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The effect of protoporphyrin on the susceptibility of human erythrocytes to oxidative stress: exposure to hydrogen peroxide

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Abstract

Binding of protoporphyrin caused a perturbation of the erythrocyte membrane, as reflected by a change in cell shape from discoid to echinocyte, and a concomitant increase in mean cellular volume and K^+-loss. Protoporphyrin-induced changes could be prevented by the presence of $BaCl_2$, whereas binding of protoporphyrin was not affected. Exposure of erythrocytes to hydrogen peroxide leads to K^+-leakage and lipid peroxidation. In de presence of protoporphyrin, H_2O_2-induced K^+-leakage was enhanced, whereas lipid peroxidation was inhibited. The increase in H_2O_2-induced K^+-leakage by protoporphyrin was not affected by diamide or various K^+ channel blockers, but could be prevented by the addition of $BaCl_2$. The inhibition of lipid peroxidation, on the other hand, was not affected by $BaCl_2$. These results indicate that the enhancement of H_2O_2-induced K^+-leakage was most likely caused by the change in cell shape. Addition of chlorpromazine and promethazine, positively charged molecules that induce stomatocytosis, did not cause an enhancement of H_2O_2-induced K^+-leakage.

Keywords: Erythrocyte; Oxidative stress; Lipid peroxidation; Potassium ion leakage; Protoporphyrin; Porphyria

1. Introduction

Oxidative stress is thought to play an important role in many physiological and pathological phenomena. Erythrocytes have been used extensively as a model to investigate oxidative damage, with special emphasis on membrane damage [1–5]. An important consideration in this context is the absence of DNA and subcellular structures in human red blood cells, excluding several mechanisms of cell injury. On the other hand, the presence of hemoglobin often plays an important role in various reactions. Erythrocyte membrane damage can be reflected, e.g., by leakage of cellular constituents, such as K^+, to the medium and lipid peroxidation. It has been found that erythrocytes are more susceptible to oxidative stress in disorders with intrinsic membrane defects, such as sickle cell anemia [6,7]. This increased susceptibility of sickle cells has been attributed to the instability of sickle hemoglobin and to impaired protective mechanisms, but also to the loss of membrane structural integrity that accompanies the change in cell shape.

We found that exposure of erythrocytes to protoporphyrin resulted in a change in cell shape, which is described in this paper. Porphyrin accumulation occurs in a group of diseases called the porphyrias. The porphyrias are caused by various enzyme deficiencies in the heme synthesis [8]. In erythropoietic protoporphyria the enzyme ferrochelatase, which catalyses the insertion of ferrous iron into protoporphyrin, is affected and protoporphyrin accumulates in liver and erythroid cells [8,9]. The major clinical manifestation of this disease is a pronounced hypersensitivity of the skin to visible light, caused by the sensitizing properties of protoporphyrin accumulated in the skin. Furthermore, it has been found that liver disease occurs more frequently than normal in patients with erythropoietic protoporphyria, most likely due to massive accumulation of protoporphyrin in liver tissue [8]. Hemolysis has only occasionally been reported in erythropoietic protoporphyria, usually in association with liver disease and it has been suggested that there might be a causal relationship between liver disease and hemolysis [10].

Abbreviations: TBARS, thiobarbituric acid reactive substances; TBA, thiobarbituric acid; PBS, phosphate-buffered saline; MCV, mean cellular volume; BHT, butylated hydroxytoluene.

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Since the susceptibility of cells to oxidative damage depends on the integrity of the membrane structure, it is possible that protoporphyrin, by changing the shape of the cell, also affects its susceptibility to oxidative stress. Oxidative damage as a result of exposure to light in the presence of porphyrins, has been studied extensively [11–13]. However, not much is known about the effects of protoporphyrin on erythrocytes in the dark. In the present paper we have investigated the susceptibility of erythrocytes towards hydrogen peroxide in the presence of protoporphyrin and it is shown that the susceptibility of the cells is indeed changed by the presence of protoporphyrin.

2. Materials and methods

Protoporphyrin IX and uroporphyrin III were purchased from Porphyrin Products (Logan, UT). Bilirubin IX was obtained from ICN Biochemicals (Zoetermeer, The Netherlands). ^{14}C-labelled inulin came from Amersham, UK. All other reagents were of analytical grade and used without further purification. All solutions were prepared in water that had been passed through a Millipore Milli-Q ultrapurification system and were made up freshly before use. Stock solutions (1 mM) of porphyrins and bilirubin were made in 4 mM NaOH. To avoid photoexcitation of the porphyrins and bilirubin the incubation mixtures were prepared and handled under dim light.

