On the Inhibitory Effect of C₁₇-Sulfoconjugated Catechol Estrogens upon Lipid Peroxidation of Rat Liver Microsomes¹⁾

Kaori TAKANASHI,* Kazuhiro WATANABE, and Itsuo YOSHIZAWA

Hokkaido College of Pharmacy, 7-1, Katsuraoka-cho, Otaru, Hokkaido 047-02, Japan.

Received February 20, 1995; accepted May 29, 1995

The antioxidant effect of C₁₇-sulfoconjugated catechol estrogens was examined under ascorbic acid- or NADPH-dependent lipid peroxidation in rat liver microsomes and compared with that of various estrogens and α -tocopherol. Among the estrogens tested, a free catechol estrogen such as 4-hydroxyestradiol showed the strongest effect, followed by 2-hydroxyestradiol, 2-methoxyestradiol and estradiol. Next to these steroids, 2-hydroxyestradiol 17-sulfate, followed by 4-methoxyestradiol, 4-hydroxyestradiol 17-sulfate and estrone also showed a strong inhibitory effect, which was greater than that of α -tocopherol. Among the C₁₇-sulfates, the guaiacols (2- and 4-methoxyestradiol 17-sulfate) showed a slightly lower effect than α -tocopherol, but estradiol 17-sulfate had almost no effect. The antioxidant activity observed in phenolic or guaiacol steroids was considered to be attributed to the catechols produced by their 2- (or 4-)hydroxylation or their *O*-demethylation, respectively, during the incubation. This was confirmed by identification of the catechols produced from phenolic or guaiacol estrogens and even from the estrogen C₃-sulfates. The mechanism of the inhibition by catechols on lipid peroxidation was speculated to involve their activity as radical scavengers, because of their strong reducing activity for 1,1-diphenyl-2-picrylhydrazyl.

The above results suggest that C₁₇-sulfoconjugated catechol estrogens (2- and 4-hydroxyestradiol 17-sulfate), although with slightly lower activity than their free catechols, are promising endogenous antioxidants. The physiological role of these estrogen conjugates during pregnancy is discussed.

Key words 2-hydroxyestradiol 17-sulfate; 4-hydroxyestradiol 17-sulfate; estrogen antioxidant; lipid peroxidation; catechol estrogen

Preeclampsia, a serious disease during human pregnancy, particularly at latter term, is considered to be caused by accumulation of lipid peroxides produced in placenta.²⁾ The placenta, in addition, has the opposite physiological activities, the production of antioxidants which decompose the placental peroxides.²⁾ Generally, a healthy pregnancy is maintained through the quantitative balance between such oxidants and antioxidants. The disintegration of this equilibrium state might cause preeclampsia.^{2,3)} Until now, it is unknown what kind of substances are placental antioxidant(s). Catechol estrogen (free form) has been considered as one of the most likely candidates acting as an endogenous antioxidant.⁴⁾ However, we suspect the effect of free catechol estrogen, because of its low plasma concentration and rapid metabolic clearance rate in rats.⁵⁾

In a series of our papers on the metabolism of estradiol 17-sulfate (E₂-17-S), we have reported the following results: (1) Plasma circulating catechol estrogen was essentially 2-hydroxyestradiol 17-sulfate (2-OH-E₂-17-S).⁶⁾ (2) The presence of 2-OH-E₂-17-S was confirmed in human serum⁷⁾ and urine.⁸⁾ (3) Human placental microsomes have the ability to convert E₂-17-S to 2-OH-E₂-17-S, accompanied by 4-hydroxyestradiol 17-sulfate (4-OH-E₂-17-S).⁹⁾ (4) Serum E₂-17-S and lipid peroxide values in late pregnancy were shown to be in a negative correlation-ship.¹⁰⁾

Judging from these results and also from the fact that free catechol estrogens have a strong antioxidant effect,¹¹⁾ we have proposed that 2- and 4-OH-E₂-17-S are considered as the most possible candidates for endogenous antioxidants during pregnancy.⁹⁾

In this study, we have examined the antioxidant effect of C₁₇-sulfoconjugated catechol estrogen together with

other steroids including free catechol estrogens. The inhibitory activity of free and C₁₇-sulfoconjugated guaiacol estrogens was also demonstrated.

