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ENHANCED PRODUCTION OF ETHYLENE FROM METHIONAL BY IRON CHELATES AND HEME CONTAINING PROTEINS IN THE SYSTEM CONSISTING OF QUINONE COMPOUNDS AND NADPH-CYTOCHROME P-450 REDUCTASE

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Abstract—The addition of iron chelates or heme containing proteins to the systems consisting of NADPH-cytochrome P-450 reductase and quinone compounds, such as vitamin K_3 (menadione), adriamycin, tetrahydropyranyladriamycin, daunomycin, aclacinomycin A, carbazilquinone, and mitomycin C, showed the enhanced production of ethylene from methional. In the vitamin K_3 system, the effective iron chlates were Fe(II)–EDTA, Fe(II)–ADP, Fe(II)–bleomycin A_2, and hemin, and the effective iron containing proteins were methemoglobin, myoglobin, ferredoxin, and partially purified cytochromes P-450, P-420, and b_5, and the reversed effects were observed by horse radish peroxidase and sulfite reductase from yeast. In the system consisting of aclacinomycin A and methemoglobin, the ethylene production was potently inhibited by radical scavengers, such as Tiron, Tris, thiourea, and KI, and weakly inhibited by some other scavengers. In the system containing vitamin K_3 and methemoglobin, the ethylene production was potently inhibited by catalase, but partially by superoxide dismutase, KCN, and NaN_3. In this system, the absorption spectrum of methemoglobin was immediately changed to oxyform and quenched with time, and catalase protected the decrement of the spectrum. The addition of hydrogen peroxide or cumene hydroperoxide to methemoglobin also produced ethylene from methional.

NADPH-cytochrome P-450 reductase (EC 1.6.2.4) is an important enzyme for drug metabolism by the catalysis of the electron transfer from NADPH to cytochrome P-450. On the other hand, recent studies indicate the direct contribution of NADPH-cytochrome P-450 reductase to drug metabolism [1–5]. In our studies, under anaerobic conditions, the reductase catalyzed the reductive glycosidic cleavage reaction of anticancer anthracyclines, such as adriamycin, aclacinomycin A and daunomycin, and the reductive activation of mitomycin C [1, 2, 6]. The intermediate form of the drugs in these reactions was suggested as semiquinone radicals of drugs which were produced by the reduction of the drugs by NADPH-cytochrome P-450 reductase [7, 8]. Under aerobic conditions, the system consisting of the reductase and above described quinone drugs generated hydrogen peroxide and hydroxyl radicals, and this process was suggested as one electron reduction of molecular oxygen by semiquinone form of the drugs, following production of superoxide anion, its dismutation to hydrogen peroxide, and further generation of hydroxyl radicals [9]. Through these studies, we found the enhanced production of ethylene from methional by iron chelates and heme containing proteins, probably part of these are the result of the action of hydroxyl radicals. In this paper, we report the basic properties of the enhanced production of ethylene from methional in the system consisting of the reductase, quinone compounds, and iron chelates or heme containing proteins.

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MATERIALS AND METHODS

NADPH-cytochrome P-450 reductase was purified from phenobarbital-induced rabbit liver microsomes as reported previously [10], and the purified preparation was dialyzed against a large volume of 0.1 M Tris–HCl, pH 7.7, containing 20% (v/v) glycerol. Yeast sulfite reductase (EC 1.8.1.2) was purified in our laboratory [11]. Spinach ferredoxin was the kind gift of Dr. G. Tamura, Chiba University (Japan), and purified by DEAE-cellulose column chromatography, and the purifed preparation showed the A_{420}/A_{270} value as 0.46 [12]. Bovine methemoglobin, equine skeletal muscle myoglobin, type I, horse radish peroxidase (EC 1.11.1.7), type VI, twice-crystallized bovine liver catalase (EC 1.11.1.6), bovine superoxide dismutase (EC 1.15.1.1), hemin, and methional were obtained from Sigma Chemical Co. (St Louis, MO). Adriamycin hydrochloride, aclacinomycin A hydrochloride [13], and carbazilquinone [14], were the kind gift of Dr. F. Arcamone, Farmitalia-carloerba S.p.A. (Italy), Dr. A. Yoshimoto, Sanraku-Ocean Co., Ltd. (Japan), and Sankyo Co., Ltd. (Japan), respectively. Tetrahydropyranyladriamycin hydrochloride [15] and metal-free bleomycin A_2 were the kind gift of Dr. T. Takeuchi, Institute of Microbial Chemistry (Japan). Daunomycin hydrochloride and mitomycin C were obtained from Meiji Seika Kaisha, Ltd. (Japan) and

