Superoxide-dependent formation of hydroxyl radical catalyzed by transferrin

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Hydroxyl radicals are generated in the hypoxanthine–xanthine oxidase system in the presence of iron-saturated transferrin isolated from human serum. This has been demonstrated by colorimetrically measuring the hydroxylation of salicylic acid and by EPR using the spin trap DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide). A Fenton-type Harber-Weiss reaction catalyzed by transferrin is proposed.

Superoxide *Hydroxyl radical* *Hydroxylation* *Transferrin* *EPR* *Spin trapping*

1. INTRODUCTION

Hydroxyl radicals ($OH^{\cdot}$) are known to be a higher reactive species than superoxide radicals ($O_2^{\cdot -}$) [1]. Indeed, many of the damaging effects of the superoxide-generating system are due to the superoxide-dependent formation of hydroxyl radicals. Hydroxyl radicals are produced by interaction between $O_2^{\cdot -}$ and H_2O_2 in the presence of iron salts [2,3]. The reactions may be represented by the following Fenton-type Harber-Weiss reaction:

$$O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad (2)$$

$$O_2^{\cdot -} + H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} + O_2 \quad (3)$$

The possibility of hydroxyl radical formation in biological systems has been proposed with the iron-containing proteins, lactoferrin [4–6] and transferrin [2,7]. Authors in [7] have shown that transferrin is used as the catalyst in the hydroxyl radical generation following superoxide production by neutrophil NADPH oxidase in the presence of H_2O_2. Here, we provide evidence that transferrin in the hypoxanthine–xanthine oxidase system may induce hydroxyl radical formation through a Fenton-type Harber-Weiss reaction.

2. MATERIALS AND METHODS

Catalase (bovine liver), superoxide dismutase (type I) and xanthine oxidase (grade I) were obtained from Sigma. Human serum transferrin (holo and apo) was obtained from Green Cross (Japan), and purified by eliminating mannitol and sodium chloride added as stabilizer through a Sephadex G-25 column (Pharmacia, disposable column PD-10). The protein concentration was measured using an extinction $E_{280}^{1\%}$ of 11.1. Transferrin (holo) contained 1.5 μg iron/mg protein. The spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich Chemicals. Ferric EDTA and diethylenetriaminepentaacetic acid (DETAPAC) were obtained from Dojindo (Japan). All other reagents were of the highest grade available.

A colorimetric assay of hydroxyl radical formation by the hypoxanthine–xanthine oxidase system was performed by measuring salicylate hydroxylation [8] with a Hitachi 320 recording spectrophotometer. X-Band EPR measurements were made using a JES-FE-3X spectrometer. EPR spec-

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tra were recorded with a field set 3370G, modulation frequency 100 kHz, modulation amplitude 1.0 G, microwave power 10 mW and microwave frequency 9.442 GHz.

3. RESULTS AND DISCUSSION

The effect of superoxide-dependent formation of hydroxyl radicals in the presence of ferrous or ferric salt, or ferric EDTA was compared with that of an iron-containing protein, transferrin, by the colorimetric assay (fig.1). Ferric EDTA induced about twice as much hydroxyl radical formation in comparison with that induced by ferrous sulphate and ferric chloride. Transferrin (holo) showed salicylate hydroxylation similar to that found with less than 20 μM ferric chloride, and did not increase with increasing transferrin concentration. Transferrin (apo) used as a control for protein effects had no effect. When iron was replaced by metmyoglobin, no hydroxylation was detected even at 100-μM concentrations.

Spin trapping of superoxide radicals produced by the hypoxanthine–xanthine oxidase reaction is reported in fig.2a, which shows the EPR signal of the superoxide spin adduct of DMPO (DMPO-OOH). When the reaction was carried out in the presence of 20 μM transferrin, a new EPR spectrum was observed (fig.2b). The spectrum of the spin adduct formed gave the parameters $a_N = a_H =$ 14.7 G. The parameters and the characteristic 1:2:2:1 quartet pattern are identical with those reported for the hydroxyl spin adduct of DMPO (DMPO-OH) [9,10].

