Biochimica et Biophysica Acta, 1036 (1990) 78–81
Elsevier

BBAGEN 20268

BBA Report

NADPH–cytochrome-*P*-450 reductase promoted hydroxyl radical production by the iron(III)-ochratoxin A complex

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(Received 21 December 1989)
(Revised manuscript received 15 June 1990)

Key words: Ochratoxin A; Iron(III); ESR; Oxygen free radical

The Fe^{3+} complex of ochratoxin A has been shown to produce hydroxyl radicals in the presence of NADPH and NADPH–cytochrome-*P*-450 reductase. ESR spin-trapping experiments carried out in the presence of the hydroxyl radical scavenger ethanol and the spin trap DMPO (5,5-dimethyl-1-pyrroline-1-oxide) produced ESR spectra characteristic of the hydroxyl radial-derived carbon-centered DMPO-alkoxyl radical adduct. Thus hydroxyl radicals produced by the Fe^{3+}-ochratoxin A complex in the presence of an enzymatic reductase may be partly responsible for ochratoxin A toxicity.

Ochratoxin A (Fig. 1) is a mycotoxin produced by some species of the genera *Aspergillus* and *Penicillium* and its occurrence in food and feed is widespread [1]. It is nephrotoxic to all single-stomached animals and is linked to outbreaks of nephropathy in pigs and chickens [1,2]. Ochratoxin A has been shown to be a liver and kidney carcinogen in mice and is strongly suspected of being the main etiologic agent responsible for endemic Balkan nephropathy and associated urinary tract tumors in humans [3]. It has been recently shown [4] that the administration of ochratoxin A enhanced lipid peroxidation both in vitro and in vivo. The lipid peroxidation induced by ochratoxin A in a liver microsomal system in the presence of NADPH is almost completely abolished by the presence of the strong Fe^{3+} chelator Desferal which indicates a requirement for chelatable iron in the lipid peroxidation process. Ochratoxin A has a phenolic group that is located β to a carbonyl group which is a structural feature similar to the cardiotoxic anthracyclic quinone antitumor drug doxorubicin, which is known to form a strong complex with Fe^{3+} [5]. An iron-based oxidative stress produced through an enzymatic reductive activation is thought to be, at least, partly responsible for the cardiotoxicity of doxorubicin.

The addition of anhydrous $FeCl_3$ in methanol to ochratoxin A in methanol results in the rapid formation of a reddish-brown complex with a 1 : 1 stoichiometry and absorption peaks at 342 and 483 nm (ε at 483 nm approx. 1.6 $mM^{-1} \cdot cm^{-1}$) [6]. The association constant for formation of the Fe^{3+}-ochratoxin A complex was determined fluorometrically to be $2 \cdot 10^8$ M^{-1} in methanol, indicating that ochratoxin is capable of forming a strong complex with Fe^{3+} even in the micromolar concentration range.

The NADPH–cytochrome-*P*-450 reductase system strongly stimulates liposomal lipid peroxidation in the presence of the Fe^{3+}-ochratoxin A complex [6]. The Fe^{3+} was shown to undergo reduction to Fe^{2+} and subsequent reoxidation which was accompanied by O_2 consumption. Though free hydroxyl radicals did not appear to participate in lipid peroxidation, this study does, however, show that the Fe^{3+}-ochratoxin A complex produces the extremely damaging hydroxyl radical in the presence of the NADPH–cytochrome-*P*-450 reductase system.

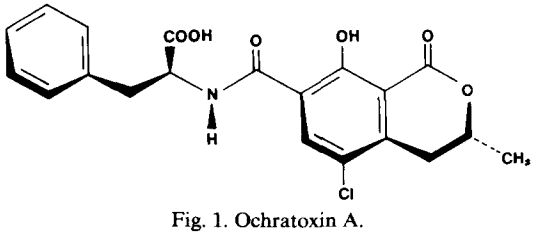

Fig. 1. Ochratoxin A.

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Ochratoxin A was isolated and purified from a culture of *Aspergillus ochraceus* NRRL 3174 as described previously [7] and was dissolved in methanol. The flavoprotein NADPH–cytochrome-*P*-450 reductase was purified from liver microsomes isolated from phenobarbital pre-treated rats as described in Ref. 8. Briefly, microsomes were treated with the detergent CHAPS, in combination with protamine sulfate, followed by centrifugation. The reductase was solubilized from the CHAPS-insoluble pellet by a combination of the detergents sodium cholate and Lubrol PX, and further purified to homogeneity by 2′, 5′-ADP-agarose chromatography. The purified reductase showed a single band on SDS-PAGE and had a specific activity of 18000 units per mg protein. (1 unit of enzyme activity is that amount which catalyzes the reduction of 1 nmol of ferricytochrome *c* per min at 22° C). Anhydrous ferric chloride (BDH) was freshly dissolved in methanol. The spin trap DMPO (5,5-dimethyl-1-pyrroline-1-oxide; Sigma) was purified over charcoal and assayed as described in Ref. 9. The ESR spectra were run in 20 mM Tris-HCl buffer (pH 8.5) (BDH ultrapure). The catalase (bovine liver) and superoxide dismutase (bovine erythrocyte) were from Sigma and were dialyzed before use.