Kyowa Hakko Kogyo Co., Ltd. (Japan), respectively. Partially purified cytochromes P-450 and b_5 were obtained from phenobarbital-induced rabbit liver microsomes according to the method described by Imai and Sato [16]. After passage on 8-aminooctyl Sepharose 4B column (3.2 × 22 cm), cytochromes P-450 and b_5 were eluted together, then the fractions containing both cytochromes were applied on DEAE-Sephadex A-50 column (1.8 × 20 cm) which was previously equilibrated with 0.1 M Tris–HCl, pH 7.7, containing 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 0.5% (w/v) Emulgen 913, and eluted the same buffer containing 0.12 M KCl. The separated cytochromes P-450 and b_5 fractions were pooled and dialyzed against a large volume of 0.1 M potassium phosphate buffer, pH 7.3, containing 20% (v/v) glycerol. The dialyzed fractions were concentrated by means of a collodion bag. Cytochrome P-420 was obtained by the heat treatment of cytochrome P-450 at 60° for 5 min. Determination of cytochromes was according to the method of Omura and Sato [17]. Porapack type Q was obtained from Waters Associated Inc. (U.S.A.). NADPH and cumene hydroperoxide were obtained from Oriental Yeast Co. (Japan) and Nakarai Chemical Co., Ltd. (Japan), respectively. All other chemicals were analytical grade. Hemin was dissolved in 0.1 N NaOH before use. Boiled methemoglobin was prepared by the heat treatment of methemoglobin at 100° for 5 min. Metal chelates were prepared by mixing the equal concentration of the components, before use. Ultraviolet and visible spectra were measured with a Shimadzu UV-200S double beam spectrophotometer. The incubation mixture, in a final volume of 1.0 ml, consisted of 0.1 M potassium phosphate buffer, pH 7.5, 0.1 mM NADPH, 20 μM vitamin K_3, 1.5 μM methemoglobin, and 0.05 units of NADPH-cytochrome P-450 reductase. In some experiments, 40 μg of superoxide dismutase and catalase were added.

Ethylene production. The ethylene production was measured using gas chromatography as described previously [9]. The basic assay mixture, in a final volume of 1.0 ml in a 11.5 ml test tube with a silicone rubber stopper, consisted of 0.1 M potassium phosphate buffer, pH 7.5, 2 mM NADPH, 1 μl of methional, 0.1 mM quinone compound, 10 μM metal chelate or iron containing protein and 0.07–0.09 units of NADPH-cytochrome P-450 reductase. In the case of hydroperoxide-supported systems, hydroperoxide compound was added in place of NADPH, quinone compound, and the reductase. In some experiments, the quinone compound and iron-containing substance were varied and radical scavengers were added as indicated in the text. Reactions were carried out at 37° for 30 min with reciprocal shaking at 120 rpm and stopped by the immersion of the test tube in an ice bath or by rapid freezing using Cryo-Cool II. The gaseous phase, 0.5 ml, was analyzed with a Shimadzu GC-3BF flame ionization gas chromatograph equipped with 3 × 1000 mm column packed with Porapack Q. The quantity of ethylene released during each incubation was calculated from the height of the peak using calibration chromatograms. The ethylene production was pH dependent, and showed optimum between pH 7 and 8, and this pH optimum was in good agreement with the property of the cytochrome *c* reduction of the reductase. Under the assay conditions used in the present study, the ethylene production proceeded linearly for at least 1 hr.