Heparinized human blood was centrifuged shortly after collection. The erythrocytes were washed three times and resuspended at 10% hematocrit in PBS. Erythrocytes were exposed to H_2O_2 (20 mM) at 30° C in the presence of 2 mM sodium azide, to inhibit endogenous catalase activity. In some experiments erythrocytes were washed twice with PBS and once with 10 mM Tris-HCl pH 7.4, containing 100 mM $BaCl_2$. Subsequently, the erythrocytes were resuspended at 10% hematocrit in 10 mM Tris-HCl pH 7.4, containing 100 mM $BaCl_2$. Preincubation of erythrocytes with 0.1 mM bumetanide was performed at 30° C for 30 min as described by Johnson and Tang [14]. Cells were preincubated with 1 mM furosemide for 10 min at 30° C and with 0.1 mM ouabain for 5 min at room temperature [15].

Hemoglobin-free ghosts were prepared by the gradual osmotic lysis method of Weed et al. [16]. Protein was determined as described by Lowry et al. using albumin as a standard [17].

Binding of porphyrins to erythrocytes was determined by extraction of protoporphyrin into ethyl acetate and subsequently into HCl. The latter step is necessary to remove heme, which interferes with the fluorescence measurements. A 10% erythrocyte suspension was incubated with protoporphyrin (final concentrations: 0–50 μM) at 30° C. After incubation (0–30 min) the cells were washed twice with PBS and resuspended at 1% hematocrit in PBS. 1 ml of this suspension was extracted with 4 ml acetic acid/ethyl acetate (1:4). To 1 ml of the ethyl acetate layer 3 ml of 3 M HCl were added. After centrifugation the fluorescence of the HCl layer was determined (excitation 405 nm; emission 632 nm). By this method 90% of the added protoporphyrin could be recovered. Fluorescence data were obtained using an Aminco SPF 500 spectrofluorometer.

K^+-leakage from red blood cells was determined with a flame photometer (Clinical Flame Photometer 410 c, Corning) and expressed as percentage of total efflux evoked by lysis of the cells in distilled water. The mean cellular volume was measured by centrifuging 0.3 ml of a 10% erythrocyte suspension during 20 min at 3000 rpm in a Hamburger-type hematocrit tube, correcting the packed cell volume for trapped medium. Trapped medium between packed cells was determined by adding 2% ^{14}C-labelled inulin to the medium, prior to centrifugation. 0.05 ml of packed cells was resuspended in 2 ml PBS and the radioactivity of the supernatant of this suspension was measured by scintillation counting. Cell morphology was assessed by light microscopy of cell suspensions after fixation of the cells in PBS + 2% glutaraldehyde for 1 h.

Lipid peroxidation was assayed by measuring the generation of TBARS [18,19]. Briefly, reactions were stopped by addition of 0.75 ml of 28% (w/v) trichloroacetic acid and 1.3% (w/v) of sodium arsenite to 1.5 ml samples. After centrifugation, 1.5 ml of the supernatant were added to 0.75 ml of 1% TBA in 0.05 M NaOH containing 0.1 mM BHT. Subsequently, samples were heated at 100° C for 15 min, and after cooling the absorption at 532 nm was measured (extinction coefficient for malondialdehyde, $1.56 \cdot 10^5$ M^{-1} cm^{-1}). Absorption measurements were carried out using a Beckman DU 64 spectrophotometer.

For all experiments, variability did not exceed 10%, with three independent experiments for each data point.

3. Results

3.1. Effect of protoporphyrin on erythrocytes

Incubation of erythrocytes with protoporphyrin (10–50 μM) at 30° C resulted in rapid binding of protoporphyrin to the erythrocytes (Fig. 1). Binding amounted to 90% of the protoporphyrin added to the incubation mixture. Uroporphyrin, a more hydrophilic porphyrin, did not bind to the erythrocytes at all (results not shown). Addition of protoporphyrin to the erythrocytes resulted in K^+-leakage and in an increase in cellular volume (Table 1). The amount of K^+ released from the cells enhanced with increasing protoporphyrin concentrations and reached a maximum after incubation for 30 min. Protoporphyrin-induced increase in MCV and K^+-leakage was not affected by the addition of EGTA, ouabain, furosemide or bumetanide or by substituting sucrose for NaCl (results not shown). Replacement of PBS by 10 mM Tris-HCl pH 7.4,