The reddish-brown Fe^{3+}-ochratoxin A complex was pre-formed by adding $FeCl_3$ dissolved in methanol to ochratoxin A also in methanol. To this was added aqueous air-saturated buffer, 2 mol of NaOH per mol of ochratoxin A (to neutralize the two acidic protons on ochratoxin A), protective enzyme (or not), NADPH–cytochrome-*P*-450 reductase, NADPH and DMPO. Due to the high concentrations of ochratoxin A and Fe^{3+} present, a small amount of reddish-brown precipitate was present in the ESR tube. Methanol from $FeCl_3$ and ochratoxin A stock solution was also present, generally in the order of 5% (v/v), and was shown to have no significant effect on the ESR spectra. The ESR spectra were recorded at room temperature in identical quartz capillary tubes on an X-band Bruker ESP-300 spectrometer using a 100 kHz modulation frequency. The spectra shown are the arithmetic sum of five consecutive spectra collected over a total time of 3.5 min. The radical concentration was determined by double integration of one of the peaks of the baseline-corrected spectra using 100 μM $MnSO_4$ as a standard [10].

The ESR spectrum shown in Fig. 2 has the characteristic 1:2:2:1 4-line spectrum found for DMPO-OH [11]. The hyperfine splitting constants were measured from Fig. 2A to be $A_N = A_H = 14.9$ G, which are identical to those reported by Finkelstein et al. [11]. In the absence of NADPH–cytochrome-*P*-450 reductase (Fig. 2B), the amount of DMPO-OH produced is very significantly reduced, indicating that the reductase is largely responsible for the production of DMPO-OH.

While the 4-line spectrum shown in Fig. 2A of DMPO-OH is known to be produced from the reaction

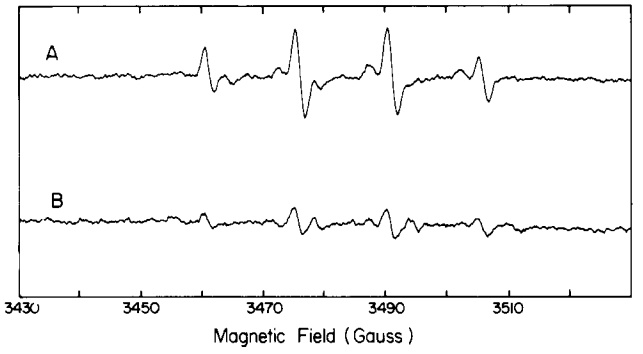

Fig. 2. (A) The ESR spectrum recorded at room temperature after Fe^{3+}-ochratoxin A (1 mM Fe^{3+}, 3 mM ochratoxin A) in Tris buffer (pH 8.5) was incubated for 55 min with NADPH–cytochrome-*P*-450 reductase (7.5 μg protein/ml), NADPH (1 mM) and DMPO (90 mM). This constitutes the 'complete reaction system'. The instrumental settings were as follows: microwave frequency 9.8 GHz, microwave power 6.3 mW, modulation amplitude 2 G, and receiver gain $4 \cdot 10^5$. (B) As in A above, but in the absence of the reductase.

of hydroxyl radical with DMPO, it is also known that DMPO-O_2^- formed from the reaction of O_2^- with DMPO decays rapidly to DMPO-OH [11]. Thus, in order to distinguish hydroxyl radical production from O_2^- production, experiments were also carried out in the presence of the hydroxyl radical scavenger ethanol. The hydroxyl radical reacts with ethanol to form an alkoxyl radical, which then reacts with DMPO to form a 1:1:1:1:1:1 6-line spectrum produced by the carbon-centered DMPO-CH(OH)-CH_3 adduct [11]. As shown in Fig. 3, when the concentration of ethanol is increased, the 4-line DMPO-OH spectrum is gradually replaced by increasing amounts of a 6-line spectrum which demonstrates that the 6-line spectrum is ethanol derived. The hyperfine splitting constants of the 6-line spectrum were measured to be $A_N = 15.9$ G and $A_H =$

