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Effect of Estrogens on the Oxidative Damage Induced by Ferrylmyoglobin

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The effect of estrogens, including estrone (E_1), estradiol-17β (E_2), estriol (E_3) and 2-hydroxyestradiol (2-OH-E_2), on the oxidative damage induced by ferrylmyoglobin (ferrylMb) was investigated. These estrogens inhibited lipid peroxidation induced by ferrylMb. The ability of 2-OH-E_2 to inhibit lipid peroxidation was much greater than the other estrogens. Furthermore, 2-OH-E_2 trapped 2,2′-azobis-(2-amidinopropane)-dihydrochloride peroxyl radicals more rapidly, and among these estrogens only 2-OH-E_2 reacted with 2,2-diphenyl-1-picrylhydrazyl. These results suggest that the ability of 2-OH-E_2 to inhibit lipid peroxidation is becaCTuse it scavenges lipid peroxyl and carbon-centered radicals. Estrogens, except for 2-OH-E_2, partially prevented the inactivation of alcohol dehydrogenase (ADH) induced by ferrylMb. Of interest, however, the exposure of sulfhydryl (SH) enzymes to ferrylMb in the presence of 2-OH-E_2 dramatically increased the inhibition of the enzyme activity. Ascorbic acid (ASA) and reduced glutathione (GSH) strongly inhibited the inactivation of ADH induced by ferrylMb in the presence of 2-OH-E_2. During the reaction of ferrylMb with ASA or GSH in the presence of 2-OH-E_2, large amounts of oxymyoglobin were formed, suggesting the involvement of the semiquinone from 2-OH-E_2 in the reduction of metmyoglobin. Presumably, the semiquinone formed from 2-OH-E_2 oxidizes the SH group of enzymes to facilitate the rapid inactivation of the SH enzymes induced by ferrylMb. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Myoglobin (Mb) is a muscle protein containing heme that is involved in the intracellular transport and storage of oxygen. FerrylMb forms from the reaction of metMb with H_2O_2 [1]; it induces lipid peroxidation [2, 3] and oxidizes the sulfhydryl (SH) groups of proteins [4]. Galalis *et al.* [5] suggested that ferrylMb has a crucial role in ischemia and reperfusion cellular injury.

Lipid peroxidation of membranes has been implicated in numerous pathological and toxicological conditions [6]. The fact that estrogens inhibit membrane lipid peroxidation is well known [7, 8]. We demonstrated [9] that estradiol-17β (E_2) inhibits lipid peroxidation only at the initial stage, and that 2-hydroxyestradiol (2-OH-E_2) inhibits lipid peroxidation at the initial and the propagation stages. Ruiz-Larrea *et al.* [10] recently demonstrated that 2-OH-E_2 acts as a potent Fe^{3+} reductant during iron-catalyzed lipid peroxidation. However, the exact mechanisms by which lipid peroxidation is prevented has not been clarified. In addition, we previously indicated [4] that ferrylMb inactivates SH enzymes, such as alcohol dehydrogenase (ADH), creatine kinase (CK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In this study, we investigated the effect of estrogens on the oxidative damage induced by ferrylMb and demonstrated that estrogens strongly inhibit lipid peroxidation, but only 2-OH-E_2 among the estrogens dramatically facilitates the inactivation of the SH enzymes induced by ferrylMb.

MATERIALS AND METHODS

Material

Estrone (E_1), E_2, estriol (E_3) and 2-OH-E_2, metmyoglobin (from horse) and CK (EC.2.7.9.2) (from rabbit heart) were obtained from Sigma, St. Louis, MO, U.S.A., 2,2′-azobis-(2-amidinopropane)-dihydrochloride (AAPH) was from Wako Pure, Osaka, Japan, and *R*-phycoerythrin was from Molecular Probes, U.S.A. ADH (EC.1.1.1.1) (from yeast) was obtained from Oriental Yeast, Tokyo, Japan, and GAPDH (EC.1.2.1.12) (from rabbit muscle) was

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from Boehringer–Mannheim–Yamanouchi Tokyo, Japan. Other reagents were analytical-grade products from a commercial supplier.

