The mechanism of potentiation of horseradish peroxidase-catalysed oxidation of NADPH by porphyrins

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Several porphyrins, including HpD (haematoporphyrin derivative), potentiate the oxidation of NADPH by horseradish peroxidase/ H_2O_2 . To elucidate the mechanism of potentiation, the following observations are relevant. 1. During peroxidase-catalysed NADPH oxidation, $\text{O}_2^{\cdot-}$ (superoxide radical) is generated, as judged from superoxide dismutase-inhibitable cytochrome *c* reduction. 2. This generation of $\text{O}_2^{\cdot-}$ is suppressed by HpD. 3. Peroxidase-catalysed NADPH oxidation is stimulated by superoxide dismutase and by anaerobic conditions. 4. Under anaerobic conditions HpD has no influence on peroxide-catalysed NADPH oxidation. Previous studies have shown that horseradish peroxidase is inhibited by $\text{O}_2^{\cdot-}$. Thus the experimental results indicate that the potentiating effect of HpD can be explained by its ability to inhibit $\text{O}_2^{\cdot-}$ generation in the horseradish peroxidase/ H_2O_2 /NADPH system.

INTRODUCTION

Like other photosensitizers, porphyrins can be activated by visible light, yielding highly reactive photoexcited species. The photosensitizing properties of porphyrins have been studied extensively and are now well established. Recent interest in porphyrins as photosensitizers is increased by the application of HpD in photodynamic therapy of cancer [1].

Much less is known about the reactivity of porphyrins in the absence of light, although some experimental observations indicate that porphyrins may interfere with biological processes in the dark. For instance, as shown by Berns *et al.* [2], HpD has a growth-inhibiting effect on the established kangaroo-rat cell line PTK2, without added light. Further, haematoporphyrin has a pronounced potentiating effect on the HRP-catalysed oxidation of NADPH, again in a non-photodynamic manner [3]. It was suggested that the enzymic generation of haematoporphyrin transients, which are usually generated photodynamically, might be responsible for this potentiation [3]. If so, such-like reactions might constitute a general, light-independent, cytotoxicity of porphyrins, with possible consequences in the fields of photodynamic therapy and inborn errors of metabolism which lead to accumulation of porphyrins in various cells and tissues. In this context the non-photodynamic potentiation of HRP-catalysed oxidation of NADPH was studied in some detail.

MATERIALS AND METHODS

HpD was purchased from Photofrin Medical, Raritan, NJ, U.S.A., as a sterile solution ($5 \text{ mg} \cdot \text{ml}^{-1}$) in iso-osmotic NaCl. Dilutions were made in 50 mM-sodium phosphate, pH 7.4. HRP, NADPH, SOD and xanthine oxidase were obtained from Boehringer, Mannheim, Germany. All other reagents were of analytical grade and used without further purification.

Solutions were prepared in double-distilled water. All experiments were conducted in 50 mM-sodium phosphate buffer, pH 7.4, at room temperature. Reaction mixtures were prepared and handled under dim light, to avoid photooxidation of porphyrins.

NADPH and guaiacol oxidation were measured spectrophotometrically at 340 nm and 436 nm respectively. Cytochrome *c* reduction was monitored at 550 nm. Optical absorbance was measured on a Beckman DB spectrophotometer connected to a Vitatron 2001 recorder with lin/log module. Chemiluminescence was monitored using a Nuclear-Chicago Mark I scintillation spectrometer, operated in the out-of-coincidence-summation mode. Anaerobic conditions were obtained by bubbling argon (containing less than 0.3 p.p.m. oxygen) through the solution.

RESULTS

The stimulation of HRP/ H_2O_2 -catalysed oxidation of NADPH by HpD is shown in Fig. 1(a). In control experiments it appeared that, when HRP was omitted from the reaction mixture, HpD did not stimulate the very slow autooxidation of NADPH (result not shown). With other porphyrins, namely haematoporphyrin and protoporphyrin, a similar potentiation was found (result not shown). NaN_3 , at a final concentration of 10 mM, had no influence on the velocity of NADPH oxidation by the HRP/ H_2O_2 /HpD system.

As described by Kachar *et al.* [4], the oxidation of NADH in an HRP/ $\text{Mn}^{2+}/\text{O}_2$ system at pH 4.2 is potentiated by eosin, and this reaction is accompanied by photon emission. In preliminary experiments both the potentiating effect of eosin and the accompanying photon emission could be confirmed. In contrast, with the system HRP/ H_2O_2 /NADPH/HpD no photon emission occurred.

Guaiacol is oxidized by HRP in the presence of H_2O_2 .

Fig. 1. (a) Potentiation of HRP-catalysed oxidation of NADPH by HpD and (b) effect of HpD on HRP-catalysed oxidation of guaiacol

(a) The reaction mixture contained (final concns.): sodium phosphate, 50 mM, pH 7.4; NADPH, 0.15 mM; H_2O_2 , 0.10 mM; HRP, 0.10 mg $\cdot$ ml $^{-1}$. Further additions: a, none; b, HpD (10 μ M); c, HpD (20 μ M); d, HpD (30 μ M). (b) The reaction mixture contained: sodium phosphate, 50 mM, pH 7.4; guaiacol, 0.15 mM; H_2O_2 , 0.10 mM; HRP, 0.03, μ g $\cdot$ ml $^{-1}$. Further additions: a, none; b, HpD (20 μ M).

In contrast with the results with NADPH as substrate, HpD inhibited HRP-catalysed guaiacol oxidation, as shown in Fig. 1(b). Apparently the potentiating effect of HpD on HRP-catalysed oxidation exhibits some degree of substrate specificity.