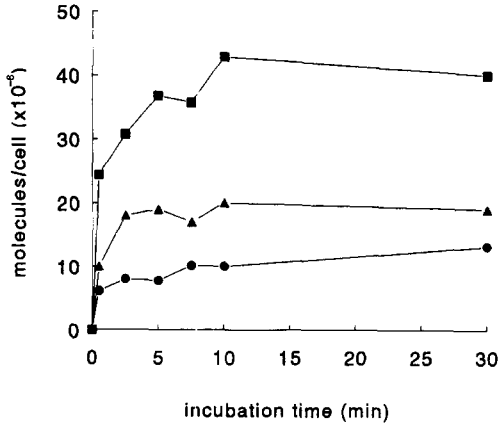

Fig. 1. Binding of protoporphyrin to erythrocytes. Erythrocytes were incubated for 0–30 min with protoporphyrin at 30° C. Subsequently protoporphyrin was extracted as described in Materials and methods. (●) 10 μM protoporphyrin; (▲) 25 μM protoporphyrin; (■) 50 μM protoporphyrin.

containing 100 mM $BaCl_2$, prevented protoporphyrin-induced cell swelling and K^+-leakage (Table 1). However, binding of protoporphyrin to the erythrocytes was not affected under these conditions and again approx. 90% of the porphyrin molecules was bound to the cells (Table 1).

Incubation of erythrocytes with protoporphyrin also resulted in morphological changes. In PBS the discoid cells became echinocytic after exposure to protoporphyrin (Fig. 2A). Incubation with uroporphyrin did not induce changes in cell shape. When these experiments were repeated in 10 mM Tris-HCl pH 7.4, containing 100 mM $BaCl_2$, the shape of the erythrocytes did not change upon incubation with protoporphyrin, but remained discoid (Fig. 2B).

The localisation of protoporphyrin in the red cell membrane was further studied in ghosts (Fig. 3). The absorption spectrum of protoporphyrin in PBS is typical of aggregated species: the absorbance maximum is blue-shifted (λ_{max} = 360 nm), broadened and the absorbance is low [20]. In the presence of red cell membranes the absorbance maximum shifted to 412 nm, which is characteristic for the monomeric form of the porphyrin. This indicates that protoporphyrin molecules were localised in the membrane. In Tris-buffered $BaCl_2$ the absorbance maximum of protoporphyrin was slightly shifted to 377 nm, but still broad and low (Fig. 3). In the presence of red cell membranes the absorbance increased, but there was no change in the absorption maximum, showing that the localisation of protoporphyrin in/at the membrane was completely different in the presence of $BaCl_2$.

We compared the effect of protoporphyrin on MCV and K^+-leakage with the effect of several other compounds, amongst them some well-known radical scavengers (Table 2). Erythrocytes were incubated with these compounds for 30 min at 30° C and the effect on cell volume and K^+-leakage was determined. Of these compounds both chlorpromazine and promethazine caused an increase in MCV and K^+-leakage during the preincubation period (Table 2). No K^+-leakage or increase in MCV could be observed when cells were incubated with uroporphyrin, bilirubin, BHT or diphenylamine.

3.2. Effect of protoporphyrin on H_2O_2-induced K^+-leakage and lipid peroxidation

In further experiments erythrocytes were always incubated with protoporphyrin for 30 min at 30° C prior to the addition of H_2O_2. Removal of unbound protoporphyrin before the addition of H_2O_2 did not influence the results, indicating that the observed effects were only due to membrane-bound protoporphyrin. H_2O_2 was therefore added directly after incubation with the porphyrins, without removing unbound porphyrin molecules. Incubation of erythrocytes with H_2O_2 resulted in lipid peroxidation and K^+-leakage, as has been shown previously [1–3]. In the presence of protoporphyrin, peroxide-induced lipid peroxidation was inhibited concentration-dependently (Fig. 4). The effect of protoporphyrin on peroxide-induced K^+-leakage, on the other hand, was quite different. Exposure of the cells to 20 mM H_2O_2 for 15 min resulted in a K^+-leakage of approx. 20%. Incubation with increasing concentrations of protoporphyrin resulted in enhancement of the peroxide-induced K^+-leakage (Fig. 4). The data shown in Fig. 4 are corrected for K^+-leakage induced by protoporphyrin alone.

