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Superoxide dismutase enhances the formation of hydroxyl radicals in the reaction of 3-hydroxyanthranilic acid with molecular oxygen

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Superoxide dismutase (SOD) enhanced the formation of hydroxyl radicals, which were detected by using the e.s.r. spin-trapping technique, in a reaction mixture containing 3-hydroxyanthranilic acid (or *p*-aminophenol), Fe^{3+} ions, EDTA and potassium phosphate buffer, pH 7.4. The hydroxyl-radical formation enhanced by SOD was inhibited by catalase and desferrioxamine, and stimulated by EDTA and diethylenetriaminepenta-acetic acid, suggesting that both hydrogen peroxide and iron ions participate in the reaction. The hydroxyl-radical formation enhanced by SOD may be considered to proceed via the following steps. First, 3-hydroxyanthranilic acid is spontaneously auto-oxidized in a process that requires molecular oxygen and yields superoxide anions and anthranilyl radicals. This reaction seems to be reversible. Secondly, the superoxide anions formed in the first step are dismuted by SOD to generate hydrogen peroxide and molecular oxygen, and hence the equilibrium in the first step is displaced in favour of the formation of superoxide anions. Thirdly, hydroxyl radicals are generated from hydrogen peroxide through the Fenton reaction. In this Fenton reaction Fe^{2+} ions are available since Fe^{3+} ions are readily reduced by 3-hydroxyanthranilic acid. The superoxide anions do not seem to participate in the reduction of Fe^{3+} ions, since superoxide anions are rapidly dismuted by SOD present in the reaction mixture.

INTRODUCTION

Of the active oxygen species hydroxyl radicals seem to be the most potent oxidant. Hydroxyl radicals are generated through the Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + OH^- + Fe^{3+}$$

The Fe^{2+} ions in the above reaction can be supplied through the reduction of Fe^{3+} ions by superoxide anions (iron-catalysed Haber–Weiss reaction). Thus SOD has been considered to act as a protective reagent against the cytotoxicity of active oxygen species, since SOD dismutates the superoxide anions [1] that can reduce Fe^{3+} ions. In the present paper we attempt to evaluate the influence of SOD on the auto-oxidation of 3-HAT, a tryptophan metabolite.

It is known that in several pathological conditions certain metabolites of tryptophan are eliminated in the urine in greater amounts than in normal human beings [2,3]. For example, the presence of considerable quantities of 3-HAT and 3-hydroxykynurenine in the urine of patients with cancer of the bladder has been found by several authors [2,4]. On the other hand, both 3-HAT and cinnabarinic acid are known carcinogens that have been linked to bladder and breast carcinomas [5–7]. Indeed, it has been shown that experimental cancer of the bladder [6] can be induced by 3-HAT and 3-hydroxykynurenine. In addition, a high incidence of bladder tumours was demonstrated in rats fed with DL-tryptophan and 2-acetamidofluorene, whereas no bladder tumours were present in those animals fed with only supplemental 2-acetamidofluorene [8]. It is also recognized that *p*-aminophenol is a nephrotoxic agent, causing renal necrosis and inhibition of renal microsomal and mitochondrial enzyme activities in the rat [9], and a potent teratogen in the hamster [10]. In addition to reports on the neoplastic diseases induced by the aminophenols, Rogers & Evangelista [11] reported that 3-HAT, 3-hydroxykynurenine and *o*-aminophenol inhibit leucine-stimulated insulin release from rat pancreatic islets. Further, Werner *et al.* [12] showed that interferon γ induces the excretion of 3-HAT by human macrophages *in vitro*. To understand the above actions by 3-HAT, it is necessary to clarify the chemical properties of 3-HAT, particularly its oxidation, in detail.

