechanisms of hepatocyte killing by H_2O_2. The first pathway was related to peroxidation of membrane phospholipids, whereas the second, as yet unknown mechanism was independent of lipid peroxidation [6].

Erythrocytes have been used extensively as a model, to investigate oxidative membrane damage. Peroxide-induced membrane damage in red blood cells is reflected, e.g. by lipid peroxidation and increased passive cation permeability. Experimental evidence indicates a close interdependence between hemoglobin oxidation and lipid peroxidation [7,8]. Utilizing tBHP to induce oxidative stress in red blood cells, disturbed membrane permeability and, ultimately, hemolysis seemed to be the consequence of lipid peroxidation [9,10]. With H_2O_2 as oxidant, however, it appeared that lipid peroxidation could be inhibited, without affecting the increased passive cation permeability of the

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Abbreviations: tBHP, *t*-butyl hydroperoxide; BHT, butylated hydroxytoluene; HbCO, carbonmonooxyhemoglobin.

membrane [11], arguing against a strict interdependence of these two processes. Apparently different exogenous peroxides may have dissimilar pathways, leading to increased passive cation permeability.

Considering the importance to elucidate the mechanism of oxidative membrane damage at the molecular level, H_2O_2- and tBHP-induced membrane damage in erythrocytes was investigated in detail. It will be shown that there are significant differences between H_2O_2- and tBHP-induced membrane damage in red blood cells. Further it could be demonstrated for the first time that K^+ leakage, induced by H_2O_2, is most likely caused by oxidation of membrane SH groups.

Methods

Heparinized human blood was centrifuged shortly after collection. The red blood cells were washed three times and resuspended at 10% hematocrit in buffered isotonic NaCl solution. HbCO containing cells were prepared by exposure of the suspension to pure CO gas. Hemoglobin-free ghosts were prepared by the gradual osmotic lysis method of Weed et al. [12]. Pelleted ghosts were washed three times with 10 mM sodium phosphate buffer (pH 7.4) and resuspended in this buffer at a concentration of 1 mg protein/ml. All incubations were carried out at 30°C. Experiments with H_2O_2 and red blood cells or ghosts were done in the presence of 1.2 mM sodium azide, to inhibit endogenous catalase activity.

Linolenic acid solutions were prepared in 1% Tween 20, as described by Tien et al. [13].

K^+ efflux from red blood cells was determined with a flame photometer and expressed as percentage of total efflux, evoked by lysis of the cells in distilled water. Lipid peroxidation was assayed by measuring thiobarbituric acid reactive products, as described by Stocks and Dormandy [14]. Histidine was determined according to Sokolovsky and Vallee [15], tryptophan as described by Spies and Chambers [16], tyrosine according to Uehara et al. [17], methionine according to McCarthy and Paille [18], phenylalanine as described by Ambrose [19], SH groups by the method of Sedlak and Lindsay [20] and free NH_2 groups according to Udenfriend et al. [21]. The amounts of hemoglobin and methemoglobin were determined as described by Sutton et al. [22].

Results

Peroxide effects on solubilized substrates and ghosts

Incubation of cysteine (2 mM) with H_2O_2 (5 mM) in 10 mM sodium phosphate buffer (pH 7.4) resulted in a fast disappearance of free SH groups. Oxidation of SH groups was complete after 5 min and appeared to be insensitive to BHT (0.1 mM), isopropanol (50 mM) and superoxide dismutase (10^3 units/ml). No decrease of NH_2 groups was observed, even with prolonged incubation (2 h).

In similar experiments histidine, tryptophan, tyrosine, phenylalanine and methionine appeared to be unaffected by H_2O_2, after an incubation period of 2 h. Also after addition of hemoglobin (25 μM) to the reaction mixture no oxidation of these amino acids occurred.

Incubation of linolenic acid with H_2O_2 did not result in formation of thiobarbituric acid reactive products. Lipid peroxidation was observed, however, when hemoglobin (25 μM) was present in the incubation medium. BHT (0.1 mM) caused 95% inhibition of this peroxidation in the presence of hemoglobin, whereas isopropanol and superoxide dismutase had no influence. With tBHP instead of H_2O_2 similar results were obtained.

The results obtained when ghosts were incubated with tBHP or H_2O_2 were essentially the same as with solubilized substrates. The only difference was that SH oxidation was not complete, but resulted in a decrease of free SH groups to about 40% of the initial value. The results are summarized in Table I.