MATERIALS AND METHODS

Steroids and Chemicals The following steroids were synthesized in this laboratory by known methods; 2-hydroxyestradiol (2-OH-E₂),¹²⁾ 4-hydroxyestradiol (4-OH-E₂),¹²⁾ 2-methoxyestradiol (2-OMe-E₂),¹³⁾ 4-methoxyestradiol (4-OMe-E₂),¹³⁾ E₂-17-S,¹³⁾ 2- and 4-OH-E₂-17-S,¹³⁾ 2-methoxyestradiol 17-sulfate (2-OMe-E₂-17-S),¹³⁾ 4-methoxyestradiol 17-sulfate (4-OMe-E₂-17-S),¹³⁾ 6 α -hydroxyestradiol (6 α -OH-E₂),¹²⁾ 6 β -hydroxyestradiol (6 β -OH-E₂),¹²⁾ and 7 α -hydroxyestradiol (7 α -OH-E₂).¹²⁾ Estrone (E₁), estradiol (E₂), estriol (E₃), estrone sulfate (E₁-S) and estradiol 3-sulfate (E₂-3-S) were purchased from Steraloids (N. H., U.S.A.). The purity of E₁-S and E₂-3-S was checked by HPLC prior to use. NADPH and adenosine 5'-diphosphate (ADP) were obtained from Oriental Yeast Inc. (Osaka, Japan) and 2-thiobarbituric acid (TBA) and α -tocopherol (α -Toc) from Merck (Darmstadt, Germany). The Determiner LPO-kit, Sep-Pak C₁₈ cartridges and Column Guard were purchased from Kyowa Medics (Tokyo, Japan), Waters Assoc. (Milford, Mass, U.S.A.) and Millipore Co. (Bedford, Mass, U.S.A.), respectively. All other reagents including ascorbic acid (AsA) and solvents were of reagent grade.

Animals Wistar strain male rats (body weights: 200—250 g) were used, and hepatic microsomes were prepared by the method of the previous paper.¹⁴⁾

Conditions of Lipid Peroxidation¹⁵⁾ AsA-Dependent Microsomal Lipid Peroxidation: Ice-cooled reaction vessels contained microsomal protein (0.5 mg/ml), AsA

* To whom correspondence should be addressed.

(0.5 mM) and estrogens or α -Toc (0–25 μ M) which were diluted with 50 mM Tris-HCl buffer solution (pH 7.4) to 2 ml as a final volume and incubated for 30 min at 37 °C under aerobic conditions.

NADPH-Dependent Microsomal Lipid Peroxidation: Ice-cooled reaction vessels contained microsomal protein (0.5 mg/ml), NADPH (0.1 mM), ADP-Fe³⁺ complex (0.3 mM ADP–0.015 mM FeCl₃) and estrogens or α -Toc (0–50 μ M) which were diluted with 50 mM Tris-HCl buffer solution (pH 7.4) to 2 ml as a final volume and incubated for 10 min at 37 °C under aerobic conditions.

Determination of Lipid Peroxides TBA Method: Lipid peroxides were determined by the method of Buege and Aust.¹⁶⁾ To 1 ml of the reaction mixture, 30 μ l of 2% butylated hydroxytoluene and 2 ml of TCA-TBA-HCl reagent (15% trichloroacetic acid; 0.375% TBA; 0.25 N HCl) were added. The mixture was heated for 15 min in boiling water. After being cooled in ice water, the mixture was centrifuged for 10 min at 1500 $\times g$. The absorbance of the supernatant at 535 nm was determined (model 200-20, Hitachi, Tokyo) against a blank that contained all reagents except estrogens or α -Toc.

Hemoglobin-Methylene Blue (Hb-Mb) Method¹⁷⁾: Lipid peroxide was measured using a Determiner LPO-kit.

