The influence of porphyrins on iron-catalysed generation of hydroxyl radicals

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Uroporphyrin I, haematoporphyrin and haematoporphyrin derivative had no effect on $O_2^{\cdot-}$ generation during oxidation of hypoxanthine by xanthine oxidase and on the formation of hydroxyl radicals ($OH^{\cdot}$) in the hypoxanthine/xanthine oxidase/ Fe^{3+} -EDTA/deoxyribose system. On the other hand, these porphyrins strongly inhibited $O_2^{\cdot-}$ formation in a horseradish peroxidase/ H_2O_2 /NADPH mixture, whereas they augmented $OH^{\cdot}$ generation in this system after addition of Fe^{3+} -EDTA. Experimental evidence suggests that these observations should be ascribed to the formation of a porphyrin anion radical in the horseradish peroxidase/NADPH system. The formation of this anion radical was confirmed by e.s.r. spectroscopy. This radical is apparently unable to reduce cytochrome *c*, but it can replace $O_2^{\cdot-}$ in the $OH^{\cdot}$ -generating Haber–Weiss reaction.

INTRODUCTION

In previous studies it was shown that HRP-catalysed oxidation of NADPH was strongly potentiated by haematoporphyrin [1] and by eosin [2]. In the non-photodynamic HRP/NADPH/eosin system the reaction was accompanied by photon emission with the spectrum of eosin fluorescence, indicating elevation of eosin to the excited singlet state [2]. On the basis of this observation it was suggested that the potentiating effect of haematoporphyrin on HRP-catalysed oxidation of NADPH might be based on the enzymic generation of haematoporphyrin in the excited singlet state, decaying to the excited hematoporphyrin triplet state, which can generate singlet oxygen by energy transfer. As singlet oxygen is known to oxidize NADPH, this mechanism would explain the potentiating effect of haematoporphyrin in this system [1]. In the HRP/ H_2O_2 /NADPH/porphyrin system it appeared, however, that no photon emission occurred, excluding a similar mechanism for the potentiation of HRP-catalysed oxidation of NADPH by porphyrins [3]. Instead it appeared that, during HRP-catalysed oxidation of NADPH, $O_2^{\cdot-}$ was generated and that porphyrins strongly suppressed this $O_2^{\cdot-}$ formation. As $O_2^{\cdot-}$ inhibits HRP [4], the potentiating effect of haematoporphyrin can, most probably, be explained along these lines [3].

In the simultaneous presence of $O_2^{\cdot-}$, H_2O_2 and a suitable Fe complex, $OH^{\cdot}$ will be formed via the Haber–Weiss reaction [5]. As spontaneous dismutation of $O_2^{\cdot-}$ yields H_2O_2 , an $O_2^{\cdot-}$ -forming system becomes an $OH^{\cdot}$ -generating system after the addition of, e.g., Fe^{3+} -EDTA. The mechanism of porphyrin-induced suppression of $O_2^{\cdot-}$ -generation in the HRP/ H_2O_2 /NADPH system is still unknown. Therefore the influence of porphyrins on two $O_2^{\cdot-}$ -dependent $OH^{\cdot}$ -generating systems, namely hypoxanthine/XO/ Fe^{3+} -EDTA and HRP/ H_2O_2 /NADPH/ Fe^{3+} -EDTA was investigated, as an

attempt to elucidate the mode of action of porphyrins in non-photodynamic radical reactions. The experimental results are described in the present paper.

MATERIALS AND METHODS

HRP, SOD, XO, catalase and NADPH were purchased from Boehringer, Mannheim, Germany. HPD was obtained from Photofrin Medical, Raritan, NJ, U.S.A. as a 5 mg·ml⁻¹ sterile solution. Uroporphyrin I and haematoporphyrin were purchased from Sigma. All other reagents were analytical grade and used without further purification.