The ring structure (*o*-aminophenol) of 3-HAT, a tryptophan metabolite occurring in the kynurenine pathway, is normally opened by 3-HAT oxygenase. If 3-HAT is not opened initially by the oxygenase, it may be auto-oxidized in a process that involves the production of organic and oxygen free radicals and that eventually yields the red pigment cinnabarinic acid [13–16]. *p*-Aminophenol is also oxidized very readily by peroxidases such as horseradish peroxidase [17,18] and prostaglandin endoperoxide synthase [19–21] to form *p*-aminophenoxyl free radicals, the one-electron oxidation product of *p*-aminophenol. The formation of the organic and oxygen free radicals might be closely related to a high incidence of bladder carcinoma. Hence we attempted to detect the oxygen-derived free radicals in the reaction of 3-HAT with molecular oxygen by using the e.s.r. spin-trapping technique.

Abbreviations used: SOD, superoxide dismutase; 3-HAT, 3-hydroxyanthranilic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DETAPAC, diethylenetriamine-*NNN'N''N''*-penta-acetic acid.

MATERIALS AND METHODS

Materials

DMPO was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.; it was further purified by passage through a charcoal column [22], and the concentration of DMPO in the solution was determined by measurement of u.v. absorbance at 234 nm (ϵ 7700 $M^{-1} \cdot cm^{-1}$) [23]. Bovine erythrocyte SOD (EC 1.15.1.1) and bovine liver catalase (EC 1.11.1.6) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). EDTA, L-3-hydroxykynurenine, *m*-hydroxybenzoic acid, *o*-aminobenzoic acid, *o*-phenanthroline and $FeCl_3$ were from Wako Pure Chemical Industries (Osaka, Japan). Desferrioxamine was from CIBA–GEIGY (Japan). DETAPAC was from Nakarai Chemicals (Kyoto, Japan). 3-HAT, *o*-aminophenol, *m*-aminophenol and *p*-aminophenol were from Tokyo Kasei Kogyo (Tokyo, Japan). Hydrogen peroxide (31 %, w/v) was from Mitsubishi Gasu Co. (Tokyo, Japan). Cinnabarinic acid was prepared chemically from 3-HAT in accordance with Prinz & Savage [24]. All other chemicals used were commercial products of the highest grade available.

Reaction mixture containing SOD

The SOD reaction mixture contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of SOD (35000 units/ml, in 10 mM-potassium phosphate buffer, pH 7.4), 10 μl of 0.1 M-EDTA (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The $FeCl_3$ was added after addition of EDTA to prevent the formation of $FePO_4$, which is insoluble [25]. The reactions were initiated by adding 3-HAT, and were carried out at 25 °C for 4 h under aerobic conditions in the dark, unless otherwise indicated. The reaction was also carried out under anaerobic conditions in the dark; the anaerobic conditions were established by using a rotary pump (Mitamura Riken Co., Tokyo, Japan) with Thunberg tubes.

Reaction mixture containing hydrogen peroxide

The hydrogen peroxide reaction mixture contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of 0.1 M-hydrogen peroxide (in water), 10 μl of 0.1 M-EDTA (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were initiated by adding hydrogen peroxide and were carried out at 25 °C for 5 min. At the concentration (2.8 mM) of hydrogen peroxide used here, the rate of hydroxyl-radical formation reached a plateau.

E.s.r. measurement

The e.s.r. spectra were obtained by using an FX2XG instrument (JEOL, Tokyo, Japan) with a 100 kHz modulation frequency. Aqueous samples were aspirated into a Teflon tube centred in a microwave cavity. The e.s.r. spectrometer settings were as follows: microwave power, 5 mW; modulation amplitude, 0.1 mT; time constant, 1 s; scan range, 10 mT; scan time, 4 min. The spectra were taken at 25 °C. The magnetic fields were calculated by the splitting of MnO (ΔH_{3-4} = 8.69 mT).