Formation of ferrylmyoglobin

FerrylMb was prepared by incubating metMb with a tenfold molar excess of H_2O_2 in 10 mM Hepes, at pH 7.4 containing 0.15 M NaCl at 37°C for 5 min. After the incubation, catalase (20 nM) was added to the reaction mixture to remove excess H_2O_2.

Lipid peroxidation

Rat liver microsomes were prepared by the method of Pederson and Aust [11]. The formation of thiobarbituric acid-reactive substances (TBARS) was determined by the method of Buege and Aust [12] with minor modifications. The reaction mixture contained microsomes (0.1 mg protein/ml) and 0.15 M NaCl in 1.0 ml of 10 mM Hepes buffer at pH 7.4 unless otherwise noted. The peroxidation reaction was started by adding ferrylMb (100 μM) and was stopped by adding 1.0 ml of 30% trichloroacetic acid. After centrifuging for 10 min at 1500 × g, the precipitate was discarded. The TBARS formation was assayed by measuring the absorption at 535 nm after heating for 30 min at 100°C, and the absorbance was expressed as nmol TBARS/mg protein, using $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [13]. The protein was measured by the bicinchoninic acid assay using bovine serum albumin as a standard [14].

Fluorescence of R-phycoerythrin

The loss of fluorescence of *R*-phycoerythrin was measured at 540 nm excitation and 575 nm emission [15]. The reaction mixture contained 17 nM *R*-phycoerythrin, 40 mM AAPH and estrogens in 75 mM phosphate buffer at pH 7.0.

Determination of enzyme activities

The ADH (2.9 μM), CK (2.5 μM) and GAPDH (2.8 μM) were incubated with ferrylMb (100 μM). The GAPDH activity was measured by the method of Prinsz *et al.* [16], the ADH activity was determined by the method of Bonnischen and Brink [17], and the CK activity was measured at 25°C using a Wako Pure Chemical Industries creatine kinase kit.

RESULTS

Inhibition of lipid peroxidation by estrogens

Figure 1 shows that the estrogens, including E_1, E_2, E_3 and 2-OH-E_2, inhibited the lipid peroxidation induced by ferrylMb in a concentration dependent manner. Among these estrogens, 2-OH-E_2 was a very effective inhibitor of the lipid peroxidation induced by ferrylMb. The IC_{50} of 2-OH-E_2, E_2, E_3 and E_1 was about 0.29, 18, 17 and 52 μM, respectively.

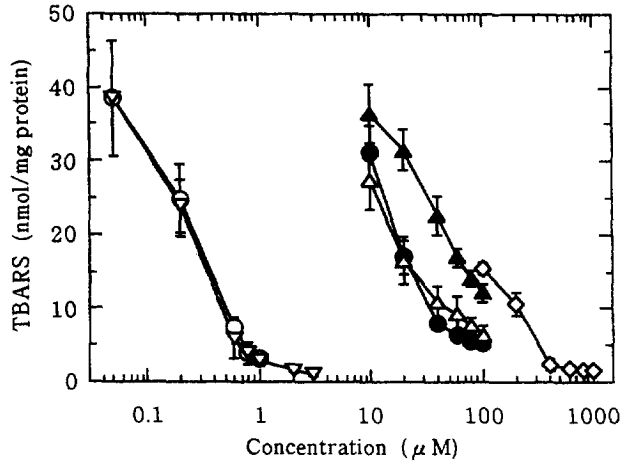

Fig. 1. Inhibitory effect of estrogens on lipid peroxidation induced by ferrylMb. After incubation for 60 min at 37°C, lipid peroxidation was measured as described in Section 2. Estrogens were added to the reaction mixture before the start of the reaction. Each point represents the mean ± SD of four experiments. (○) 2-OH-E_2; (△) E_1; (•) E_2; (▲) E_3; (▽) BHT and (◇) α-tocopherol