Table 1. Ethylene production in various systems

System	Ethylene produced (pmole/system)
Complete	33,600
Aclacinomycin A	12,400
Methemoglobin	7710
Aclacinomycin A Methemoglobin	1380
Enzyme	231
NADPH	47

Assay methods are described in Materials and Methods, and aclacinomycin A and 9.2 μM methemoglobin were used as a quinone compound and a heme containing protein, respectively.

RESULTS

Ethylene production

The requirements of the components for the ethylene production using aclacinomycin A as a quinone compound and methemoglobin as a heme containing protein are shown in Table 1. The highest production was observed in the complete system which contains both aclacinomycin A and methemoglobin, in addition to NADPH-cytochrome P-450 reductase and NADPH. The quinone compound minus system, which contains methemoglobin, the reductase, and NADPH, also produced about 30–40% ethylene compared with the complete system. As reported previously [9], the system which contains quinone compound, the methemoglobin minus system, was weaker than the quinone minus system. The minus system of both methemoglobin and quinone compound showed a weak ethylene production. Minus NADPH or the reductase from the complete system produced a trace amount of ethylene.

Enhanced production of ethylene by hemin, methemoglobin, and myoglobin

The effects of hemin and heme-containing proteins, methemoglobin and myoglobin, on the ethylene production in the vitamin K_3-containing system were tested. As shown in Fig. 1, hemin and methemoglobin showed the enhancement of the ethylene production with dependent concentration, and the optimum around 10 μM. The effects were decreased with further increment of the heme concentration, and in the case of methemoglobin, the decrement was slower than that of hemin. Horse heart myoglobin was also effective and less intense concentration dependency was observed.

Effects of various iron chelates and heme containing proteins on the ethylene production

The effects of various iron containing compounds on the ethylene production in the vitamin K_3-containing system were tested. As shown in Table 2, iron-chelates, such as Fe(II)–EDTA, Fe(II)–bleo-

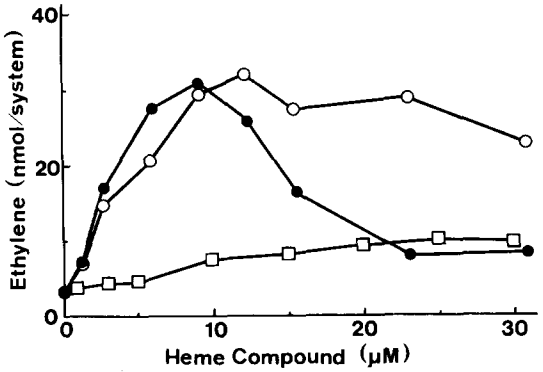

Fig. 1. Enhanced production of ethylene by hemin, methemoglobin, and myoglobin. ●, Hemin; ○, methemoglobin; □, myoglobin.

mycin A_2, Fe(II)–ADP, and hemin, were effective in the enhancement of ethylene production, but not so effective as methemoglobin. We tested other metal–EDTA chelates varying metals, such as Li(II), Mg(II), Al(III), Ca(II), Mn(II), V(III), Co(II), Cr(III), Fe(III), Ni(II), Cu(II), Zn(II), As(III), Se(IV), Mo(VI), Cd(II), Sn(II), and W(VI). Among these metal–EDTA chelates, the only effective chelate was Fe(III)–EDTA, which showed about 80% ethylene production compared with Fe(II)–EDTA, and other metal–EDTA chelates showed 15–30%. Among proteins, boiled methemoglobin showed a higher value than methemoglobin. Myoglobin was effective and cytochrome *c* was slightly effective. Spinach ferredoxin, which is a non-heme iron–sulfur protein, was also effective on the ethylene production. Horse radish peroxidase and sulfite reductase from yeast, were not effective and the ethylene production was reduced. Using dihydroxyfumaric acid as the substrate for horse radish peroxidase, Halliwell [18] reported the hydroxyl radical generation in the study of *p*-coumaric acid hydroxylation, and we observed the ethylene production by horse radish peroxidase and sulfite reductase in the other systems (unpublished results). So, there may exist a different mechanism for the production of ethylene in both enzymes. Fractions containing cytochromes P-450, P-420, and b_5, although these cytochromes were not purified, showed the enhancement of the ethylene production, and the combination of the fractions of cytochromes P-450 and b_5 was also effective, but not additively.