Cinnabarinic acid formed in the reaction of 3-HAT with SOD

The reaction mixture contained, in a total volume of 310 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT and 10 μl of SOD (35000 units/ml, in 10 mM-potassium phosphate buffer, pH 7.4). The reactions were initiated by adding SOD and were carried out at 25 °C under aerobic conditions. Samples (10 μl) of the reaction mixture were subjected to h.p.l.c. on a Jasco Trirotor V apparatus with a variable-wavelength u.v.–visible detector. The u.v.–visible detector was set at 450 nm. A column (150 mm × 4.6 mm internal diam.) packed with TSK ODS gel (5 μm particle size) (Toyo-Soda, Tokyo, Japan) was used with 10 mM-phosphoric acid in 40 % (v/v) methanol as a mobile phase at a flow rate of 2.0 ml/min. The column was kept at 40 °C throughout. Identification of cinnabarinic acid formed in the reaction mixture was based on the retention time (t_R 6.5 min). The h.p.l.c. peak height of the product was compared with those of three different concentrations of cinnabarinic acid standards to determine the quantities formed. The concentration of the cinnabarinic acid standard was determined by measuring the absorbance at 450 nm (ϵ 17000 $M^{-1} \cdot cm^{-1}$) [15].

Reduction of Fe^{3+} ions by 3-HAT

The reaction mixtures contained, in a total volume of 920 μl, 450 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 450 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 15 μl of 1 mM-$FeCl_2$ (in 1 mM-HCl) or 1 mM-$FeCl_3$ (in 1 mM-HCl) and 5 μl of 0.1 M-*o*-phenanthroline (in ethanol). After 1 min reaction at 25 °C, visible spectra of the reaction mixtures were measured. The reaction mixture with *o*-phenanthroline omitted was present in the reference cell. All spectrophotometric measurements were carried out with 10 mm-light-path cells, with a Hitachi 100-50 spectrometer.

RESULTS

Hydroxyl-radical formation enhanced by SOD

To investigate the influence of SOD on the autoxidation of 3-HAT, the formation of hydroxyl radicals was examined in the reaction mixture containing 3-HAT and SOD by using the e.s.r. spin-trapping technique (Fig. 1). Characteristic signals were detected in the complete reaction mixture (Fig. 1*a*) after 4 h reaction under aerobic conditions in the dark. Hyperfine splitting constants (a_H = 1.50 mT, a_N = 1.50 mT) of the signals indicated that the signals are due to the spin adduct of DMPO with hydroxyl radicals [26]. The signals were not observed under anaerobic conditions (Fig. 1*b*). This result showed that molecular oxygen is essential to the formation of the hydroxyl radicals. No signals were detected in the absence of SOD (Fig. 1*c*), suggesting that SOD enhances the formation of hydroxyl radicals in the reaction of 3-HAT with molecular oxygen.

The hydroxyl-radical formation enhanced by SOD was also examined in the reaction mixture containing other 3-HAT-related compounds, namely 3-hydroxykynurenine, *o*-aminophenol, *m*-aminophenol, *p*-aminophenol, *m*-hydroxybenzoic acid and *o*-aminobenzoic acid (Table 1). Of these compounds, the formation of hydroxyl radicals was most effectively enhanced by SOD

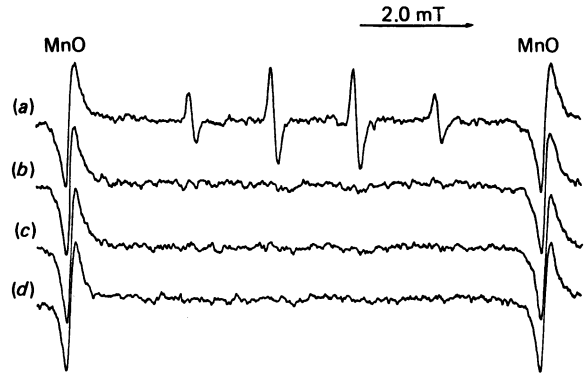

Fig. 1. E.s.r. spectra obtained for the reaction mixture containing 3-HAT and SOD

The reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of SOD (35000 units/ml, in 10 mM-potassium phosphate buffer, pH 7.4), 10 μl of 0.1 M-EDTA (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were initiated by adding 3-HAT and carried out at 25 °C for 4 h in the dark under aerobic conditions except for (*b*). E.s.r. conditions were as described in the Materials and methods section. (*a*) Complete reaction mixture; (*b*) complete reaction mixture under anaerobic conditions; (*c*) in the absence of SOD; (*d*) in the absence of 3-HAT.

in the reaction mixture containing *p*-aminophenol. No signals of the DMPO-OH were detected in the reaction mixtures containing 3-hydroxykynurenine, *o*-aminophenol, *m*-aminophenol, *m*-hydroxybenzoic acid and *o*-aminobenzoic acid. In the absence of SOD, no signals of DMPO-OH were detected in any of the reaction mixtures containing the 3-HAT-related compounds except for *p*-aminophenol.

The pH-dependence of the formation of hydroxyl radicals was examined in the reaction mixture containing 3-HAT and SOD. With increasing pH the formation of hydroxyl radicals increased (Fig. 2). In the absence of SOD, no signals of hydroxyl radicals were detected up to pH 8.0 and weak signals were detected at pH 8.5.

The effects of the chelators EDTA, DETAPAC and desferrioxamine on the formation of hydroxyl radicals were examined in the reaction mixture containing 3-HAT and SOD (Table 2). The formation of hydroxyl radicals was stimulated remarkably by EDTA and slightly by DETAPAC, and inhibited completely by desferrioxamine. These findings indicate that iron ions are essential to the formation of hydroxyl radicals enhanced by SOD. Catalase (560 units/ml) suppressed the formation of DMPO-OH completely, suggesting that hydrogen peroxide participates in the reaction. Addition of ethanol (2.8%, v/v) decreased the peak height of the DMPO-OH to 31% and resulted in the detection of signals characteristic of the spin adduct of DMPO with hydroxyethyl radicals [26]. The effects of catalase and ethanol on the formation of the DMPO-OH confirmed that the spin adduct DMPO-OH is formed through direct reaction of DMPO with hydroxyl radicals, not through decomposition of DMPO-OOH, the spin adduct of DMPO with superoxide radicals [26]. Hence the

Table 1. Hydroxyl-radical formation enhanced by SOD in reaction mixtures containing 3-HAT (or related compounds) and SOD

Reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of SOD (35000 units/ml, in 10 mM-potassium phosphate buffer, pH 7.4), 10 μl of 0.1 M-EDTA (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were carried out for 4 h at 25 °C under aerobic conditions in the dark. E.s.r. conditions were as described in the Materials and methods section. Signal intensities are evaluated by the peak height of the second signal of the quartet of DMPO-OH, and the values are means ± S.D. for three determinations. The signal intensity of the hydroxyl radicals formed with *p*-aminophenol in the absence of SOD is presented in parentheses. For all reagents other than *p*-aminophenol no signals of hydroxyl radicals were detected in the reaction mixtures without SOD.

Reagent	Peak height of DMPO-OH (cm)	(%)
3-HAT	5.4 ± 0.1	100
3-Hydroxykynurenine	0	0
o-Aminophenol	0	0
m-Aminophenol	0	0
p-Aminophenol	8.7 ± 0.8 (1.7)	161
m-Hydroxybenzoic acid	0	0
o-Aminobenzoic acid	0	0
None	0	0