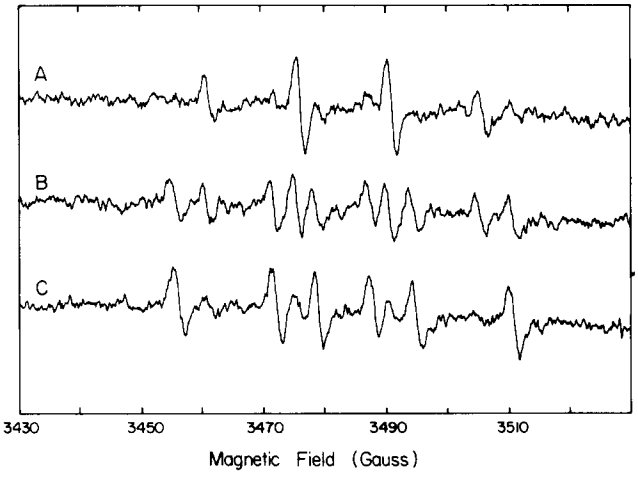

Fig. 3. The ESR spectra recorded on the complete reaction system after 20 min in the presence of increasing amounts of ethanol. (A) No added ethanol; (B) 3% (v/v) ethanol; and (C) 5% (v/v) ethanol. Except as noted above, the reaction mixture and the instrumental settings were identical to those described in Fig. 2A.

23.1 G, which are very close to previously reported values [11–13] for the carbon-centered DMPO-CH(OH)-CH_3 radical. The results of Figs. 2 and 3 thus confirm the production of hydroxyl radical in the reaction system.

As shown in Fig. 4B, in the absence of either any added Fe^{3+} or ochratoxin A, a significant amount of 4-line spectrum due to DMPO-OH is produced. Since the NADPH–cytochrome-*P*-450 reductase system is known to produce O_2^- on its own [14,15], the DMPO-OH is likely being produced either from decomposition of DMPO-O_2^- or directly from OH· [11], or both. The addition of catalase, which catalyzes the decomposition of H_2O_2 to O_2, to the complete reaction system almost completely abolished the production of the hydroxyl-radical derived 6-line spectrum (Fig. 4C). In fact, upon the addition of catalase, visible evolution of a gas that was assumed to be O_2 occurred. These results indicate that H_2O_2 is required for the production of hydroxyl radical. When ochratoxin A was omitted from the complete system in a control experiment (Fig. 4D), very little 6-line spectrum was observed. This result demonstrates that complexation of Fe^{3+} by ochratoxin A is necessary for hydroxyl radical production.

The effect of superoxide dismutase on radical production is shown in Fig. 5B. The concentration of the DMPO-CH(OH)-CH_3 radical was increased by a factor of 1.9, while the concentration of DMPO-OH was reduced to 60% of the value found for the complete system. Both these results are consistent with an increased rate of conversion of O_2^- into H_2O_2. The increased rate of production of H_2O_2 results in an increased rate of OH· radical production, while the increased rate of loss of O_2^- from the system results in less DMPO-O_2^- being formed and subsequently less DMPO-OH formed from DMPO-O_2^-. The effect of addition of $FeSO_4$ to a mixture of ochratoxin A and H_2O_2 in the absence of the reductase and NADPH is

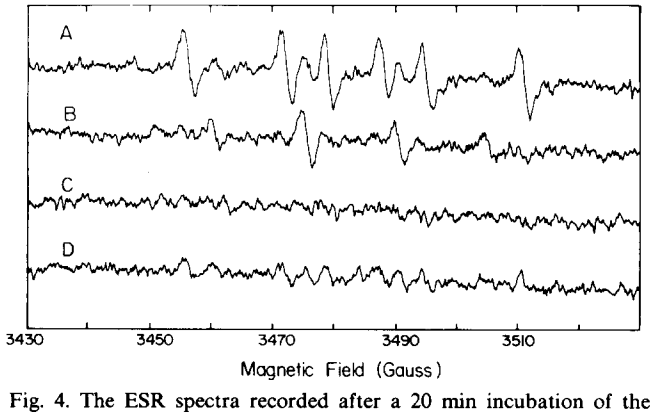

Fig. 4. The ESR spectra recorded after a 20 min incubation of the reaction system in 5% (v/v) ethanol as described in the legend of Fig. 2A except as noted: (A) complete reaction system; (B) in the absence of any added Fe^{3+} or ochratoxin A; (C) added catalase at a final concentration of 0.1 mg/ml; and (D) in the absence of any ochratoxin A.