In further experiments red cell ghosts were treated with either 5 mM diamide or 5 mM H_2O_2. Subsequently 10 mM dithiothreitol or 50 mM sodium arsenite was added. Incubation was continued for 45 min, followed by washing the ghosts four times with 10 mM phosphate buffer (pH 7.4). In three independent experiments incubation of ghosts with diamide during 10 min reduced the number of free SH groups to 32($\pm$5)% of the initial value. It is well-documented by Deuticke et al. [23–26] that the SH groups are oxidized to disulfides under these conditions. In accordance, subsequent treatment with dithiothreitol restored

TABLE I

LIPID PEROXIDATION AND OXIDATION OF SULFHYDRYL GROUPS BY H_2O_2 IN GHOSTS

The complete reaction mixtures contained, in final concentrations, 10 mM sodium phosphate (pH 7.4), 5 mM H_2O_2, 25 μM hemoglobin and 1 mg/ml ghost protein. Incubation was carried out at 30°C during 2 h. Lipid peroxidation is expressed in A_{532}/mg ghost protein and SH oxidation in nmol/mg ghost protein. Number of sulfhydryl groups in untreated ghosts: 97.2 nmol/mg ghost protein.

Reaction mixture	Additions	SH groups oxidized	A_{532}
Complete	–	58.3	0.75
Omit Hb	–	57.1	0.04
Complete	BHT (0.1 mM)	59.4	0.05
Complete	isopropanol (50 mM)	56.9	0.69
Complete	superoxide dismutase (10^3 units/ml)	57.9	0.74

the number of free SH groups to the original level, whereas arsenite had no influence. Incubation during 10 min with H_2O_2 resulted in a decrease of free SH groups to 38(±8)%. After subsequent treatment with dithiothreitol the SH groups increased to 90(±4)% of the initial value, whereas arsenite caused an increase to 69(±6)%. This indicates a sulfhydryl oxidation pattern, different from that obtained with diamide. It has been shown before that oxidation of SH groups in proteins by H_2O_2 does not only yield disulfides, but also sulfenic acids, which can be easily further oxidized by H_2O_2 to sulfonic acid residues [27–29]. Sulfenic acid residues are reduced by dithiothreitol but also, rather slowly, by relatively high concentrations of arsenite [27–29]. Therefore these results suggest that at least a fraction of the membrane SH groups is oxidized to sulfenic acid residues by H_2O_2. When incubation of the ghosts with H_2O_2 was continued to 60 min, no further decrease of SH groups occurred. After subsequent treatment with dithiothreitol the SH groups increased to 73(±5)% of the initial value, whereas the arsenite effect had disappeared. These results probably indicate that after prolonged incubation with H_2O_2 the principal oxidation products of sulfhydryl groups are disulfides and sulfonic acid residues. Finally a ghost suspension was first incubated during 10 min with 5 mM diamide and subsequently, during 60 min with 5 mM H_2O_2. Free SH groups were decreased to 28% of the original value. This sulfhydryl oxidation could be fully reversed by dithiothreitol, but arsenite had no measurable effect. In control experiments it appeared that no reaction occurred between diamide and H_2O_2, as judged from the unchanged optical absorbance of H_2O_2 at 240 nm. This indicates that the disulfides, generated by diamide, are resistent to further oxidation by H_2O_2, in accordance with previous observations [30].

Peroxide effects on intact erythrocytes

In intact red blood cells H_2O_2 (30 mM) induced lipid peroxidation, K^+ leakage and an immediate conversion of hemoglobin into methemoglobin. These effects were not influenced by isopropanol and thiourea. BHT and conversion of cellular hemoglobin to HbCO strongly inhibited lipid peroxidation, without a significant effect on K^+ leakage (Fig. 1). Treatment of erythrocytes with diamide enhances the permeability of the membrane for a number of solutes [23,24]. Diamide-induced K^+ leakage and its reversal by dithiothreitol are shown in Fig. 2. The K^+ leakage