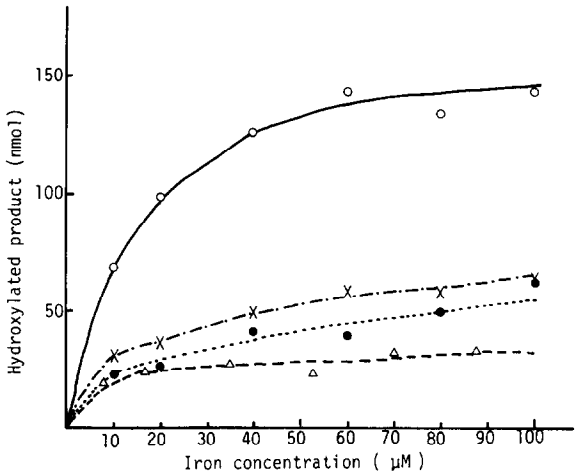

Fig.1. Effect of iron compounds on hydroxylation of aromatic compounds by the hypoxanthine–xanthine oxidase system. Reaction mixtures contain 2.5 mM salicylic acid, 0.25 mM hypoxanthine and various concentrations of the iron compound in a total volume of 2 ml 0.15 M phosphate buffer (pH 7.4). The reaction was initiated by adding 50 μl of xanthine oxidase solution (0.4 units/ml) and incubating at 25°C for 90 min. (○) Fe^{3+}-EDTA; (×) $FeSO_4$; (●) $FeCl_3$; (△) transferrin.

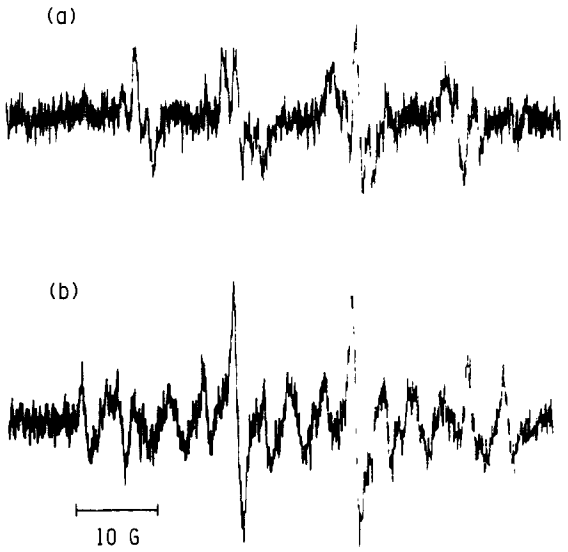

Fig.2. EPR spectra of DMPO-OOH and DMPO-OH generated from the hypoxanthine–xanthine oxidase reaction. Reaction mixtures contain: (a) control: 25 μM hypoxanthine, 0.085 units/ml xanthine oxidase, 1 mM DETAPAC, 0.175 M DMPO in 50 mM potassium phosphate buffer, pH 7.4; (b) plus transferrin. As for (a) plus 20 μM transferrin.

The production of the signal as seen in fig.2b is not conclusive evidence of the generation of hydroxyl radicals since the same spin adduct may arise from the direct oxidation of DMPO [10]. When hydroxyl radical scavenger, mannitol, was added to the hypoxanthine–xanthine oxidase–transferrin solution, the spectrum obtained (fig.3a) was identical to the superoxide spin adduct signal (fig.2a) and no hydroxyl spin adduct was observed. When catalase or superoxide dismutase was used instead of mannitol, little signal was observed.

The results obtained clearly demonstrate that the hydroxyl radical formation by the hypoxanthine–xanthine oxidase reaction in the presence of

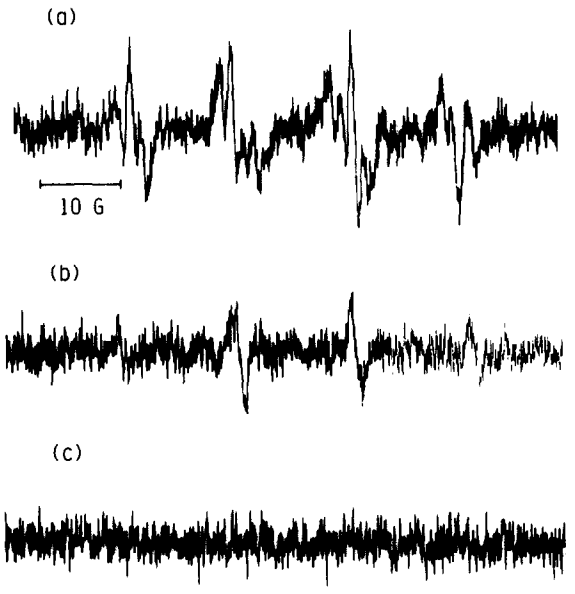

Fig.3. Effect of mannitol, superoxide dismutase and catalase on DMPO-OH generated from the hypoxanthine–xanthine oxidase reaction in the presence of transferrin. Reaction mixtures contain as described in fig.2b: (a) plus 50 mM mannitol; (b) plus 500 units superoxide dismutase; (c) plus 1000 units catalase.

transferrin occurs through a Fenton-type Haber-Weiss reaction. Human serum contains about 20 μM iron bound to transferrin. A Fenton-type reaction by an iron-containing protein such as transferrin appears to play an important role in in vivo hydroxyl radical formation.

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