Confirmation of the Incubation Product(s) Incubations under the same conditions as AsA- and NADPH-dependent lipid peroxidation were carried out on E₂, 2-OMe-E₂, E₂-17-S, 2-OMe-E₂-17-S, E₁-S and E₂-3-S at their concentration of 25 μ M.

After AsA (1 mg) was added, the reaction vessels were heated in boiling water for 1 min. The mixture was centrifuged at 1500 $\times g$ for 10 min. The supernatant diluted with distilled water (1 ml) was passed through Sep-Pak C₁₈ cartridge. After washing the cartridge with 2 ml of distilled water, the steroid-containing fraction was obtained by elution with methanol (4 ml). The eluate was evaporated under a nitrogen stream at 40 °C to give the residue, which was dissolved in methanol (200 μ l). The methanolic solution, after being passed once through a Column Guard, was subjected to HPLC. The steroidal by-products were determined by the absolute calibration method established preliminarily for the corresponding steroids.

HPLC HPLC was carried out in a model CCPE equipped with an EC-8011 electrochemical detector (Tosoh, Tokyo) at 0.9 V vs. Ag/AgCl reference electrode or UV detector UV-8011 (Tosoh) at 280 nm. A column packed with ODS-80T_MCTR (100 $\times$ 4.6 mm i.d., 5 μ m, Tosoh) was used and maintained at 40 °C in a column heater. The following solvent systems were used as mobile phases; system A: 0.5% NH₄H₂PO₄ (pH 3.0)–methanol (50:50, v/v) and system B: 0.5% NH₄H₂PO₄ (pH 3.0)–methanol (40:60, v/v).

Reducing Activity for 1,1-Diphenyl-2-picrylhydrazyl (DPPH) The reducing activity of the steroids for DPPH was measured by the method of Uchiyama *et al.*¹⁸⁾ To a mixture of DPPH (100 μ M) in 60% aqueous ethanol and 40 mM acetic acid buffer solution (pH 5.5), estrogen or α -Toc (10–50 μ M) was added to 5 ml as a final volume, and the mixture was allowed to stand for 30 min at 28 °C. After cooling the reaction mixture, the absorbance at

517 nm was determined.

RESULTS

Comparison of the Conditions between AsA- and NADPH-Dependent Lipid Peroxidation for Antioxidant Effect of Various Estrogens The antioxidant effect of various estrogens and α -Toc was determined under AsA- and NADPH-dependent lipid peroxidation in rat liver microsomes. The results obtained by both systems are compared in Table 1, where the TBA values are expressed as the steroid concentration (μ M) for 50% inhibition (IC₅₀).

The steroid group showing the strongest inhibitory effect in AsA-dependent system (a) was 2-OH-E₂, 4-OH-E₂ and 2-OMe-E₂, the effect of which exceeded that of α -Toc. Next to them, 2-OH-E₂-17-S, 4-OH-E₂-17-S and 4-OMe-E₂ showed a strong effect, followed by 2-OMe-E₂-17-S, 4-OMe-E₂-17-S, E₂ and E₁. A weak effect was observed in such steroids as E₃, E₂-17-S, E₂-3-S and E₁-S. No activity was detected in the B-ring hydroxylated estrogens, 6 α -OH-E₂, 6 β -OH-E₂ and 7 α -OH-E₂.

The antioxidant effect using the same steroids was tested under NADPH-dependent lipid peroxidation (b), the results of which are shown in Table 1(b).

Among the steroids tested, E₁, 2-OH-E₂, 4-OH-E₂ and 2-OH-E₂-17-S exhibited the strongest inhibitory effect, which by far exceeded that of α -Toc. Next to these steroids, E₂, 2-OMe-E₂ and 4-OH-E₂-17-S showed also a stronger effect than α -Toc. The effects of E₃, 2-OMe-E₂-17-S, 4-OMe-E₂-17-S, E₂-3-S and E₁-S were almost of the same degree as that of α -Toc. No inhibitory effect was recognized in other steroids.