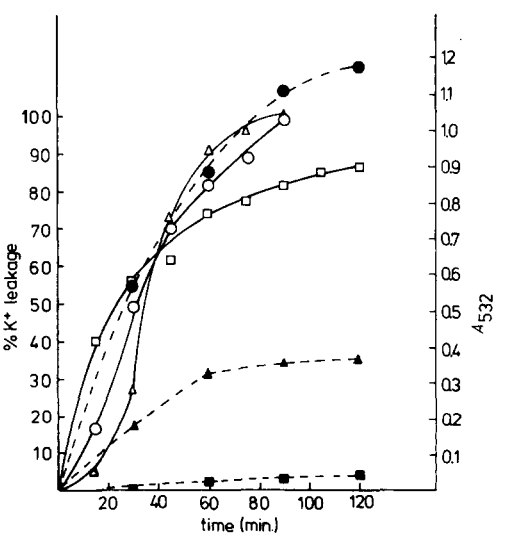

Fig. 1. K^+ leakage (open symbols) and thiobarbituric acid reactivity (A_{532}, closed symbols) during incubation of a 10% erythrocyte suspension with 30 mM H_2O_2. ○ and ●, no further additions; □ and ■, 0.1 mM BHT added; △ and ▲, cells pretreated with CO.

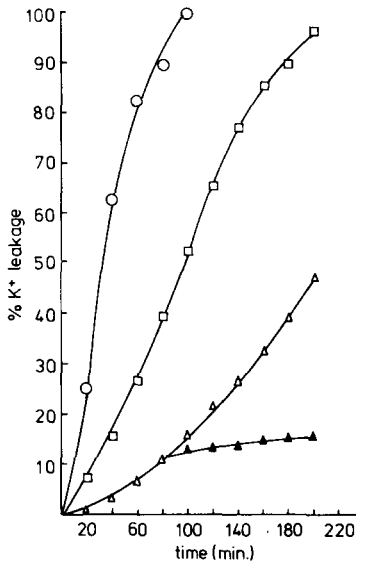

Fig. 2. K^+ leakage from red blood cells in the presence of 10 mM diamide (△), 10 mM diamide and 50 mM dithiothreitol, added after 80 min (▲), 30 mM H_2O_2 (○) and 30 mM H_2O_2 plus 10 mM diamide (□).

provoked by H_2O_2 was strongly counteracted by diamide (Fig. 2). Dithiothreitol did not inhibit the K^+ leakage, caused by H_2O_2 when added after 10, 20 or 30 min. Experiments designed to study the possible reversal of K^+ leakage by arsenite after short incubation periods with H_2O_2 were unsuccessful, as sodium arsenite itself caused cell lysis.

Incubation of erythrocytes with tBHP also induced K^+ leakage and lipid peroxidation. BHT strongly inhibited both processes, whereas conversion of cellular hemoglobin to HbCO augmented K^+ loss as well as lipid peroxidation (Fig. 3). Both processes were unaffected by isopropanol. Thiourea on the other hand inhibited K^+ leakage

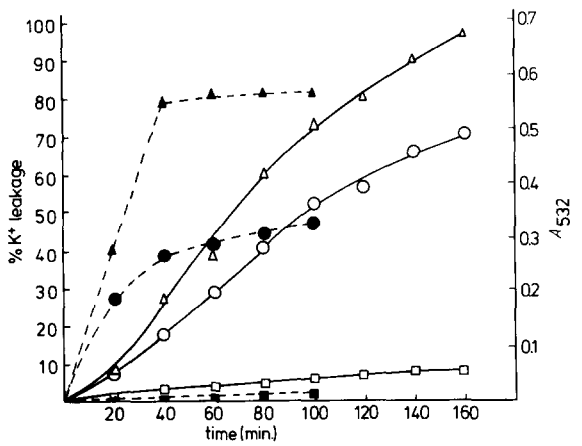

Fig. 3. K^+ leakage (open symbols) and thiobarbituric acid reactivity (closed symbols) during incubation of a 10% erythrocyte suspension with 2 mM tBHP. ○ and ●, no further additions; □ and ■, 0.1 mM BHT added; △ and ▲, cells pretreated with CO.