During oxidation of NADPH by HRP/ H_2O_2 , $O_2^{\cdot-}$ was generated, as judged from SOD-sensitive reduction of cytochrome *c* (Fig. 2). With increasing concentrations of HpD the generation of $O_2^{\cdot-}$ was progressively diminished (Fig. 2). With guaiacol as substrate no cytochrome *c* reduction was observed (results not shown).

Increasing concentrations of SOD progressively increased the velocity of HRP-catalysed oxidation of NADPH (Fig. 3). At low SOD concentrations the potentiating effects of SOD and HpD on NADPH oxidation were more or less additive. At higher SOD concentrations the effect of HpD vanished. The highest velocity of HRP/ H_2O_2 -catalysed oxidation of NADPH was obtained under strictly anaerobic conditions. Under these experimental conditions HpD has no effect on the velocity of the reaction (Fig. 3).

Finally, in a solution containing 1 mM-hypoxanthine,

Fig. 2. Reduction of cytochrome *c* by the HRP/ H_2O_2 /NADPH/HpD system

The reaction mixture contained: sodium phosphate, 50 mM, pH 7.4; NADPH, 1 mM; H_2O_2 , 1 mM; HRP, 0.05 mg $\cdot$ ml $^{-1}$; cytochrome *c*, 0.05 mM. Further additions: a, none; b, SOD (3 kunits $\cdot$ ml $^{-1}$); c, HpD (10 μ M); d, HpD (20 μ M); e, HpD (30 μ M).

Fig. 3. HRP-catalysed oxidation of NADPH under various experimental conditions

For the standard reaction mixture, see the legend to Fig. 1(a). Further additions: a, none; b, SOD (0.3 kunits $\cdot$ ml $^{-1}$); c, SOD (0.3 kunit $\cdot$ ml $^{-1}$) and HpD (20 μ M); d, SOD (3 kunits $\cdot$ ml $^{-1}$); e, SOD (3 kunits $\cdot$ ml $^{-1}$) and HpD (20 μ M); f, anaerobic conditions with and without HpD (20 μ M).

0.1 unit of xanthine oxidase/ml and 0.05 mM-cytochrome *c*, reduction of cytochrome *c* occurred, due to the well-known enzymic generation of $O_2^{\cdot-}$ in this system. Cytochrome *c* reduction in this system was not affected by HpD in concentrations up to 30 μ M. Both in the absence and in the presence of HpD, cytochrome *c*

reduction was completely abolished by 3 kunits of SOD/ml.

DISCUSSION

Potentiation of HRP-catalysed oxidation of NADPH by haematoporphyrin was first described by Bodaness [3] and confirmed in the present study. Moreover, it was shown that other porphyrins, namely protoporphyrin and HpD, have a similar potentiating effect. One of the possibilities suggested by Bodaness [3] to explain this potentiation was raising of the porphyrin to the excited singlet state, which then decays to the excited porphyrin triplet by intersystem crossing. This triplet could subsequently generate singlet oxygen, a species known to oxidize NADPH. However, the present study excludes this possibility. With the system HRP/H₂O₂/NADPH/HpD, no photon emission occurred, as should be expected when the porphyrin in the excited singlet state is an intermediate. Moreover, NaN₃, a potent singlet-oxygen scavenger [5], had no influence on the potentiating effect of HpD, excluding the involvement of singlet oxygen. In this respect the system differs basically from the system HRP/Mn²⁺/O₂/NADH/eosin, in which photon emission, indicating the generation of eosin in the excited singlet state, was first described by Kachar *et al.* [4] and confirmed in the present studies.

As shown in Fig. 2, O₂^{-•} is generated during HRP/H₂O₂-catalysed oxidation of NADPH. In the presence of HpD, the concentration of O₂^{-•} is apparently strongly decreased, either by decreased generation or by increased degradation, for instance via HpD-catalysed dimutation. The latter possibility is excluded by the experiments with the hypoxanthine/xanthine oxidase system. Cytochrome *c* reduction by this system was not affected by HpD and was completely inhibitable by SOD both in the absence and in the presence of HpD. Thus the results shown in Fig. 2 should be ascribed to an HpD-induced inhibition of O₂^{-•} generation during HRP-catalysed NADPH oxidation.

As it has been shown previously that peroxidases may be inhibited by O₂^{-•} [6], it is conceivable that the velocity

of HRP/H₂O₂-catalysed oxidation of NADPH is self-limited, via feedback inhibition by O₂^{-•}, generated by the enzymic reaction itself. If so, the potentiation of the reaction by HpD can be explained by the apparent ability of this drug to inhibit O₂^{-•} generation during HRP-catalysed NADPH oxidation. The following experimental results are in accordance with a such-like mechanism. 1. During HRP-catalysed oxidation of guaiacol, no O₂^{-•} was generated. This reaction was not potentiated (but rather inhibited) by HpD. 2. The proposed mechanism implies that SOD should stimulate HRP-catalysed oxidation of NADPH, by dismutation of the inhibitory O₂^{-•}. This was borne out experimentally, as shown in Fig. 3. 3. Under anaerobic conditions the velocity of HRP-catalysed oxidation of NADPH was much higher than under aerobic conditions (Fig. 3) and was unaffected by HpD. As O₂^{-•} generation is impossible under anaerobic conditions, these observations are in accordance with the proposed mechanism.

As yet it is not clear how HpD inhibits O₂^{-•} generation in the HRP/H₂O₂/NADPH system. Further experiments to elucidate this point are required.

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