All solutions were prepared in 50 mM-phosphate buffer, pH 7.4, double-distilled water being used. The reaction mixtures were prepared and handled under dim light to avoid photoexcitation of HPD. Anaerobic conditions were obtained by bubbling argon (< 0.3 p.p.m. O_2) through the solution.

The hypoxanthine/XO system contained, in final concentrations, 1 mM-hypoxanthine and 0.05 unit of XO/ml. The HRP/NAD(P)H system contained 1.5 mM-NADPH or NADH, 1.5 mM- H_2O_2 and 20 units of HRP/ml. When present, other reagents were added at the following final concentrations: Fe^{3+} -EDTA, 0.01 mM; catalase, 250 units/ml; deoxyribose, 1 mM; mannitol, 20 mM; and cytochrome *c*, 0.05 mM.

$O_2^{\cdot-}$ -induced cytochrome *c* reduction was measured at 550 nm. $OH^{\cdot}$ -induced degradation of 2-deoxyribose was measured with the thiobarbituric acid method as described by Gutteridge [6]. Thiobarbituric acid-reactive products were measured at 532 nm.

E.s.r. spectra were obtained at room temperature with an IBM ER-200 e.s.r. spectrometer operating at 9.7 GHz with 100 kHz modulation frequency and equipped with an ER-4103 TM cavity. The solutions were deoxygenated by bubbling with N_2 in the dark and then transferred to

Abbreviations used: HRP, horseradish peroxidase; XO, xanthine oxidase; SOD, superoxide dismutase; HPD, haematoporphyrin derivative; $OH^{\cdot}$, hydroxyl radical; $O_2^{\cdot-}$, superoxide anion; $O_2^{\cdot}$, superoxide radical.

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Fig. 1. Influence of HPD and SOD on OH[·] generation in a hypoxanthine/XO/Fe³⁺-EDTA/deoxyribose mixture

○, Standard reaction mixture; ●, 40 μM-HPD added; △, SOD (1 kunit/ml) added; ▲, 40 μM-HPD and SOD (1 kunit/ml) added.

the quartz flat cell by means of a rapid-sampling device [7]. The *g*-factor was measured relative to Fremy's salt [peroxylamine disulphonate, $g = 2.0057$, $a^N = 1.3$ mT (13.0 G)] [8].

RESULTS

Influence of porphyrins on the hypoxanthine/XO system

Incubation of deoxyribose in the presence of hypoxanthine, XO and Fe³⁺-EDTA resulted in SOD-sensitive generation of thiobarbituric acid-reactive products. Deoxyribose degradation in this system and its inhibition by SOD are unaffected by HPD, as shown in Fig. 1. Omission of Fe³⁺-EDTA from the reaction mixture or addition of catalase or the OH[·] scavenger mannitol completely abolished deoxyribose degradation. With uroporphyrin I and haematoporphyrin, identical results were obtained.

The formation of thiobarbituric acid-reactive products in this system and the reduction of cytochrome *c* in a hypoxanthine/XO mixture were equally sensitive to SOD. Again, addition of uroporphyrin I, haematoporphyrin or HPD to the reaction mixtures had no influence on the velocity of the various reactions (Fig. 2).

Influence of porphyrins on the HRP/H₂O₂/NADPH system

As shown previously, in a HRP/H₂O₂/NADPH mixture O₂^{·-} is also generated [3]. Therefore it would be expected that addition of Fe³⁺-EDTA to this system would result in the formation of OH[·]. This was confirmed experimentally, as shown in Fig. 3. Degradation of

Fig. 2. Inhibition of O₂^{·-} and OH[·] generation in the hypoxanthine/XO system by SOD

O₂^{·-} generation was measured in a hypoxanthine/XO/cytochrome *c* mixture, and formation of OH[·] was measured in a hypoxanthine/XO/Fe³⁺-EDTA/deoxyribose mixture, as described in the Materials and methods section. Results are expressed as percentages of the velocities observed in the absence of SOD. ○, ●, Velocity of cytochrome *c* reduction; △, ▲, velocity of formation of thiobarbituric acid-reactive products; ○, △, no HPD added; ●, ▲, 40 μM-HPD added.