Measurement of the Inhibition Effect Inhibitions (%) of twelve kinds of estrogens, which showed an effect equal to or stronger than that of α -Toc, were determined by the Hb-Mb method under NADPH-dependent lipid peroxida-

Table 1. IC₅₀ Values of α -Tocopherol and Various Estrogens on Microsomal AsA(a)- and NADPH(b)-Dependent Lipid Peroxidation in Rat Liver (TBA Method)

Compound	IC ₅₀ (μ M)	
	a	b
α -Toc	2.7	63
E ₁	32	2.2
E ₂	10	6.6
E ₃	56	100
2-OH-E ₂	1.3	2.2
4-OH-E ₂	0.5	2.1
2-OMe-E ₂	2.0	6.2
4-OMe-E ₂	8.3	14
6 α -OH-E ₂	—	>100
6 β -OH-E ₂	—	>100
7 α -OH-E ₂	—	>100
E ₂ -17-S	100	>100
2-OH-E ₂ -17-S	6.6	2.2
4-OH-E ₂ -17-S	7.9	6.5
2-OMe-E ₂ -17-S	30	67
4-OMe-E ₂ -17-S	21	38
E ₂ -3-S	>100	49
E ₁ -S	>100	44

Each figure represents mean value (n = 5).

tion (Table 2). Among the steroids tested, 4-OH-E₂ was the strongest antioxidant. Next to it, 2-OH-E₂, 2-OMe-E₂ and E₂ also exhibited strong effects, which were greater than that of α -Toc. Two C₁₇-sulfoconjugated catechol estrogens had 2–5 times stronger inhibition than that of α -Toc. The activities of E₁-S, 2- and 4-OMe-E₂-17-S were less than that of α -Toc. E₂-17-S showed the lowest inhibitory activity.

Confirmation of the Incubation Product(s) Considering the relationships between the structure of the steroids tested and their antioxidant effect, it was estimated that the inhibitory activity of each steroid includes the effect by antioxidant by-product(s) produced nonenzymatically or enzymatically during the incubation. To ascertain this, each steroid was incubated under the same conditions as AsA- or NADPH-dependent lipid peroxidation, and the reaction mixtures obtained were checked by HPLC. As was expected, peak(s) corresponding to the by-product appeared in the chromatogram of each steroid. The amount of the important by-product(s) was determined quantitatively and is shown in Table 3.

Under AsA-dependent lipid peroxidation, formation of 2-OH-E₂ (0.2%) from E₂ was confirmed. Hydroxylation of E₂-17-S at C₂ was negligible. As for guaiacols, *O*-demethylation of 2-OMe-E₂ and 2-OMe-E₂-17-S occurred in the range of *ca.* 0.5%. In the case of estrogen 3-sulfates

(E₂-3-S and E₁-S), almost of them were hydrolyzed to their original phenols, E₂ and E₁, respectively, a part of which might be converted further to their catechols. Formation of E₂ from E₁-S as well as that of E₁ from E₂-3-S was observed at a yield of 5%. No hydrolysis of the sulfoconjugated group at C₁₇ in E₂-17-S and 2-OMe-E₂-17-S occurred.

A similar side reaction was also observed under NADPH-dependent lipid peroxidation and the yields of by-products were greater than those under AsA-dependent condition. About 12% of E₂ was converted to 2-OH-E₂ and even the sulfate (E₂-17-S) to 2-OH-E₂-17-S (0.1%), which is ten times higher than the yield under AsA-dependent condition. Considerable *O*-demethylation of guaiacols was observed: *O*-demethylation of 2-OMe-E₂ to 2-OH-E₂ occurred to about 10 fold greater degree than in the case under AsA-dependent condition and that of 2-OMe-E₂-17-S to the catechol to a degree similar to that under AsA-dependent condition. Although hydrolysis of the C₃-sulfates occurred in a range almost equal to that under AsA-dependent condition, the amount of catechols (2-OH-E₁ and 2-OH-E₂) produced from E₁ and E₂, respectively, was greater than that under AsA-dependent condition. The formation of E₂ from E₁-S and that of E₁ from E₂-3-S were 10% and 5%, respectively. In this case too, no hydrolysis of C₁₇-sulfates was observed.