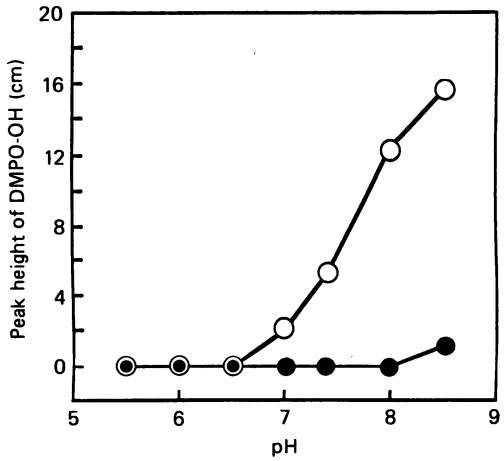

Fig. 2. pH-dependence of the formation of hydroxyl radicals in the reaction mixture containing 3-HAT and SOD

The reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of SOD (35000 units/ml, in 10 mM-potassium phosphate buffer, pH 7.4), 10 μl of 0.1 M-EDTA (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were initiated by adding 3-HAT and carried out at 25 °C for 4 h. E.s.r. conditions were as described in the Materials and methods section. ○, Complete reaction mixture; ●, in the absence of SOD.

Table 2. Effects of certain chelators on the formation of hydroxyl radicals in the reaction mixture containing 3-HAT and SOD

Reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of SOD (35000 units/ml, in 10 mM-potassium phosphate buffer, pH 7.4), 10 μl of 0.1 M-chelator (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were carried out for 4 h at 25 °C under aerobic conditions in the dark. E.s.r. conditions were as described in the Materials and methods section. Signal intensities are evaluated by the peak height of the second signal of the quartet of DMPO-OH, and the values are means ± S.D. for three determinations.

Chelator	Peak height of DMPO-OH (cm)	(%)
EDTA	5.4 ± 0.1	320
DETAPAC	2.9 ± 0.4	170
Desferrioxamine	0	0
None	1.7 ± 0.2	100

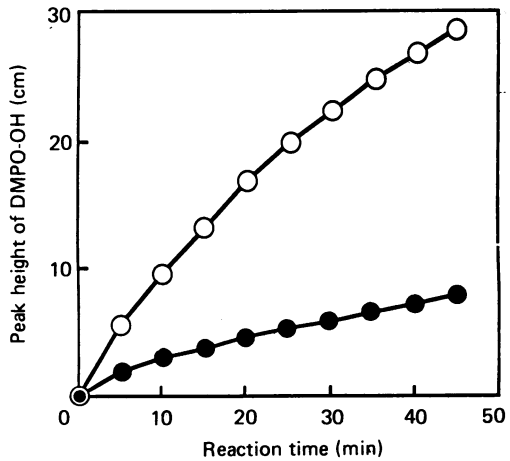

Fig. 3. Formation of hydroxyl radicals in the reaction mixture containing 3-HAT and hydrogen peroxide

The reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of 0.1 M-hydrogen peroxide (in water), 10 μl of 0.1 M-EDTA (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were initiated by adding hydrogen peroxide and carried out at 25 °C. E.s.r. conditions were as described in the Materials and methods section. ○, Complete reaction mixture; ●, in the absence of 3-HAT.

Fenton reaction [27] is presumed to participate in the reaction.

Effects of 3-HAT on hydroxyl-radical formation in the Fenton reaction

The effects of 3-HAT on the formation of hydroxyl radicals were examined in the Fenton reaction mixture containing hydrogen peroxide and Fe^{3+} ions. A time-course study indicated that the formation of hydroxyl radicals was enhanced by 3-HAT, although a slight formation of hydroxyl radicals was observed even in the absence of 3-HAT (Fig. 3). The enhancement was also observed in reaction mixtures containing 3-hydroxykynurenine, *o*-aminophenol and *p*-aminophenol, but not in reaction mixtures containing *m*-aminophenol, *m*-hydroxybenzoic acid and *o*-aminobenzoic acid (Table 3). Hence reagents containing the common *o*- or *p*-aminophenol chemical structure stimulated the formation of hydroxyl radicals.