deoxyribose was abolished by SOD, mannitol and by anaerobic conditions. Addition of uroporphyrin I, haematoporphyrin or HPD to the HRP/H₂O₂/NADPH/Fe³⁺-EDTA/deoxyribose mixture resulted in an increased generation of thiobarbituric acid-reactive products. Moreover, in the presence of porphyrins, high concentrations of SOD caused only a partial inhibition of the reaction, whereas formation of thiobarbituric acid-reactive products occurred even under anaerobic conditions (Fig. 3). In control experiments it appeared that omission of HRP, NADPH or Fe³⁺-EDTA from the porphyrin-containing reaction mixtures, or addition of mannitol, blocked deoxyribose degradation.

The effects of porphyrins were studied in more detail in the following experiments. As shown in Fig. 4, addition of increasing concentrations of HPD to an HRP/H₂O₂/NADPH/cytochrome *c* mixture resulted in a progressively decreasing reduction of cytochrome *c*. Addition of increasing concentrations of HPD to an HRP/H₂O₂/NADPH/Fe³⁺-EDTA/deoxyribose mixture, by contrast, resulted in a gradually increasing generation of thiobarbituric acid-reactive products (Fig. 4). The influence of various concentrations of SOD on cytochrome *c* reduction and deoxyribose degradation in the absence and presence of HPD is depicted in Fig. 5. Cytochrome *c* reduction in the absence and presence of HPD and deoxyribose degradation in the absence of HPD were inhibited by SOD to about the same extent.

Fig. 3. Influence of HPD and SOD on OH[•] generation in an HRP/H₂O₂/NADPH/Fe³⁺-EDTA/deoxyribose mixture

○, Standard reaction mixture; ●, 40 μM-HPD added; △, SOD (1 kunit/ml) added; ▲, 40 μM-HPD and SOD (1 kunit/ml) added; □, anaerobic conditions; ■, anaerobic conditions and 40 μM-HPD added; ▽, 20 mM-mannitol added.

Fig. 4. Influence of HPD on O₂^{•-} and OH[•] generation in an HRP/H₂O₂/NADPH system

O₂^{•-} generation was measured after addition of cytochrome *c* to the reaction mixture and formation of OH[•] after addition of Fe³⁺-EDTA and deoxyribose. Results are expressed as percentages of the velocities measured in the absence of HPD. ●, Velocity of cytochrome *c* reduction; ▲, velocity of formation of thiobarbituric acid-reactive products.

Fig. 5. Influence of SOD on the formation of O₂^{•-} (b) and OH[•] (a) in an HRP/H₂O₂/NADPH system

O₂^{•-} generation (b) was measured after addition of cytochrome *c* to the reaction mixture and formation of OH[•] (a) after addition of Fe³⁺-EDTA and deoxyribose. △, OH[•] generation, standard mixture; ▲, OH[•] generation, 10 μM-HPD added; □, OH[•] generation, anaerobic conditions, 10 μM-HPD added; ○, O₂^{•-} generation, standard mixture; ●, O₂^{•-} generation, 10 μM-HPD added.

Degradation of deoxyribose in the presence of HPD was less sensitive to low concentrations of SOD and, moreover, the inhibition levelled off at about 25% at high SOD concentrations. Degradation of deoxyribose in the presence of HPD under anaerobic conditions was not inhibited by SOD (Fig. 5). With uroporphyrin I and haematoporphyrin similar results were obtained.

E.s.r. spectrometry

The e.s.r. spectrum of the uroporphyrin I anion radical, generated anaerobically in the presence of

Fig. 6. E.s.r. spectrum of the uroporphyrin I anion radical

The reaction mixture contained 1.2 mM-uroporphyrin I, 1.5 mM-H₂O₂, 3 mM-NADH and HRP (20 units/ml) in 50 mM-phosphate buffer, pH 7.0. (a) Complete reaction mixture; (b) HRP omitted; (c) uroporphyrin I omitted; (d) H₂O₂ omitted. Instrumental conditions: 20 mW microwave power, 0.5 mT modulation amplitude, 2 s time constant and 0.3 mT/min scan rate.