DPPH Reducing Activity of Various Estrogens To investigate the inhibition mechanism for peroxidation by steroidal antioxidants, their reducing activity for DPPH was determined and compared with that of α -Toc, the results of which are shown in Table 4. Both catechol or guaiacol estrogens, independent of the presence or absence of the C₁₇-conjugate group, decreased the absorbance of DPPH at 517 nm in proportion to the added amount of each steroid. Among the steroids tested, estrogen catechols reduced DPPH effectively to almost the same degree as α -Toc. Other estrogens showed no reducing activity.

DISCUSSION

Antioxidant Effect of C₁₇-Sulfoconjugated Estrogens Lipid peroxidation of rat liver microsomes is known to occur not only in the NADPH-dependent but also in the AsA-dependent reaction system,¹⁵⁾ each of which is

Table 2. IC₅₀ Values of α -Tocopherol and Various Estrogens in Rat Liver Microsomal NADPH-Dependent Lipid Peroxidation (Hb-Mb Method)

Compound	IC ₅₀ (μ M)
4-OH-E ₂	0.7
2-OH-E ₂	1.2
2-OMe-E ₂	1.3
E ₂	1.7
2-OH-E ₂ -17-S	2.2
4-OMe-E ₂	4.8
4-OH-E ₂ -17-S	5.3
E ₁	8.4
α -Toc	10
E ₁ -S	15
2-OMe-E ₂ -17-S	18
4-OMe-E ₂ -17-S	26
E ₂ -17-S	49

Each figure represents mean value (*n* = 3).

Table 3. Amount of the Main By-products Formed from Various Estrogens in AsA(a)- and NADPH(b)-Dependent Lipid Peroxidation in Rat Liver Microsomes

Estrogen	By-product (yield, %)											
	E ₁		2-OH-E ₁		E ₂		2-OH-E ₂		2-OMe-E ₂		2-OH-E ₂ -17-S	
	a	b	a	b	a	b	a	b	a	b	a	b
E ₂	ND	ND	ND	ND	97 ^{a)}	87 ^{a)}	0.2	12	ND	ND	ND	ND
E ₂ -17-S	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	<0.01	0.1
2-OMe-E ₂	ND	ND	ND	ND	ND	ND	0.2	2.6	96 ^{a)}	92 ^{a)}	ND	ND
2-OMe-E ₂ -17-S	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.5	0.5
E ₁ -S	90	84	0.1	0.4	5.0	10	ND	ND	ND	ND	ND	ND
E ₂ -3-S	5.0	5.0	ND	ND	91	85	0.05	0.6	ND	ND	ND	ND

Incubation conditions: estrogen concentration, 25 μ M; incubation time, 30 min (*n* = 3). Each value represents mean value (*n* = 3). ND, not detected. a) Amount of the steroid recovered.

Table 4. Comparison of the Reducing Activities of DPPH

Compound	Decrease in absorbance at 517 nm		
	Added amount		
	10 μ M	25 μ M	50 μ M
α -Toc	0.23	0.55	0.73
E ₁	0	0	0
E ₂	0	0	0
E ₃	0	0	0
2-OH-E ₂	0.57	0.72	0.75
4-OH-E ₂	0.24	0.66	0.73
2-OMe-E ₂	0.33	0.54	0.69
4-OMe-E ₂	0.13	0.31	0.46
6 α -OH-E ₂	0	0	0
6 β -OH-E ₂	0	0	0
7 α -OH-E ₂	0	0	0
E ₂ -17-S	0	0	0
2-OH-E ₂ -17-S	0.21	0.29	0.70
4-OH-E ₂ -17-S	0.26	0.41	0.73
2-OMe-E ₂ -17-S	0.22	0.28	0.55
4-OMe-E ₂ -17-S	0.17	0.21	0.43
E ₂ -3-S	0	0	0
E ₁ -S	0	0	0

Each figure represents mean value of duplicate experiments.

defined as enzymatic and nonenzymatic reaction, respectively. Because E₂-17-S was converted to the 2- and 4-catechol products by microsomal enzyme cytochrome P-450 under NADPH-generation system,^{14,19} occurrence of the similar metabolism is expected in the present NADPH-dependent lipid peroxidation system, but not so expected in the AsA-dependent reaction system. Comparison of the antioxidant effect of various estrogens under the conditions between AsA- and NADPH-dependent system, thus, might make possible to recognize the molecular species of the most effective antioxidant. As was expected, a significant difference of the antioxidant effect of each estrogen was observed between AsA- and NADPH-dependent system, that is discussed in the followings.