Effects of anticancer quinone drugs on the ethylene production in the methemoglobin containing system

Using the methemoglobin-containing system, the quinone compounds were varied (Table 3). In comparison with vitamin K_3, the anticancer quinone drugs, such as adriamycin, tetrahydropyranyl-adriamycin, daunomycin, aclacinomycin A, carbazilquinone, and mitomycin C, were all effective for the generation of ethylene from methional. Among the compounds tested, carbazilquinone was the most effective, and mitomycin C showed the weakest effect. These results were in good agreement with the results obtained in the hydroxyl radical generation in the system without methemoglobin [9] and the substrate specificity of the oxidase activity of NADPH-cytochrome P-450 reductase [2].

Effects of radical scavengers on ethylene production

Effects of radical scavengers at the concentration of 10–1000 mM on the ethylene producing system which consist of aclacinomycin A and methemoglobin, were tested (Table 4). Tiron, which is superoxide anion radical scavenger, inhibited the ethylene production potently. Tris, thiourea and KI,

Table 2. Effect of iron containing compounds on ethylene production

Compound	Ethylene produced (pmole/system)	Relative production* (%)
Methemoglobin	32,500	100
Methemoglobin	4520	13.9
$FeSO_4$	7830	24.1
Fe(II)–EDTA	21,900	67.4
Fe(II)–ADP	7350	22.6
Fe(II)–bleomycin A_2	16,800	51.6
Hemin	19,500	60.0
Boiled methemoglobin	43,600	134
Myoglobin	13,100	40.3
Ferredoxin	22,900	67.7
Cytochrome c	7090	21.8
Peroxidase	553	1.7
Sulfite reductase	98	0.3
Cytochrome P-450†	29,400	90.5
Cytochrome P-420†	23,000	70.8
Cytochrome b_5†	21,400	65.8
Cytochrome P-450† + cytochrome b_5†	30,200	92.9

Vitamin K_3 was used as a quinone compound. The concentrations of iron containing compounds were 10 μM, except Fe(III)–ADP 25 μM, methemoglobin 9.2 μM, and cytochromes P-450, P-420, and b_5 4.5 μM.

* Relative production was expressed using methemoglobin as 100%.

† As described in the text, partially purified cytochromes were used.

Table 3. Effect of anticancer quinone drugs on ethylene production

Sample	Ethylene produced (pmole/system)	Relative production* (%)
Vitamin K_3	41,500	100
Adriamycin	33,400	80.5
Tetrahydropyranyladriamycin	31,800	76.6
Daunomycin	32,100	77.3
Aclacinomycin A	38,100	91.8
Carbazilquinone	41,600	100
Mitomycin C	21,000	50.6

9.2 μM methemoglobin was used as a heme containing protein.
* Relative production was expressed using vitamin K_3 as 100%.

which are hydroxyl radical scavengers, strongly inhibited the ethylene production about 27–86%. Ethanol, dimethyl sulfoxide, formate, benzoate and mannitol, which are also hydroxyl radical scavengers, inhibited the ethylene production about 16–35%. Triethylenediamine, which is singlet oxygen scavenger, inhibited the ethylene production about 10–30%. By these scavengers, the cytochrome *c* reductase activity of the enzyme was scarcely inhibited, except 23% inhibition by mannitol and 56% inhibition by Tiron, both at a concentration of 100 mM. These results indicate that the ethylene production is dependent on superoxide anion and suggest that some parts of the ethylene production are the result of the action of hydroxyl radicals on methional.

Effects of superoxide dismutase, catalase, KCN and NaN_3 on the ethylene production

The effects of superoxide dismutase and catalase on the ethylene production in the system containing methemoglobin and vitamin K_3 were tested (Table 5). By the addition of superoxide dismutase, the ethylene production was inhibited slightly, whereas, by catalase and by the addition of both superoxide dismutase and catalase, potent inhibition was observed. The ligands which bind to ferric state hemoglobin, such as KCN and NaN_3, were tested. As observed in Table 5, KCN, 0.1–1.0 mM, showed a weak concentration dependent inhibition, and NaN_3, 0.5–5.0 mM, inhibited the ethylene production slightly.