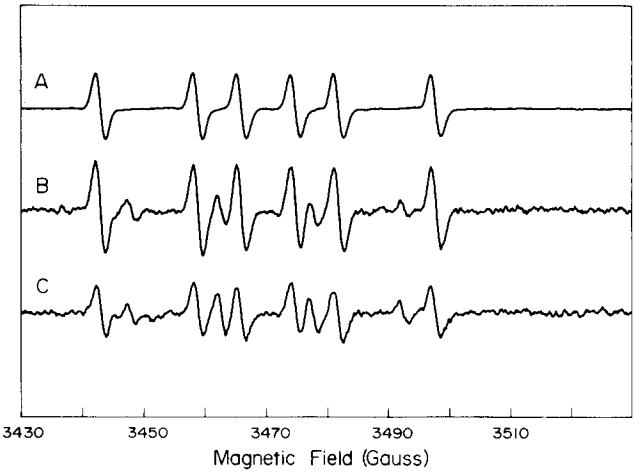

Fig. 5. The ESR spectra recorded after a 20 min incubation in Tris (pH 8.5) buffer in 5% (v/v) ethanol and DMPO (90 mM). In addition: (A) contained ochratoxin A (3.0 mM), H_2O_2 (200 μM) and $FeSO_4$ (1.0 mM), added in that order; (B) contained the complete reaction system described in the legend of Fig. 2A plus superoxide dismutase (0.1 mg/ml); and (C) is the complete reaction system for comparison. The concentration of DMPO-CH(OH)-CH_3 is 1300, 220 and 120 μM in A, B and C, respectively. Trace A, was recorded at a gain of one-eighth of that used for B and C.

shown in Fig. 5A. The concentration of DMPO-CH(OH)-CH_3 increased by a factor of 11 compared to the complete system in Fig. 5C. This result shows that the Fe^{2+}-ochratoxin A complex is capable of directly reducing H_2O_2 to produce hydroxyl radical.

A variety of toxic compounds, such as doxorubicin, CCl_4 and ethyl hydrazine, have been shown by ESR spin-trapping experiments to produce hydroxyl radicals in microsomal system [5,12–14]. The NADPH–cytochrome-*P*-450 reductase system in the presence of iron and EDTA has also been shown by ESR spin-trapping experiments to result in the production of hydroxyl radicals [14]. This result was confirmed under our reaction conditions in an experiment conducted as shown in Fig. 4A in which ochratoxin A had been omitted but which contained 0.25 mM EDTA (ESR spectrum not shown). Thus it seems likely that hydroxyl radical is produced by an iron-based Fenton-type chemistry in which Fe^{2+}-ochratoxin A reduces H_2O_2. Thus in a mechanism similar to that described in Ref. 14, hydroxyl radical is ultimately produced by the following reactions:

$$E_{ox} + NADPH \rightarrow E_{red} + NADP^+ \quad (1)$$

$$E_{red} + O_2 \rightarrow O_2^- + E_{ox} \quad (2)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$O_2^- + Fe^{3+}\text{-ochratoxin A} \rightarrow Fe^{2+}\text{-ochratoxin A} + O_2 \quad (4)$$

$$Fe^{2+}\text{-ochratoxin A} + H_2O_2 \rightarrow Fe^{3+}\text{-ochratoxin A} + OH^- + OH\cdot \quad (5)$$

in which E_{ox} and E_{red} are the oxidized and reduced forms, respectively, of NADPH–cytochrome-*P*-450 re-

ductase. The direct reduction of Fe^{3+}-ochratoxin A by E_{red} may also be occurring simultaneously with the reaction of Eqn. 4. In this mechanism ochratoxin A solubilizes Fe^{3+} by forming a complex. The Fe^{3+}-ochratoxin A complex accepts an electron to form Fe^{2+}-ochratoxin A which then reduces H_2O_2 given hydroxyl radical.

While it has previously been shown that lipid peroxidation induced by ochratoxin A is strongly iron dependent [4], the lack of any protection offered by catalase and several hydroxyl radical scavengers [6] suggests that hydroxyl radical production through the reaction of Eqn. 5 is not a significant factor in the vitro lipid peroxidation. However, these results do not preclude hydroxyl radical production by the Fe^{3+}-ochratoxin A complex, as demonstrated in this study, also having an important role in the toxicity of ochratoxin A. There are several reports of ochratoxin A-induced DNA damage both in vivo [16] and in cultured cells [17]. Production of the extremely reactive hydroxyl radical by the reaction of Eqn. 5 is a possible cause of this damage.

The support of the Natural Sciences and Engineering Council of Canada (B.B.H.) and the Kidney Foundation of Canada (A.D.R.) is gratefully acknowledged. The skilled technical assistance of Vivien Clark is also gratefully acknowledged.

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