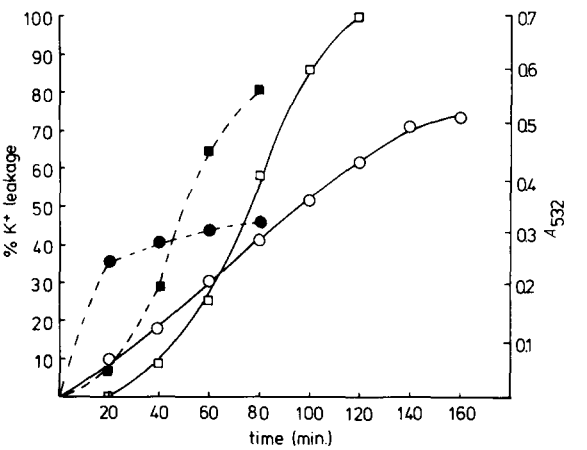

Fig. 4. K^+ leakage (open symbols) and thiobarbituric acid reactivity (closed symbols) during incubation of a 10% erythrocyte suspension with 2 mM tBHP. ○ and ●, no further additions; □ and ■, 1.2 mM sodium azide added.

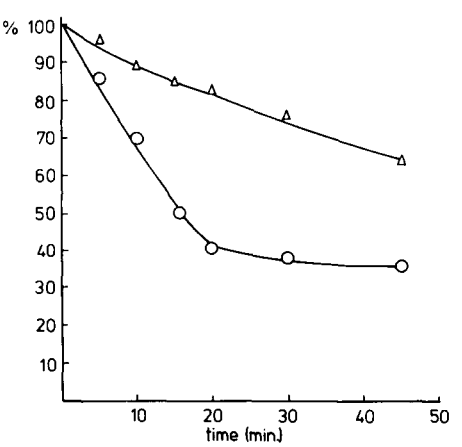

Fig. 5. Concentration of oxyhemoglobin during incubation of a 10% erythrocyte suspension with 2 mM tBHP. ○, no further additions; △, 1.2 mM sodium azide added. Concentrations are expressed in % of the initial oxyhemoglobin concentration.

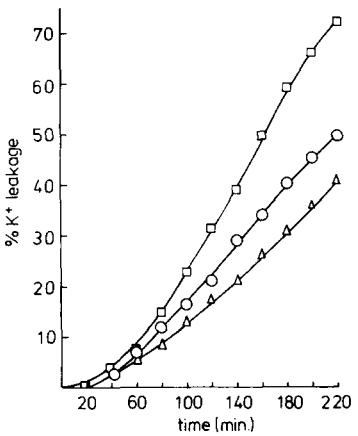

Fig. 6. K^+ leakage from red blood cells in the presence of 10 mM diamide (△), 2 mM tBHP (○) and 10 mM diamide plus 2 mM tBHP (□).

(from 62% to 29% after an incubation period of 120 min), without affecting lipid peroxidation. Raising the tBHP concentration from 2 mM to 8 mM augmented the velocity of K^+ leakage from about 27% to 80% in 60 min, without increasing lipid peroxidation. Sodium azide had a remarkable effect on tBHP-induced lipid peroxidation and K^+ loss. At first both processes were inhibited but after prolonged incubation periods they were stimulated (Fig. 4). Concomitantly, the tBHP-induced conversion of hemoglobin to methemoglobin was inhibited (Fig. 5). Addition of diamide had no clear effect on tBHP-induced K^+ loss: the effects of diamide and tBHP were more or less additive (Fig. 6). Dithiothreitol did not inhibit the tBHP-induced K^+ leakage, when added after 80 min.

Discussion

The peroxide-induced effects on solubilized substrates and ghosts were not influenced by superoxide dismutase or the $OH^{\cdot}$ scavenger isopropanol, indicating that neither superoxide anion nor hydroxyl radicals are involved in the described phenomena. These model experiments further demonstrated that H_2O_2 and tBHP directly oxidized SH groups, without affecting other oxidizable amino acid residues. Especially the experiments on ghosts are relevant in this context. It has been shown that under certain experimental conditions free radical metabolites are generated during oxidation of SH groups [31]. If generated during peroxide-induced SH oxidation, such free radical metabolites might have triggered oxidation of other amino acid residues in a secondary reaction. The experimental results do not support such a mechanism.

Lipid peroxidation only occurred in the presence of hemoglobin, indicating an essential intermediary role of hemoglobin in this process. A close interdependence between hemoglobin oxidation and lipid peroxidation has been described before [8].

In intact cells BHT strongly suppressed the lipid peroxidation caused by H_2O_2, without a similar effect on K^+ leakage (Fig. 1). This observation is in accordance with previous studies by Snyder et al. [11] and demonstrates that the two processes are not interrelated. This conclusion is emphasized by the effect of CO, presented in Fig. 1. Again H_2O_2-induced lipid peroxidation is inhibited, whereas K^+ leakage is hardly affected.