The fate of the protoporphyrin molecules during these reactions was also determined. Erythrocytes were exposed to H_2O_2 in the presence of 50 μM protoporphyrin. After incubation for 120 min, protoporphyrin was extracted from the incubation mixture and the fluorescence of the HCl

Table 1
Interaction between protoporphyrin and erythrocytes

Porphyrin concentration	PBS			Tris/$BaCl_2$		
	binding	MCV	%K^+-loss	binding	MCV	%K^+-loss
10 μM	7.8 (91%)	114	10	7.5 (88%)	100	2
25 μM	18.2 (85%)	115	12	19.1 (89%)	102	2
50 μM	38.2 (89%)	115	20	38.5 (90%)	102	4

A 10% erythrocyte suspension was incubated with protoporphyrin (10–50 μM) for 30 min at 30° C in PBS or in 10 mM Tris-HCl pH 7.4, containing 100 mM $BaCl_2$. Binding, MCV and K^+-leakage were determined as described in Materials and methods. Numbers in parentheses give percentage of total amount of protoporphyrin. MCV is expressed as percent of initial value. Initial MCV is 100% and initial K^+-leakage is 2%. Binding is expressed as 10^6 molecules/cell

layer was determined. This was compared with the fluorescence of protoporphyrin extracted from an erythrocyte suspension not exposed to H_2O_2. In both cases no effect on the fluorescence of protoporphyrin could be observed, indicating that protoporphyrin was not degraded during incubation of the erythrocytes with H_2O_2 (data not shown).

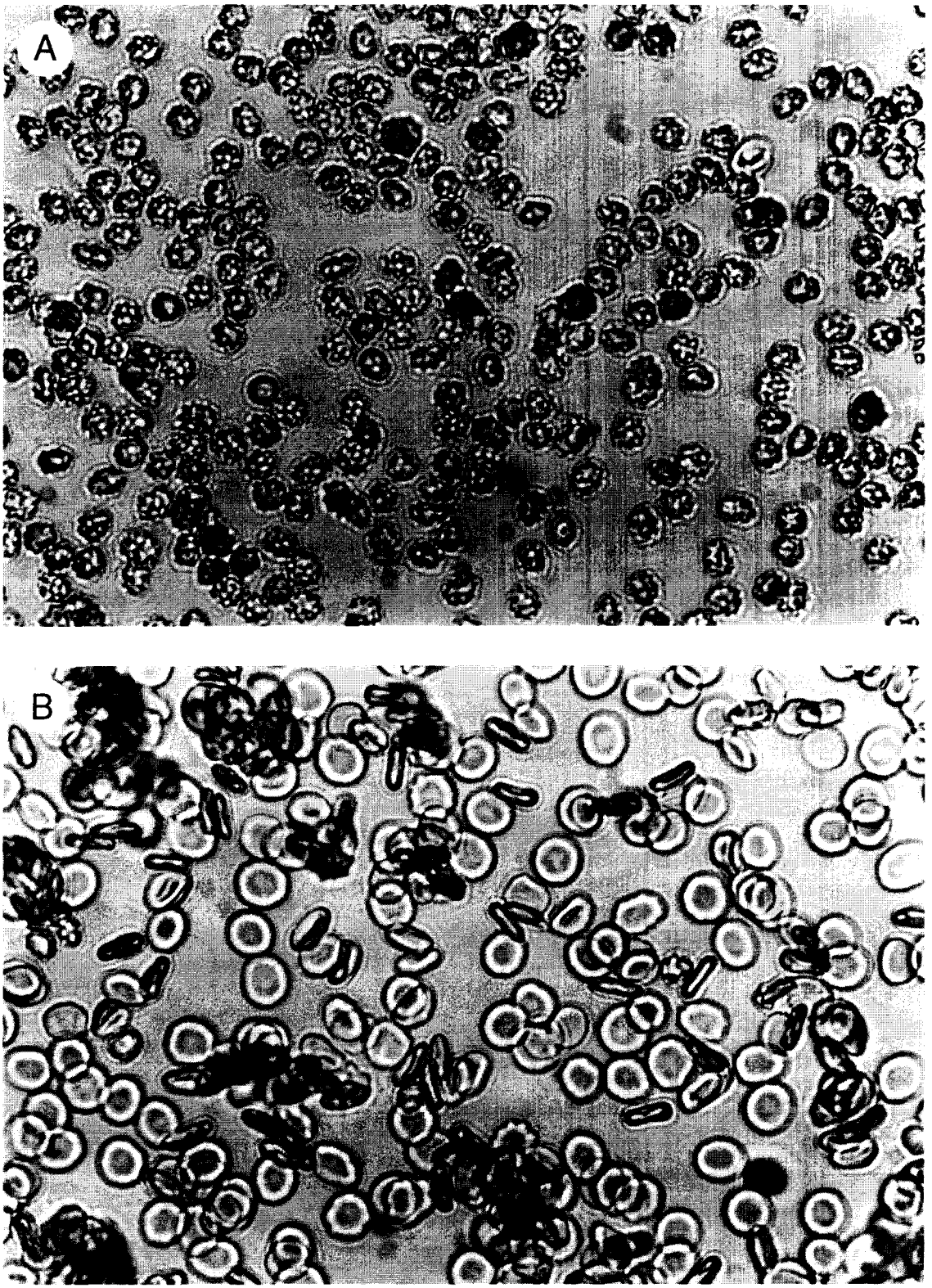

Fig. 2. Protoporphyrin-induced changes in cell shape. Light micrographs of erythrocytes (10% suspension) exposed to 20 μM protoporphyrin in PBS (A) or in Tris-HCl/$BaCl_2$ (B). Enlargement: 40 ×.