Thus, the IC_{50} of 2-OH-E_2 was low at about a two order of magnitude over that of E_1, E_2 and E_3. This value of 2-OH-E_2 was compared with that of other known antioxidants, including butylated hydroxytoluene (BHT) and α-tocopherol. Among these antioxidants, BHT had a very powerful inhibitory activity to the ferrylMb-induced lipid peroxidation. The IC_{50} of BHT was about 0.3 μM. This value of BHT was the same as that of 2-OH-E_2. Evidently, 2-OH-E_2 was the most powerful inhibitor of ferrylMb-induced lipid peroxidation.

The mechanism of lipid peroxidation inhibited by estrogens was examined by measuring the decrease in ferrylMb. Figure 2 shows that adding 2-OH-E_2 at

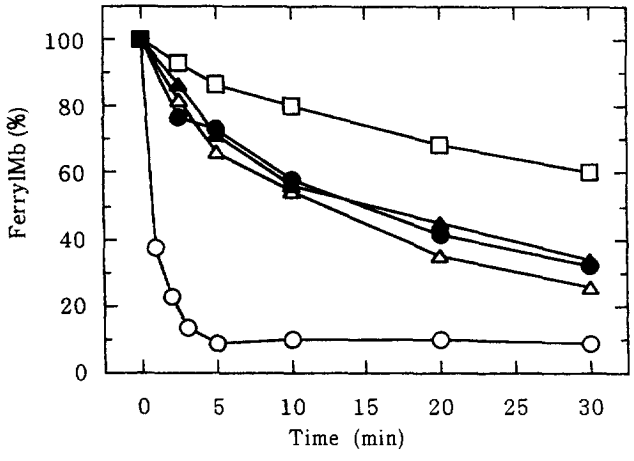

Fig. 2. Decrease in ferrylMb induced by estrogens. The reaction mixture contains 100 μM ferrylMb and 0.15 M NaCl in 10 mM HEPES at pH 7.4. Estrogens (10 μM) were added to the reaction mixture before the start of the reaction. Each point represents the mean of three experiments. The variation was less than 10%. (□) no addition; (○) 2-OH-E_2; (△) E_1; (•) E_2 and (▲) E_3

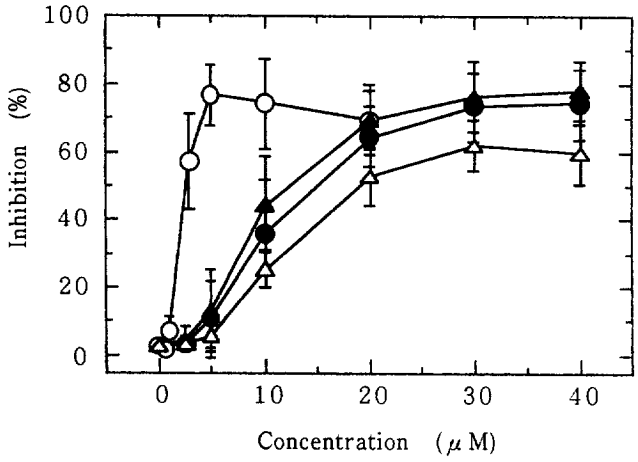

Fig. 3. Inhibition of fluorescent decomposition of *R*-phycoerythrin by estrogens. Experimental conditions were described in Section 2. Each point represents the mean ± SD of five experiments. (○) 2-OH-E_2; (△) E_1; (•) E_2 and (▲) E_3

10 μM immediately converted ferrylMb to metMb; after 2 min, the conversion of ferrylMb to metMb reached about 80%. In contrast, the other estrogens slowly promoted the reduction of ferrylMb, with conversion to metMb reaching about 70–80% after 30 min. Evidently, 2-OH-E_2 was much more effective in converting ferrylMb to metMb than the other estrogens.