Ethylene production in the hydroperoxide-supported system

As observed in Table 5, the ethylene production in the NADPH-cytochrome P-450 reductase-supported system was potently inhibited by the presence of catalase. The ethylene production in the hydroperoxide supported system, which consisted of

Table 4. Effect of radical scavengers on ethylene production

Scavenger	Concentration (mM)	Ethylene produced (pmole/system)	Relative production* (%)
Control	—	33,300	100
Tiron	100	9220	27.7
	50	10,300	30.9
	10	10,600	31.8
Tris	100	4800	14.4
	50	10,600	31.8
	10	24,300	73.0
Thiourea	100	8520	25.6
	50	12,000	36.0
	10	17,700	53.2
KI	100	13,500	40.5
	50	14,500	43.5
	10	17,400	52.3
Ethanol	1000	21,600	64.9
	500	24,000	72.1
	200	21,400	72.4
Dimethyl sulfoxide	100	22,400	67.3
Formate	100	23,400	70.3
Benzoate	100	27,500	82.6
Mannitol	100	28,000	84.1
Triethylenediamine	100	22,800	68.5
	50	24,000	72.1
	10	30,000	90.1

Aclacinomycin A and 10 μM methemoglobin were used as a quinone compound and a heme containing protein, respectively. In each sample, indicated concentration of scavenger was added.
* Relative production was expressed using control sample which does not contain the scavenger, as 100%.

Table 5. Effect of superoxide dismutase, catalase, KCN, and NaN_3 on ethylene production

Sample	Concentration (mM)	Ethylene produced (pmole/system)	Relative production* (%)
Control		36,500	100
+ Superoxide dismutase		27,400	75.1
+ Catalase		2130	5.8
+ Superoxide dismutase + Catalase		1270	3.5
+ KCN	0.1	33,900	92.9
+ KCN	0.5	28,800	78.9
+ KCN	1.0	23,600	64.7
+ NaN_3	0.5	28,400	77.8
+ NaN_3	1.0	29,100	79.9
+ NaN_3	5.0	28,000	76.7

In each sample, vitamin K_3 and 9.2 μM methemoglobin were used as a quinone compound and a heme containing protein, respectively. The concentration of superoxide dismutase and catalase was 40 μg/ml.

* Relative production was expressed using control sample as 100%.

methemoglobin and hydroperoxide compound, was tested. As shown in Table 6, compared with the enzyme system, 67% and 18% of ethylene was produced in the system containing 2.0 mM and 0.2 mM hydrogen peroxide, respectively. When cumene hydroperoxide with a higher molecular weight and more hydrophobic hydroperoxide was used, the production of ethylene was decreased.

Fate of methemoglobin in the incubation system

The spectral changes of methemoglobin under ethylene producing conditions were measured (Fig. 2). Before the addition of NADPH-cytochrome P-450 reductase, the Soret peak at 405 nm, broad absorptions at 631 nm and 500 nm corresponding α and β bands, respectively, and absorption at 340 nm which originate from NADPH, were observed (spectrum a). After the addition of the reductase, methemoglobin spectrum was changed to oxyform with the absorption peaks at 577 nm, 541 nm and 414 nm, and the intensity of the spectrum was decreased with concomitant consumption of NADPH, probably by the destruction of heme structure (spectra b, c, d, and e). By the addition of superoxide dismutase to the above system, the shift of Soret peak to 406–407 nm and a slight formation of oxyform at α and β bands were observed, and the spectrum was decreased with consumption of NADPH (dotted spectra). By the addition of catalase, the spectrum was changed to oxyform and was stable at least for 20 min, although, NADPH was consumed (dashed spectrum). By the addition of both superoxide dismutase and catalase, the spectrum of methemoglobin was scarcely changed.