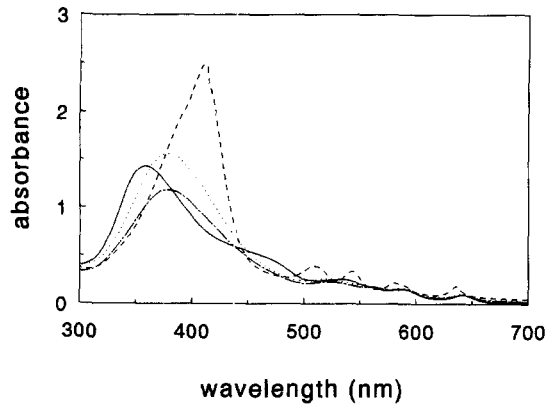

Fig. 3. Changes in the absorption spectrum of protoporphyrin. Visible absorption spectra of 25 μM protoporphyrin in PBS (–); or in PBS with ghosts (0.2 mg/ml protein) (– – –); or in 10 mM Tris-HCl pH 7.4, containing 100 mM $BaCl_2$ (–·–); or in 10 mM Tris-HCl pH 7.4, containing 100 mM $BaCl_2$ with ghosts (0.2 mg/ml protein) (· · ·). When necessary the spectra were corrected for the absorption of ghosts.

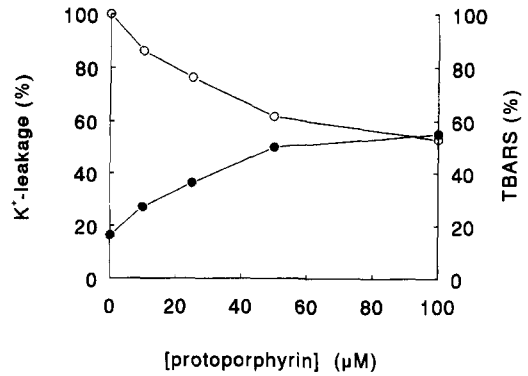

Fig. 4. The effect of protoporphyrin on H_2O_2-induced lipid peroxidation and K^+-leakage. Erythrocytes were preincubated for 30 min with protoporphyrin (0–100 μM) and subsequently exposed to 20 mM H_2O_2 in the presence of 2 mM azide at 30° C. After 15 min of incubation in the presence of H_2O_2, K^+-leakage (●) was determined as described in 'Material and methods'. Lipid peroxidation (○) is expressed as percentage of the amount of TBARS formed by $7 \cdot 10^8$ cells in 120 min without protoporphyrin present. This amounted to 18.4 nmol TBARS.

Protoporphyrin affected both peroxide-induced lipid peroxidation and K^+-loss. In further experiments we compared the effect of protoporphyrin with the effect of various radical scavengers, i.e., bilirubin, BHT, chlorpromazine, promethazine and diphenylamine, on these parameters. After incubation with these compounds for 30 min, H_2O_2 was added and the effect of these compounds on peroxide-induced K^+-leakage and lipid peroxidation was determined (Table 2). Peroxide-induced lipid peroxidation was inhibited by all compounds and the rate of inhibition was dependent on the compound used. The presence of diphenylamine resulted in a substantial increase in K^+-leakage, whereas bilirubin, BHT, chlorpromazine and promethazine inhibited peroxide-induced K^+-leakage slightly (Table 2). Uroporphyrin did not affect lipid peroxidation or K^+-leakage.