Lipid peroxidation is induced by various free radicals [18]. We examined the ability of estrogens to scavenge peroxyl radicals and 2,2-diphenyl-1-picrylhydrazyl (DPPH). In the presence of O_2, AAPH decomposed to the AAPH peroxy radical, which induces lipid peroxidation [19]. Glazer [15] assessed the ability of various compounds to react with peroxyl radicals by monitoring the degradation of *R*-phycoerythrin fluorescence. We also tested if estrogens reacted with AAPH peroxyl radicals. Figure 3 shows that estrogens prevented the degradation of *R*-phycoerythrin fluorescence in a concentration dependent manner. The ability of 2-OH-E_2 to scavenge AAPH peroxyl radicals was about five to six times greater compared with the other estrogens. In addition, the high reactivity of 2-OH-E_2 with ferrylMb may also lead to inhibiting lipid peroxidation. Only 2-OH-E_2 among estrogens effectively decolored the DPPH solution, indicating that it reacts with DPPH (Table 1).

Table 1. Scavenging of DPPH by estrogens

Additions	Concentrations (μM)	DPPH-ΔOD 517 nm/min
E_1	100	0.003
E_2	100	0.003
2-OH-E_2	10	0.621
E_3	100	0.003

Estrogens were added to ethanolic DPPH (100 μM) solution. The decrease in DPPH absorption at 517 nm after 1 min was measured. Each value represents the mean of three experiments. The variation was less than 10%.

These results suggest that the inhibition of lipid peroxidation may be due to the activity of 2-OH-E_2 scavenging peroxyl and DPPH radicals.

Enhancement of enzyme inactivation by estrogens

FerrylMb oxidizes SH groups in proteins to inactivate SH enzymes [4]. Figure 4 shows the effect of estrogens on the ADH inactivation induced by ferrylMb. The ADH activity was inhibited by about 25% by exposure to ferrylMb for 30 min. A partial inhibition by E_2 was observed in the ADH inactivation induced by ferrylMb. The same results were obtained by E_1 and E_3 (data not shown). Of interest, when ADH was exposed to ferrylMb in the presence of 2-OH-E_2, inactivation of ADH was dramatically enhanced. When 2-OH-E_2 was incubated with ADH for 30 min in the absence of ferrylMb, the enzyme activity was inhibited by only about 16% (data not shown). Evidently, 2-OH-E_2 facilitated ADH inactivation induced by ferrylMb. We further examined the ADH inactivation induced by ferrylMb in the presence of 2-OH-E_2. Figure 5shows that the facilitation of ADH inactivation depended on the concentration of 2-OH-E_2. When the molar ratio of 2-OH-E_2 to ADH subunit was about 1:1, the enzyme activity was inhibited by about 90%. The enzyme has eight free SH groups per subunit and is inhibited by thiol reagent [20]. Two of the cysteine residue are especially reactive toward chemical modification [20]. ADH inactivation may be due to modification of one reactive residue of cysteine per subunit.

We speculated that the facilitation of ADH inactivation may occur through a one electron transfer mechanism, because ferrylMb was rapidly reduced to metMb in the presence of 2-OH-E_2. If this is true,