DISCUSSION

In the present study, we found that by the presence of metal chelates or heme-containing proteins, the easy and enhanced production of ethylene from methional occurs in the systems consisting of NADPH-cytochrome P-450 reductase and the reductase plus quinone compounds. The ethylene production from methional is generally explained as the action of hydroxyl radicals [19], and the actions by other radicals are reported [20]. In our present study, the possible causes of ethylene production can be explained as (a) hydroxyl radical action, (b) peroxidase activity of heme compound, and (c) other radical and/or highly oxidative species that are produced by heme compound. The ethylene production by metal chelates can be explained as the action of hydroxyl radicals which are produced by Fenton reaction [21]. In the case of heme containing proteins, George and Irvine [22], suggested the production of hydroxyl radicals by the reaction of methemoglobin and hydrogen peroxide. In addition to the results of the effects of radical scavengers (Table 4), we observed the occurrence of the hydroxylation of salicilate in the enzyme systems

Table 6. Ethylene production in enzyme system and hydroperoxide supported system

System	Concentration (mM)	Ethylene produced (pmole/system)	Relative production* (%)
Enzyme	—	43,500	100
Hydrogen peroxide	2.0	29,000	66.7
Hydrogen peroxide	0.2	7790	17.9
Cumene hydroperoxide	2.0	7770	17.9
Cumene hydroperoxide	0.2	992	2.3

In enzyme system, vitamin K_3 and 9.2 μM methemoglobin were used as a quinone compound and a heme containing protein, respectively. In hydroperoxide systems, 9.2 μM methemoglobin and indicated concentration of hydroperoxide compound were used.

* Relative production was expressed using enzyme system as 100%.

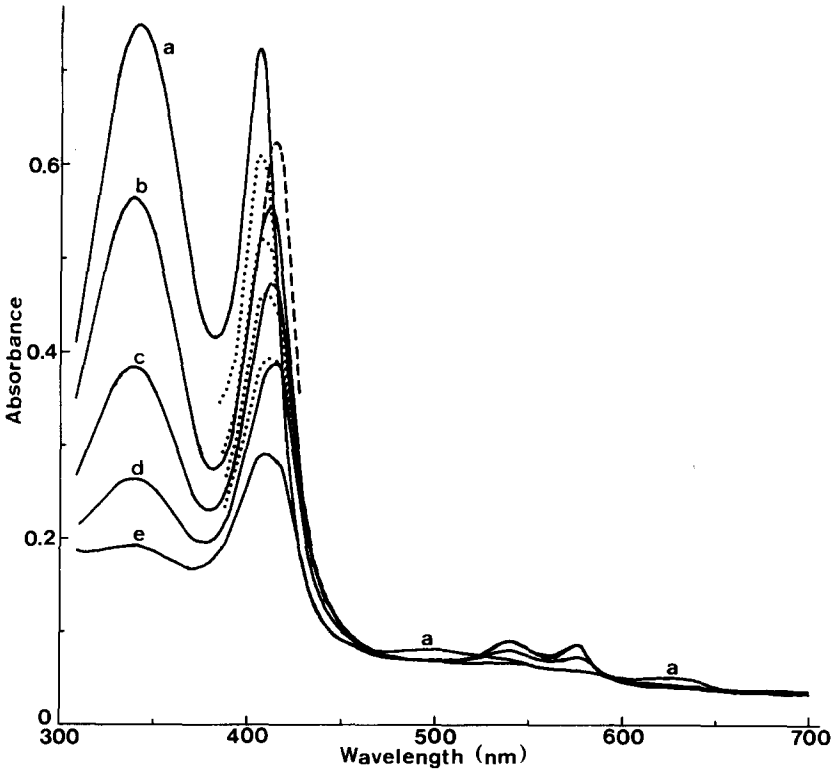

Fig. 2. Fate of methemoglobin in the incubation system. The incubation conditions are described in the text. Spectrum a is before the addition of the reductase, and spectra b to e are after the addition of the reductase, 1, 5, 10 and 20 min, respectively. Dotted spectra are in the presence of superoxide dismutase, and recorded after the addition of the reductase, 1, 5, 10 and 20 min, in the order of high intensity. Dashed spectrum is in the presence of catalase.