3.3. The mechanism of the enhancement of peroxide-induced K^+-leakage by protoporphyrin

In an attempt to further elucidate the effect of protoporphyrin on H_2O_2-induced K^+-loss and lipid peroxidation, we investigated the effect of BHT and diamide on both processes in the presence of H_2O_2 and protoporphyrin. Previous investigations showed that H_2O_2-induced lipid peroxidation can be inhibited (e.g., by BHT) without affecting the increased passive cation permeability of the membrane. Subsequently we showed, using the SH-reagent diamide, that peroxide-induced K^+-leakage was most likely caused by oxidation of SH-groups in the membrane, a process that was independent of lipid peroxidation [1]. Addition of 0.1 mM BHT completely inhibited H_2O_2-induced lipid peroxidation both in the absence and in the presence of protoporphyrin (Table 2). Peroxide-induced K^+-leakage, on the other hand, was not affected by the addition of BHT (Table 2). Furthermore, the enhancement of H_2O_2-induced K^+-leakage by protoporphyrin was not affected by the addition of BHT (data not shown). These results indicate that also in the presence of protoporphyrin, there is most likely no causal relationship between H_2O_2-induced lipid peroxidation and K^+-leakage. This is corroborated by the results obtained with diamide. Protoporphyrin-induced K^+-leakage was not affected by the addi-

Table 2
Effect of various compounds on H_2O_2-induced K^+-leakage and lipid peroxidation

Addition	% K^+-loss in 30 min	MCV (% of control)	H_2O_2-induced lipid peroxidation	H_2O_2-induced K^+-leakage (%)
None	2	100	1.274	50
Protoporphyrin (0.05 mM)	20	115	0.764 (60%)	75
Uroporphyrin (0.05 mM)	2	100	1.3 (100%)	50
Bilirubin (0.05 mM)	2	100	0.841 (66%)	42
BHT (0.1 mM)	2	100	0.008 (0%)	50
Chlorpromazine (0.2 mM)	10	110	0.833 (65%)	44
Promethazine (0.2 mM)	10	113	0.008 (0%)	40
Diphenylamine (0.1 mM)	2	100	0.086 (7%)	98

A 10% erythrocyte suspension was incubated with various compounds for 30 min at 30° C and K^+-loss and MCV were determined. Initial MCV is 100% and initial K^+-leakage is 2%. Subsequently 20 mM H_2O_2 and 2 mM sodium azide were added and K^+-leakage and lipid peroxidation were measured. Lipid peroxidation was determined after 120 min and numbers in parentheses give percentage of lipid peroxidation without scavengers present. K^+-leakage was determined after 60 min.

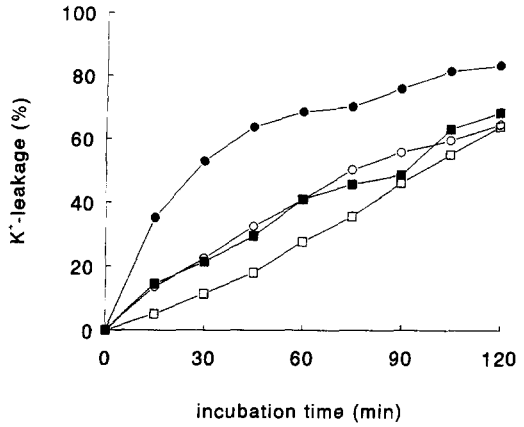

Fig. 5. The effect of diamide on K^+-leakage in the presence of protoporphyrin and H_2O_2. Erythrocytes were incubated for 30 min with 0 or 50 μM protoporphyrin at 30° C, with and without 10 mM diamide. Subsequently the cells were exposed to 20 mM H_2O_2 in the presence of 2 mM azide at 30° C. Open symbols: without protoporphyrin. Closed symbols: with protoporphyrin. (○, ●) no diamide; (□, ■) with 10 mM diamide.

tion of 10 mM diamide: protoporphyrin-induced and diamide-induced K^+-leakage were additive (data not shown). The results in Fig. 5 show that H_2O_2-induced K^+-loss was counteracted by pretreatment of the cells with diamide: the combined effect of both treatments was less than additive. Finally, in the presence of diamide H_2O_2-induced K^+-leakage was still enhanced by protoporphyrin (Fig. 5).

In subsequent experiments we investigated the effect of various K^+-channel blockers on K^+-loss in the presence of H_2O_2 and protoporphyrin. H_2O_2-induced K^+-loss was not affected by preincubation with 0.1 mM ouabain, 0.1 mM bumetanide or 0.1 mM EGTA or by substitution of NaCl with sucrose (results not shown). The addition of 1 mM furosemide inhibited H_2O_2-induced K^+-leakage for 30%. Similar results were obtained in the presence of protoporphyrin: no effect was found by incubation with ouabain, bumetanide, EGTA or sucrose, whereas with furosemide a 30% reduction in K^+-loss was found (results not shown). Replacement of PBS with a buffer containing 10 mM Tris-HCl and 100 mM $BaCl_2$, on the other hand, markedly affected K^+-leakage (Fig. 6). H_2O_2-induced K^+-leakage was slightly reduced in the presence of $BaCl_2$. However, exposure of erythrocytes to H_2O_2 and protoporphyrin in the $BaCl_2$-containing buffer did not result in an enhanced K^+-loss, as was found in PBS (Fig. 6). Lipid peroxidation was not affected by the change of buffers and in the presence of protoporphyrin peroxide-induced lipid peroxidation was still inhibited (results not shown).