Table 3. Effects of the various compounds on the formation of hydroxyl radicals in reaction mixtures containing 3-HAT (or 3-HAT-related compound) and hydrogen peroxide

Reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT or related compound (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of 0.1 M-hydrogen peroxide (in water), 10 μl of 0.1 M-EDTA (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were carried out for 5 min at 25 °C. E.s.r. conditions were as described in the Materials and methods section. Signal intensities are evaluated by the peak height of the second signal of the quartet of DMPO-OH, and the values are means ± S.D. for three determinations.

Reagent	Peak height of DMPO-OH (cm)	(%)
None	2.0 ± 0.4	100
3-HAT	5.7 ± 0.5	279
3-Hydroxykynurenine	3.7 ± 0.6	179
o-Aminophenol	7.0 ± 0.4	343
m-Aminophenol	2.8 ± 1.2	136
p-Aminophenol	17.8 ± 1.0	871
m-Hydroxybenzoic acid	2.5 ± 0.3	121
o-Aminobenzoic acid	2.5 ± 0.7	121

The formation of hydroxyl radicals in the Fenton reaction mixture was examined at various pH values (Fig. 4). The formation of hydroxyl radicals was stimulated by 3-HAT at all pH values examined, particularly at alkaline pH. In the absence of 3-HAT weak DMPO-OH signals were obtained and the signal intensity increased with increasing pH. The pH-dependence was not observed in the absence of EDTA, where very weak DMPO-OH signals were detected (results not shown).

The chelators EDTA, EDTAPAC and desferrioxamine had similar effects on hydroxyl-radical formation in the Fenton reaction to those observed in the system containing SOD. EDTA and DETAPAC stimulated hydroxyl-radical formation and desferrioxamine inhibited the reaction completely (Table 4).

Reduction of Fe^{3+} ions by 3-HAT

The formation of hydroxyl radicals enhanced by SOD seems to proceed through the Fenton reaction. Fe^{2+} ions are essential to the Fenton reaction. To investigate the supply of Fe^{2+} ions, the oxidation state of the iron ions was examined in the reaction mixture containing 3-HAT, Fe^{3+} ions and *o*-phenanthroline by using a u.v.–visible

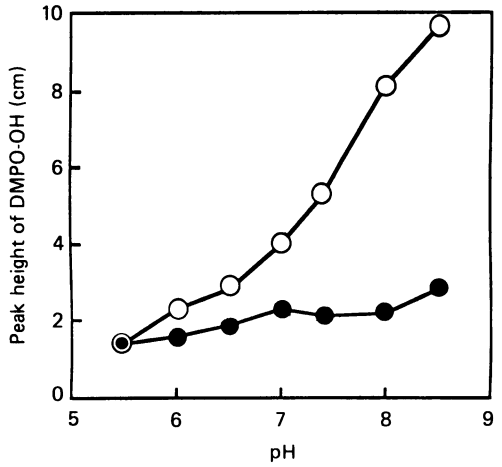

Fig. 4. pH-dependence of the formation of hydroxyl radicals in the reaction mixture containing 3-HAT and hydrogen peroxide

The reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1 M-DMPO (in water), 10 μl of 0.1 M-hydrogen peroxide (in water), 10 μl of 0.1 M-EDTA (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were initiated by adding hydrogen peroxide and carried out at 25 °C for 5 min. E.s.r. conditions were as described in the Materials and methods section. ○, Complete reaction mixture; ●, in the absence of 3-HAT.

Table 4. Effects of certain chelators on the formation of hydroxyl radicals in the reaction mixture containing 3-HAT and hydrogen peroxide

Reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5), 30 μl of 1.0 M-DMPO (in water), 10 μl of 0.1 M-hydrogen peroxide (in water), 10 μl of 0.1 M-chelator (in water) and 5 μl of 1 mM-$FeCl_3$ (in 1 mM-HCl). The reactions were carried out for 5 min at 25 °C. E.s.r. conditions were as described in the Materials and methods section. Signal intensities are evaluated by the peak height of the second signal of the quartet of DMPO-OH, and the values are means ± S.D. for the three determinations.