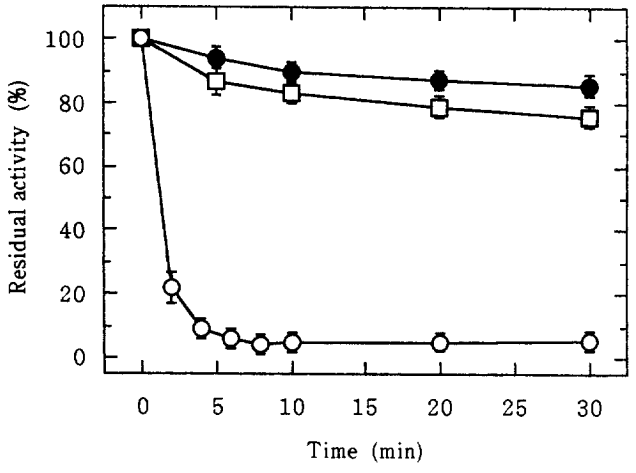

Fig. 4. Effect of estrogens on the inactivation of ADH induced by ferrylMb. The reaction mixture contains 100 μM ferrylMb, 2.9 μM ADH and 0.15 M NaCl in 10 mM HEPES buffer at pH 7.4. Estrogens (10 μM) were added to the reaction mixture before the start of the reaction. Each point represents the mean ± SD of five experiments. (□) no addition; (○) 2-OH-E_2 and (•) E_2

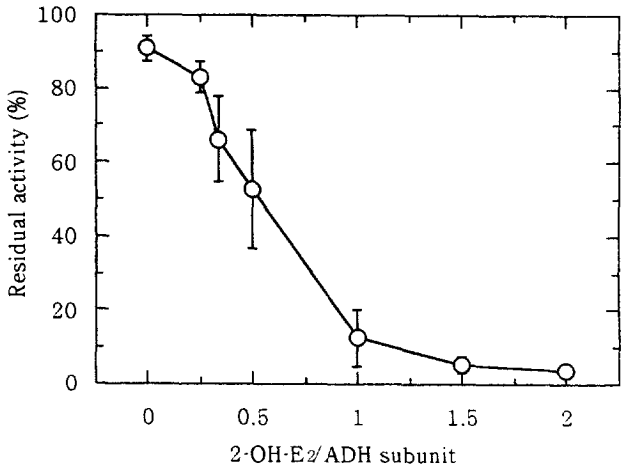

Fig. 5. Effect of 2-OH-E_2 concentrations on ADH inactivation induced by ferrylMb. The reaction mixture was incubated for 10 min at 37°C. Other experimental conditions were the same as described in Fig. 4. Each point represents the mean ± SD of five experiments

the reaction of ferrylMb with 2-OH-E_2 would produce a semiquinone radical of 2-OH-E_2. Ascorbic acid (ASA) and reduced glutathione (GSH) can scavenge the semiquinone from catechol estrogens [21]. Figure 6 shows that the inactivation of ADH was blocked by ASA or GSH in a concentration dependent manner, whereas ASA and GSH had no effect in preventing ADH inactivation until 20 μM. Furthermore, ASA and GSH produced a rapid increase in the absorption at around 580 nm during the reaction of ferrylMb with 2-OH-E_2, indicating that a large amount of oxyMb was formed from metMb in the presence of ASA and GSH (Fig. 7). Adding ASA and GSH to metMb solution very slightly converted the ferrylMb to oxyMb. These results suggest that the 2-OH-E_2 semiquinone formed

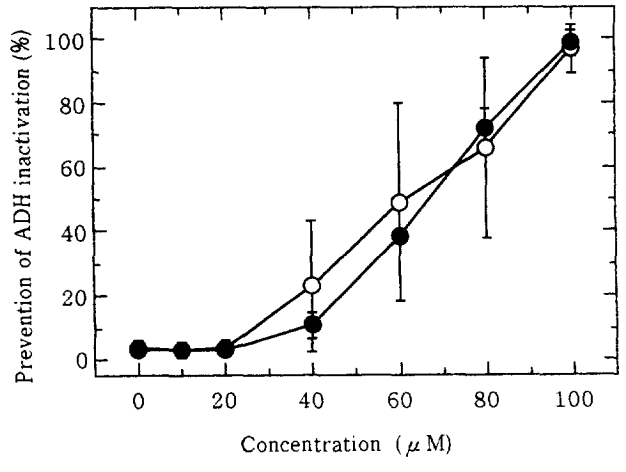

Fig. 6. Prevention of ferrylMb-induced ADH inactivation by ASA and GSH. ASA and GSH added to the reaction mixture consisted of 100 μM ferrylMb, 0.15 M NaCl and 10 μM 2-OH-E_2 in 10 mM HEPES buffer at pH 7.4. After exposure for 10 min, the ADH activity was measured. Each point represents the mean ± SD of five experiments