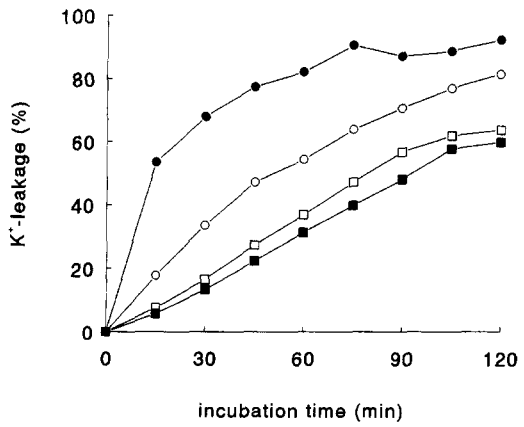

Fig. 6. The effect of protoporphyrin on peroxide-induced K^+-leakage in a Tris-HCl/$BaCl_2$ buffer. Erythrocytes were incubated for 30 min with 0 or 50 μM protoporphyrin at 30° C in PBS or in a Tris-HCl/$BaCl_2$ buffer. Subsequently the cells were exposed to 20 mM H_2O_2 in the presence of 2 mM azide at 30° C. Open symbols: without protoporphyrin. Closed symbols: with protoporphyrin. (○, ●) PBS; (□, ■) Tris-HCl/$BaCl_2$ buffer.

4. Discussion

We investigated the effect of protoporphyrin on peroxide-induced damage, using K^+-leakage and lipid peroxidation as parameters of membrane damage. The presence of protoporphyrin enhanced H_2O_2-induced K^+-leakage considerably, whereas lipid peroxidation was inhibited (Fig. 4). To inhibit lipid peroxidation, the porphyrin had to be localized in the membrane. Uroporphyrin, a porphyrin that remains in solution, did not affect lipid peroxidation at all. These results are in accordance with previous observations in rat liver microsomes [21]. In this case we found that protoporphyrin was able to inhibit lipid peroxidation induced by Fe^{3+}-ADP/ascorbate or Fe^{3+}-ADP/NADPH, whereas uroporphyrin had no effect. In the microsomal system complete inhibition was reached with protoporphyrin concentrations between 10 and 25 μM. In erythrocytes peroxide-induced lipid peroxidation could not be inhibited as effectively: at 100 μM protoporphyrin lipid peroxidation was inhibited for only 50%. Comparison with other inhibitors of lipid peroxidation showed that protoporphyrin was considerably less effective than BHT or promethazine (Table 2). Protoporphyrin was slightly more effective than bilirubin, a degradation product of protoporphyrin and a well-known radical scavenger [22,23].

Peroxide-induced K^+-leakage was enhanced by the presence of protoporphyrin (Fig. 4). In erythrocytes, K^+ can be transported via various transport systems, which can be inhibited by a number of inhibitors [24]. We investigated whether any of these systems was involved in H_2O_2-induced K^+-loss, either with or without protoporphyrin present. Protoporphyrin-induced K^+-leakage was not affected by the addition of 0.1 mM ouabain, inhibitor of the Na^+/K^+-ATPase, 0.1 mM bumetanide, inhibitor of [$Na^+/K^+/Cl^-$] cotransport, 0.1 mM EGTA, inhibitor of Ca^{2+} activated K^+-transport or 1 mM furosemide, which is an inhibitor of the [K^+/Cl^-] cotransport. The residual pathway, stimulated by replacement of NaCl with sucrose [24], was also not involved. Ouabain, bumetanide, sucrose and EGTA also failed to affect K^+-leakage when cells were incubated with H_2O_2 or with H_2O_2 and protopor-