	Peak height of DMPO-OH	
Chelator	(cm)	(%)
EDTA	5.7 ± 0.5	630
DETAPAC	13.0 ± 1.3	1440
Desferrioxamine	0	0
None	0.9 ± 0.3	100

spectrometer. The reaction mixture gave a visible spectrum with $\lambda_{max.}$ 512 nm, suggesting that the Fe^{3+} ions are reduced by 3-HAT [28].

Cinnabarinic acid formation enhanced by SOD

The effects of SOD on the formation of cinnabarinic acid from 3-HAT were examined, since the formation of hydroxyl radicals was stimulated by SOD in the reaction of 3-HAT with molecular oxygen. The reaction mixture, which contained 3-HAT and SOD, was subjected to h.p.l.c. in conjunction with a u.v.–visible detector (450 nm). A prominent peak was obtained at a retention time of 6.5 min in the h.p.l.c. elution profile of the complete reaction mixture. The peak height increased linearly throughout the reaction (Fig. 5). Authentic cinnabarinic acid was eluted at the identical position. In the absence of SOD only a weak h.p.l.c. peak was observed, suggesting that SOD enhances the formation of cinnabarinic acid from 3-HAT. The result is in agreement with that obtained previously by Dykens *et al.* [29].

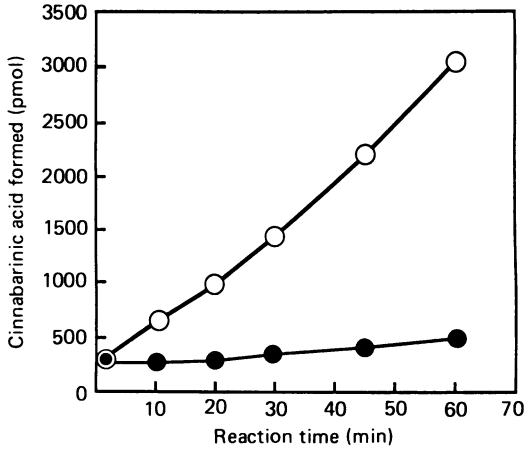

Fig. 5. Cinnabarinic acid formation enhanced by SOD

The reaction mixtures contained, in a total volume of 355 μl, 150 μl of 0.5 M-potassium phosphate buffer, pH 7.4, 150 μl of 0.5 mM-3-HAT (in 5 mM-potassium phosphate buffer, pH 5.5) and 10 μl of SOD (35000 units/ml, in 10 mM-potassium phosphate buffer, pH 7.4). The reactions were initiated by adding SOD and carried out at 25 °C under aerobic conditions in the dark. Samples (10 μl) of the reaction mixtures were subjected to h.p.l.c. ○, Complete reaction mixture; ●, in the absence of SOD.

DISCUSSION

In the present investigation it has been shown that the formation of hydroxyl radicals is enhanced by SOD during the auto-oxidation of 3-HAT. Hydroxyl radicals are known to be very reactive towards proteins, lipids and nucleic acids. Therefore hydroxyl radicals are considered to be a trigger for cell damage and to exert highly deleterious effects on cells. Thus hydroxyl radicals formed during the auto-oxidation of 3-HAT may participate in the induction of bladder carcinoma by 3-HAT. Further, the inhibition of leucine-stimulated insulin release from rat pancreatic islets [11] may be caused by hydroxyl-radical formation.

Here we present a possible mechanism for the hydroxyl-radical formation enhanced by SOD. First, 3-HAT reduces molecular oxygen to form superoxide anions (eqn. 1):

$$\text{3-HAT} + O_2 \rightleftharpoons \text{anthranilyl radical} + O_2^- \quad (1)$$

During this reaction 3-HAT is oxidized, leading to the formation of cinnabarinic acid via anthranilyl radicals.