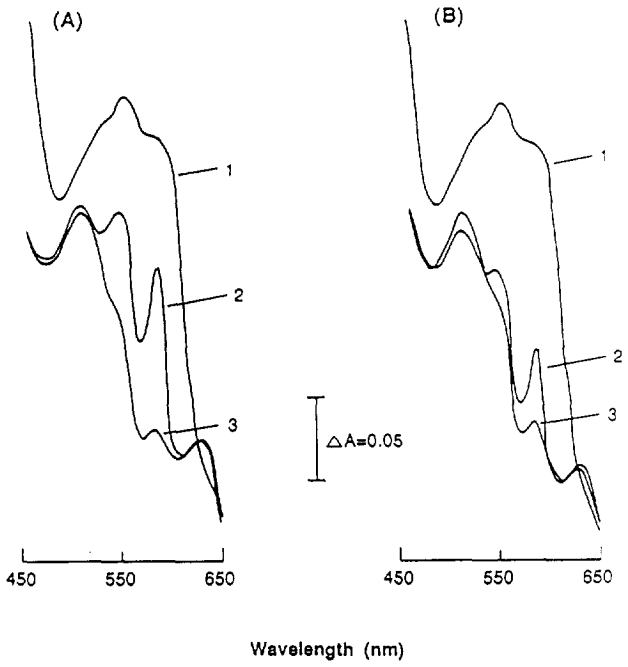

Fig. 7. Spectral change of ferrylMb induced by ASA (A) and GSH (B). Experimental conditions were the same as described in Fig. 6. After adding ASA (A) or GSH (B) for 10 min, the spectral changes of ferrylMb were recorded. (A) Curve 1, ferrylMb; curve 2, + ASA (100 μM) in the presence of 2-OH-E_2; curve 3, + ASA (100μM) in the absence of 2-OH-E_2. (B) Curve 1, ferrylMb; curve 2, + GSH (100 μM) in the presence of 2-OH-E_2; curve 3, + GSH (100 μM) in the absence of 2-OH-E_2

by one electron transfer from 2-OH-E_2 participated in forming oxyMb and caused the facilitation of the ADH inactivation induced by ferrylMb.

Also, ferrylMb in the presence of 2-OH-E_2 caused the rapid inactivation of other SH enzymes. FerrylMb inactivated CK and GAPDH about 25 and 70%, respectively (Table 2). E_1, E_2 and E_3 partially prevented the inactivation of SH enzymes induced by ferrylMb. In contrast, the exposure of SH enzymes to ferrylMb in the presence of 2-OH-E_2 caused an increase in the inhibition of the activity of the enzymes. When 2-OH-E_2 was incubated with

Table 2. Effect of estrogens on the inactivation of CK and GAPDH induced by ferrylMb

Addition	Residual activity (%)	
	CK	GAPDH
None	76.8 ± 10.4	33.1 ± 6.2
E_1	88.8 ± 6.0	54.1 ± 15.5
E_2	84.0 ± 7.5	44.7 ± 10.6
2-OH-E_2	17.5 ± 3.2	1.9 ± 0.5
E_3	86.3 ± 5.6	48.3 ± 9.2

The reaction mixture contains 100 μM ferrylMb, 0.15 M NaCl and 2.5 μM CK or 2.8 μM GAPDH in 10 mM HEPES at pH 7.4. Estrogens (10 μM) were added to the reaction mixture before the start of the reaction. CK was exposed to ferrylMb for 60 min at 37°C and GAPDH was exposed for 120 min at 25°C. Each value represents the mean ± SD of five experiments.