phyrin. With furosemide, K^+-leakage induced by H_2O_2 or by H_2O_2 in the presence of protoporphyrin was inhibited for 30%. The former is in accordance with data in the literature, where it was shown that K^+/Cl^- cotransport is involved in H_2O_2-induced K^+-loss [25]. However, none of these transport systems seemed to be involved in the enhancement of peroxide-induced K^+-leakage by protoporphyrin. This enhancement was also not affected by diamide (Fig. 5). In a previous study we used the SH-reagent diamide to show that H_2O_2-induced K^+-leakage was most likely caused by oxidation of SH-groups in the membrane [1]. Diamide causes an increased K^+-leakage by oxidation of SH groups to disulfides [26,27]. As shown in Fig. 5, the combined effects of diamide and H_2O_2 are less than the effect of H_2O_2 alone, which indicates that the oxidation of SH groups to disulfides by diamide protects these groups against oxidation by H_2O_2 [1]. However, neither the K^+-leakage induced by protoporphyrin alone nor the enhancement of H_2O_2-induced K^+-leakage by protoporphyrin was affected by pretreatment of the cells with diamide. This means that these diamide and H_2O_2 sensitive SH-groups are not involved in the enhancement of H_2O_2-induced K^+-loss by protoporphyrin.

Another possible explanation for the increase in K^+-loss might be that there is a causal relationship between the enhancement of K^+-loss and the inhibition of lipid peroxidation by protoporphyrin. A reaction between peroxide- or lipid-derived radicals and protoporphyrin could result in the formation of reactive intermediates, which could subsequently cause an increase in K^+-leakage. Such a mechanism has been proposed for diphenylamine [28]. Diphenylamine also inhibited lipid peroxidation, whereas at the same time K^+-leakage was increased (Table 2). It was suggested that a diphenylamine radical, formed upon reaction of diphenylamine with a lipid peroxyl radical, causes damage that finally leads to enhanced K^+-loss. At the same time diphenylamine is formed back again [28]. We found that protoporphyrin was also not degraded, suggesting that similar reactions might take place. However, the experiments with BHT and $BaCl_2$ argue against such a mechanism for protoporphyrin. BHT is a better radical scavenger than protoporphyrin, which is illustrated by the fact that in erythrocytes BHT inhibited lipid peroxidation much more effectively than protoporphyrin (compare Fig. 4 and Table 2). A possible reaction between lipid peroxyl radicals and protoporphyrin, and the subsequent increase in K^+-leakage, would therefore be prevented by BHT. However, the addition of BHT did not affect protoporphyrin-induced increase of K^+-loss at all, indicating that this enhancement was most likely not caused by a reaction between peroxyl radicals and protoporphyrin.

Secondly, we compared the effects of protoporphyrin on the cells in PBS and in Tris-HCl/$BaCl_2$ buffer. Incubation of erythrocytes with protoporphyrin led to binding of protoporphyrin molecules to the cell (Fig. 1). Binding of protoporphyrin to the erythrocyte caused perturbations of the membrane, as reflected by the change in cell shape, and the concomitant increase in MCV and K^+-loss (Table 1). The change in cell shape was most likely caused by selective intercalation of negatively charged protoporphyrin molecules into the outer monolayer of the membrane, resulting in expansion of the outer relative to the inner monolayer [29]. Compounds that produce echinocytes associate preferentially with the outer monolayer, probably as a result of their inability to cross the bilayer or because of charge repulsion by negatively charged inner monolayer lipids. $BaCl_2$ did not prevent protoporphyrin binding to the membrane, but prevented protoporphyrin-induced change in cell shape, as well as the increase in MCV and K^+-leakage. The localisation of protoporphyrin in the membrane was also clearly affected by the presence of the barium molecules (Fig. 3). It is feasible that the positive barium molecules shielded the carboxylic acid groups of the protoporphyrin molecules from negatively charged lipids of the inner layer, thus changing the localisation of protoporphyrin and preventing the change in cell shape. H_2O_2-induced K^+-loss was slightly decreased in the Tris-HCl/$BaCl_2$ buffer, whereas lipid peroxidation was not affected. More importantly, the presence of protoporphyrin did no longer enhance H_2O_2-induced K^+-leakage (Fig. 6). These results, therefore, strongly suggest that there is a causal relationship between protoporphyrin-induced change in cell shape and the increased K^+-loss upon exposure of the cells to H_2O_2. This effect seems to be rather specific for protoporphyrin-induced changes, which is corroborated by the following observations. Changes in cell shape can also be induced by positively charged molecules, like chlorpromazine and promethazine [30–32]. Positively charged compounds associate preferentially with the inner monolayer, inducing stomatocytosis [29]. Interestingly, both compounds induced an increase in MCV and K^+-loss as a result of the change in cell shape, but they did not cause an increase in H_2O_2-induced K^+-leakage (Table 2). Thus, the enhancement of H_2O_2-induced K^+-leakage is most likely caused by particular changes in cell shape due to binding of protoporphyrin, and is definitely not a general phenomenon.

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