The reaction seems to be reversible. It is possible for 3-hydroxykynurenine to act as a superoxide-radical scavenger when the reaction (eqn. 1) proceeds in the reverse direction. Indeed, Goshima *et al.* reported that 3-hydroxykynurenine can act as a superoxide-radical scavenger [30]. SOD readily dismutes superoxide radicals produced in the reaction (eqn. 1) to form hydrogen peroxide and molecular oxygen (eqn. 2):

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (2)$$

Thus the equilibrium (eqn. 1) is displaced in favour of the formation of superoxide ions on the addition of SOD.

The iron chelators EDTA, DETAPAC and desferrioxamine exerted an influence on the hydroxyl-radical formation enhanced by SOD, suggesting that the Fenton reaction (eqn. 3) participates in the reaction:

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

Fe^{2+} ions are essential to the Fenton reaction. However, 14 μM of Fe^{3+} ions are contained in the reaction mixture, but not Fe^{2+} ions. In the iron-catalysed Haber–Weiss reaction, Fe^{3+} ions are reduced by superoxide anions. That is not the case for the present system, since superoxide anions are readily dismuted by SOD. Fe^{3+} ions appear to be reduced by 3-HAT in the present reaction mixture (eqn. 4):

$$Fe^{3+} + \text{3-HAT} \rightarrow Fe^{2+} + \text{anthranilyl radical} \quad (4)$$

The anthranilyl radicals formed in the reaction (eqn. 4) may be further oxidized to lead to the formation of cinnabarinic acid. Since the formation of hydroxyl radicals was stimulated by increasing pH, the reduction of Fe^{3+} ions and/or molecular oxygen may be brought about through the reaction with 3-HAT anion.

The formation of hydroxyl radicals was also effectively stimulated by 3-HAT, *p*-aminophenol, *o*-aminophenol and 3-hydroxykynurenine, but not by *m*-aminophenol, *m*-hydroxybenzoic acid and *o*-aminobenzoic acid, in the reaction mixtures containing hydrogen peroxide, Fe^{3+} ions and EDTA. In these reactions the 3-HAT-related compounds seem to enhance the formation of hydroxyl radicals by reducing the Fe^{3+} ions. Both 3-hydroxykynurenine and *o*-aminophenol effectively stimulated the formation of hydroxyl radicals in the reaction mixture containing hydrogen peroxide, but not in the reaction mixture containing SOD. This is probably because both 3-hydroxykynurenine and *o*-aminophenol can reduce Fe^{3+} ions but not molecular oxygen.

The present finding that SOD stimulates the formation of hydroxyl radicals is quite surprising, since SOD is generally considered to be a scavenger of active oxygen species. SOD inhibits the formation of hydroxyl radicals by decomposing the superoxide anions that can reduce Fe^{3+} ions. However, SOD could not inhibit the formation of hydroxyl radicals if effective reducing agents that can reduce Fe^{3+} ions are present in the reaction mixture. Under such limited conditions SOD could not act as a scavenger of active oxygen species. Further, SOD may have deleterious effects rather than protective effects with respect to the cytotoxicity of active oxygen species under conditions such as those prevailing in the present system. Indeed, some investigators have recently reported that SOD enhances the cytotoxicity by active oxygen species [31,32].

In the present paper we have shown that 3-HAT stimulates the formation of hydroxyl radicals by reducing Fe^{3+} ions to Fe^{2+} ions for participation in the Fenton reaction. The formation of hydroxyl radicals enhanced by 3-HAT appears to have deleterious effects on cells. On the other hand, Werner *et al.* [12] showed that interferon γ induces the excretion of 3-HAT by human macrophages *in vitro*. Hydroxyl radicals are considered to be generated from superoxide anions, which are produced by NADPH oxidase in the plasma membrane of macrophages through the iron-catalysed Haber–Weiss reaction. The 3-HAT generated in macrophages might enhance the bactericidal activity of macrophages, since 3-HAT could increase formation of hydroxyl radicals by reducing Fe^{3+} ions.

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