NADH by the HRP-catalysed reduction of uroporphyrin I, is shown in Fig. 6(a). High porphyrin concentrations were necessary to obtain a detectable signal. Therefore uroporphyrin I was used in these measurements, allowing a final concentration of up to 1 mg/ml. The porphyrin anion radical gives an unresolvable single-line spectrum with a peak-to-peak linewidth of 0.53 mT (5.3 G) and a *g*-factor of 2.0022. Formation of the porphyrin anion radical did not occur when either HRP, H₂O₂ or uroporphyrin I were omitted from the reaction mixture (Figs. 6b, 6c and 6d). Omission of NADH resulted in the HRP-catalysed formation of the uroporphyrin I cation radical. This cation radical has different spectroscopic features and can therefore be distinguished from the porphyrin anion radical (K. M. Morehouse, H. Sipe and R. P. Mason, personal communication).

DISCUSSION

As shown in Figs. 1 and 2, porphyrins have no influence at all on the velocity of hypoxanthine/XO-induced O₂^{•-} formation, the generation of OH[•] after addition of Fe³⁺-EDTA or the sensitivity of these systems to SOD. In the HRP/H₂O₂/NADPH system, by contrast, the generation of O₂^{•-} was strongly inhibited by porphyrins, as reflected in decreased cytochrome *c* reduction (Fig. 4). Considering the lack of influence of porphyrin on cytochrome *c* reduction by hypoxanthine/

XO, this effect can not be explained by a SOD-like activity of porphyrins or by a reaction of the type:

Thus the porphyrin-induced lowering of [O₂^{•-}] during HRP-catalysed oxidation of NADPH should be attributed to an inhibited generation of this radical, whereas, simultaneously, the velocity of enzymic oxidation of NADPH is strongly increased [3].

In the absence of porphyrins the following catalytic cycle of NADPH oxidation by HRP is observed [9]:

O₂^{•-} generated in reaction (4) will react with the HRP molecule, leading to a strong inhibition of enzyme activity [4,10].

As discussed above, O₂^{•-} generation in the HRP/H₂O₂/NADPH system is depressed in the presence of porphyrins, with a concomitant increase of the rate of NADPH oxidation, as shown previously [3]. These effects could be explained by assuming the following cycle:

The formation of the proposed porphyrin anion radical was confirmed by e.s.r. spectroscopy. As shown in Fig. 6, an unresolvable single-line spectrum is found with a *g*-factor and peak-to-peak linewidth in close agreement with earlier studies, in which the porphyrin anion radical was generated photochemically [11] or microsomally catalysed [12].

The results shown in Fig. 4 indicate that the porphyrin anion radical is not able to reduce cytochrome *c*. On the other hand, the anion radical can, apparently, replace O₂^{•-} in the iron-catalysed generation of OH[•], as shown by the following observations. (1) Degradation of deoxyribose in the presence of HRP/H₂O₂/NADPH to thiobarbituric acid-reactive products increased after addition of porphyrins, despite the simultaneously occurring suppression of O₂^{•-} formation (Fig. 4). (2) Generation of OH[•] in this porphyrin-containing system was much less sensitive to SOD (Fig. 5). (3) Generation of OH[•] in the HRP/H₂O₂/NADPH system under anaerobic conditions occurred in the presence, but not in the absence, of porphyrins (Fig. 3), whereas in the presence of a porphyrin anaerobic generation of OH[•] was insensitive to SOD (Fig. 5). Presumably the porphyrin anion radical reacts with the Fe³⁺-EDTA complex in a similar way to O₂^{•-}:

thus sustaining the iron-catalysed generation of hydroxyl radicals.

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