(unpublished result). Then, at present, some parts of enhanced production of ethylene from methional by heme and hemoproteins may be explained as the action of hydroxyl radicals and some parts may be explained as the peroxidase activity of heme, other radicals, and highly oxidative species produced by heme compound. In the previous studies, the generation of peroxy-free radicals is reported in the reaction mixture consisting of hydrogen peroxide and hematin [23], and Shiga and Imaizumi [24] reported no free hydroxyl radical generation in the hydroxylation model system which consist of methemoglobin, hydrogen peroxide and organic compound. At present, the possible reaction mechanisms for the hydroxyl radical generation may be described as followingly.

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

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

$$E_{red} + O_2 \rightarrow E_{ox} + O_2^{\cdot-} \quad (3)$$

$$Q^{\cdot-} + O_2 \rightarrow Q + O_2^{\cdot-} \quad (4)$$

$$O^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow Q + H_2O_2 \quad (5)$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (6)$$

$$Fe(III) + Q^{\cdot-} \rightarrow Fe(II) + Q \quad (7)$$

$$Fe(III) + O_2^{\cdot-} \rightarrow Fe(II) + O_2 \quad (8)$$

$$Q^{\cdot-} + H_2O_2 \rightarrow Q + OH\cdot + OH^- \quad (9)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH\cdot\ OH^- \quad (10)$$

Here, E_{ox}, E_{red}, Q, and $Q^{\cdot-}$ indicate the oxidized and reduced forms of the reductase, and the quinone and semiquinone forms of quinone compound, respectively.

In the case of anticancer quinone drugs, the intercalation mechanism is known as the general action in anthracycline antibiotics such as aclacinomycin A, adriamycin, and daunomycin [25, 26], and in the case of carbazilquinone and mitomycin C, the alkylation mechanism is proposed [27]. Further actions of these drugs which may include the oxygen related radicals are reported, such as the stimulation of lipid peroxidation [28, 29], strand scission of DNA [30–32], mutagenicity [33, 34], and toxicity [35–37]. As evidenced in this paper and a previous report [9], the generation of superoxide anion, hydrogen peroxide, and hydroxyl radicals are easily produced in the systems containing these drugs and NADPH-cytochrome P-450 reductase, and the ethylene production was enhanced by the presence of metal chelates or heme-containing proteins. Then, some of the above described biological effects may be the results of the action of hydroxyl radicals which are produced by the reaction mechanisms described above.

There are some reports which may involve hydroxyl radicals that are produced by the action of heme or heme-containing proteins. In the case of cytochrome P-450, ethanol oxidation [38–40], benzene activation [41], S-oxidation [42], and lipid peroxidation [43, 44], in the case of heme, hydroxylation of organic compounds [24], heme destruction [45, 46], and lipid peroxidation [47], and in the case of prostaglandin synthesis, prostaglandin synthase reaction [48], are reported. Many of these reactions are dependent on peroxide compound and heme or heme-containing proteins, and discussed with the possible involvement of hydroxyl radicals and highly oxidative species. As evidenced in Tables 5 and 6, the easy generation of ethylene in the reaction mixture consisting of NADPH-cytochrome P-450 reductase, quinone compound, and heme protein was simplified as the reaction of hydrogen peroxide and heme proteins. So, the same reactions described above may be the result of the action of hydroxyl radicals which are produced by the reaction scheme described above.

In the present study, we used NADPH-cytochrome P-450 reductase as the enzyme that interacts with a quinone compounds. As reported previously [2, 7, 49, 50], DT-diaphorase (EC 1.6.99.2), xanthine oxidase (EC 1.2.3.2), mitochondrial NADH dehydrogenase (EC 1.6.99.3), and nuclear NADPH cytochrome *c* reductase are also active on quinone compounds. Then, *in vivo* conditions, the enzymes which are active on quinone compounds in addition to NADPH-cytochrome P-450 reductase may contribute to the hydroxyl radical and other highly oxidative species generation.

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