The lack of correlation between lipid peroxidation and K^+ leakage makes it likely that H_2O_2 induced increase of passive cation permeability is caused by SH oxidation, as observed in the model experiments. This is confirmed by the results depicted in Fig. 2. It has been demonstrated by Deuticke et al. [23,24] that diamide increases passive membrane permeability by oxidation of membrane SH groups to disulfides. As shown in Fig. 2 the combined effects of diamide and H_2O_2 on K^+ leakage are not additive and in fact even much less than the effect of H_2O_2 alone. This indicates that H_2O_2 and diamide affect the same SH groups. As shown in the results section, it seems likely that oxidation of membrane SH groups by H_2O_2 does not only yield disulfides, but also sulfenic and sulfonic acid residues, whereas disulfides are not sensitive to further oxidation by H_2O_2. Therefore the results shown in Fig. 2 may be explained as follows. Oxidation of a limited number of membrane SH groups by diamide to disulfides increases passive permeability [23,24], whereas oxidation of the same SH groups to sulfenic and sulfonic acid residues by H_2O_2 is much more effective in this respect. Under this supposition the strongly damaging H_2O_2-induced generation of sulfenic and sulfonic acid groups will be inhibited

by the less damaging diamide-induced generation of disulfides.

The pathways of tBHP-induced membrane damage are clearly different from the mechanism by which H_2O_2 inflicts membrane deterioration, as shown by the following observations. (1) Comparison of Figs. 2 and 6 indicates, that tBHP-induced K^+ loss cannot be explained along the same lines of reasoning as H_2O_2-induced K^+ leakage. (2) In the case of tBHP-induced oxidative stress BHT inhibited not only lipid peroxidation, but also K^+ leakage (Fig. 3), contrary to the results with H_2O_2. (3) Also CO has a quite different effect, aggravating both lipid peroxidation and K^+ leakage. These dissimilar effects of CO suggest different mechanisms of H_2O_2- and tBHP-induced lipid peroxidation.

With tBHP, the correlation between lipid peroxidation and K^+ leakage in the presence of both BHT and HbCO suggests a causal relationship between lipid peroxidation and K^+ leakage, in accordance with previous conclusions of Trotta et al. [10,32]. Further experiments, however, contradicted such a causal relationship. First, as mentioned in the results section, an increase of the tBHP concentration from 2 to 8 mM had a pronounced effect on K^+ leakage, without affecting lipid peroxidation. This makes a causal relationship between tBHP-induced lipid peroxidation and K^+ leakage doubtful. Further, in the presence of 30 mM H_2O_2 or 8 mM tBHP K^+ leakage proceeded with virtually the same velocity. The concomitant lipid peroxidation with H_2O_2 was, however, much more pronounced than with tBHP and could be suppressed by BHT, without affecting K^+ loss (Fig. 1). Finally, in the case of tBHP, thiourea brought about a clear dissociation between the two processes, causing inhibition of K^+ leakage without affecting lipid peroxidation. It seems improbable that this effect of thiourea can be explained by its activity as a hydroxyl radical scavenger, as another potent $OH^{\cdot}$ scavenger, viz. isopropanol, had no effect at all. More likely thiourea interacts with another tBHP-derived radical, unreactive towards isopropanol. According to Trotta et al. [10,32] such radicals are formed in a reaction between tBHP and hemoglobin (which is converted into methemoglobin in this process). In a further reaction with methemoglobin these tBHP-derived radicals can be rendered harmless, yielding *t*-butylalcohol [10,32]. The effect of sodium azide can be interpreted along these lines. Apparently azide inhibits methemoglobin formation by tBHP and thus, presumably, the generation of tBHP-derived radicals (Fig. 5). This can explain the initial inhibition of both lipid peroxidation and K^+ leakage (Fig. 4). After prolonged incubation, however, both processes are augmented by azide. This may be attributed to the lower level of scavenging of tBHP-derived radicals, due to the relatively low concentrations of methemoglobin. Thus a higher percentage of the radicals remains reactive, causing lipid peroxidation and K^+ leakage. As the effect of thiourea indicates that these two processes are not directly interdependent, further investigations will be necessary to elucidate the mechanism of tBHP-induced increased passive membrane permeability.

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