GAPDH, the activity of the enzyme was inhibited by only about 10%. These results indicate that 2-OH-E_2 facilitates the inactivation of SH enzymes induced by ferrylMb.

DISCUSSION

This study demonstrated that 2-OH-E_2 among estrogens strongly inhibits lipid peroxidation, but dramatically facilitates the inactivation of SH enzymes induced by ferrylMb. Estrogens are excellent inhibitors of NADPH-ADP-Fe^{3+}-dependent microsomal lipid peroxidation [9, 10, 22, 23]. They also strongly inhibited lipid peroxidation induced by ferrylMb. In this study, only 2-OH-E_2 among the estrogens caused a rapid conversion of ferrylMb to metMb. We previously reported that E_2 inhibited microsomal lipid peroxidation at the initial stage, but inhibited it only a little at the propagation stage, whereas 2-OH-E_2 produced a strong activity to inhibit the lipid peroxidation at both stages [9]. Lipid peroxyl radicals predominantly increases the lipid peroxidation reaction at the propagation stage [18]. In this study, 2-OH-E_2 scavenged AAPH peroxyl radicals more rapidly than the other estrogens. However, the reaction of 2-OH-E_2 with peroxyl radicals was unlikely to have a crucial role in the inhibition of lipid peroxidation, because the IC_{50} of 2-OH-E_2 was low at about two orders of magnitude over that of other estrogens. The 2-OH-E_2 seems to scavenge carbon-centered radicals at the initial stage to show a strong inhibiting activity of lipid peroxidation, because only 2-OH-E_2 among estrogens reacted with DPPH. Presumably, only 2-OH-E_2 among the estrogens is available in the defense system against lipid peroxidation induced by ferrylMb *in vivo*.

The facilitation of ADH inactivation by 2-OH-E_2 should be caused through a one electron reduction of ferrylMb, because 2-OH-E_2 markedly enhanced the conversion of ferrylMb to metMb. However, 2-OH-E_2 would be oxidized to the semiquinone radical. We, however, failed to detect the semiquinone of 2-OH-E_2 by electron spin resonance, because free radicals have a very short lifetime [21]. FerrylMb is an analogue of the peroxidase compound I or II [1]. Kalyanaraman *et al.* [24] demonstrated that horse radish peroxidase-H_2O_2-dependent oxidation of catechol and catechol estrogens forms *o*-semiquinones to act as obligate intermediates. Both ASA and GSH prevented ADH inactivation induced by ferrylMb in the presence of 2-OH-E_2. In the presence of ASA and GSH, formation of oxyMb markedly increased, indicating that metMb was further reduced to oxyMb. In the absence of ASA or GSH, metMb was not reduced, indicating that the formation of oxyMb was not due to direct reduction of metMb by the semiquinone, but by the ascorbyl or glutathionyl radical formed from the reaction with the semiquinone. Presumably, the semiquinone participates in oxidizing the SH group of enzymes to facilitate the inactivation of SH enzymes induced by ferrylMb. Although data are not shown, catechol and hydroquinone also promoted the reduction of ferrylMb to metMb to inhibit lipid peroxidation and dramatically facilitated the inactivation of SH enzymes induced by ferrylMb. However, resorcin had no effect. From these results, we speculate that two phenolic hydroxy groups at the ortho or para positions may be important to inhibit lipid peroxidation or to facilitate inactivation of SH enzymes induced by ferrylMb. Presumably, *o*- or *p*-semiquinones are involved in the oxidative damage.

Talcott *et al.* [25] indicated that semiquinone from menadione traps free radicals to inhibit lipid peroxidation. Our data suggest that not only 2-OH-E_2, but also the semiquinone, acts as an active antioxidant for lipid peroxidation. The semiquinone from 2-OH-E_2 may trap lipid carbon-centered radicals involved in lipid peroxidation at the initial stage. However, 2-OH-E_2 acted as a prooxidant for the SH groups of enzymes.

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