Effect of the Estrogens under AsA-Dependent Lipid Peroxidation: Under AsA-dependent lipid peroxidation condition, free catechol estrogens (2- and 4-OH-E₂) showed the strongest effect, followed by the C₁₇-sulfoconjugated catechols (2- and 4-OH-E₂-17-S), the effect of the latter was to the same degree as that of α -Toc. This means that the antioxidant effect may be attributed to the catechol structure. The difference in the activity observed between free and C₁₇-sulfoconjugated catechol estrogens may be based on their affinity for the microsomal membrane. Because of the high polarity of the sulfoconjugated group, the affinity of these sulfates for the membrane may be reduced, which causes their antioxidant activity to decrease. As for guaiacols, 2- and 4-OMe-E₂ showed a stronger effect than α -Toc, and their C₁₇-sulfates (2- and 4-OMe-E₂-17-S) had a lesser effect. The effect of these guaiacols (free and conjugated) is considered to be caused by their *O*-demethylation during the incubation. Similarly, the fairly strong inhibitory activity recognized in phenolic estrogens (E₁ and E₂) may be attributed to the catechols similarly produced during the incubation. The lower

inhibitory activity of E₃ and E₂-17-S may be explained by the low conversion rate to their catechols. Little activity was observed in C₃-sulfoconjugated estrogens at any concentration, which indicates negligible formation of their catechols.

The formation of catechol product(s) from each steroid was demonstrated by HPLC of the incubation mixtures (Table 3). Under incubation conditions, conversion of phenolic estrogens to their catechols, *O*-demethylation of guaiacols and even the catechol formation from the C₃-sulfates, probably through two metabolic steps involving hydrolysis and subsequent 2-oxidation of the phenols, were evidently confirmed. Because the conversion of these steroids to their catechols was not so great, the antioxidant effect observed with phenolic and guaiacol estrogens may be caused by their own effect.

Finally, ring-B hydroxylated estrogens showed no inhibitory effect. This may be explained by the fact that they are hardly hydroxylated under such conditions because of their higher polarity than E₃.²⁰

Effect of Estrogens under NADPH-Dependent Lipid Peroxidation: The antioxidant effect of the same steroids tested under NADPH-dependent lipid peroxidation condition showed the effect except the ring-B hydroxylated estrogens. Because the reaction conditions are nearly the same as that of microsomal oxidation for estrogens,¹⁴ the antioxidant effect observed with phenolic or guaiacol estrogens should be caused by their conversion to the catechols. Similarly, the effect by the C₃-sulfates may be explained by their sequential conversion; nonenzymatic hydrolysis of the conjugated group, followed by aromatic hydroxylation of the hydrolyzates produced. These were also demonstrated by HPLC of the incubation products (Table 3); the catechol formation from phenolic or guaiacol estrogens in the reaction exceeded that in the AsA-dependent system. Thus, catechol formation from phenols or guaiacols was considered to be influenced by the C₁₇-sulfoconjugate group; 2-hydroxylation of E₂ was 12%, whereas that of E₂-17-S was 0.1%. Similarly, the *O*-demethylation of 2-OMe-E₂ and 2-OMe-E₂-17-S was 2.6% and 0.5%, respectively. E₁-S and E₂-3-S were hydrolyzed during the incubation to E₁ and E₂ as the main hydrolyzate, accompanied by E₂ and E₁, respectively. This indicates the occurrence of a redox reaction at the C₁₇ by 17 β -hydroxysteroid dehydrogenase²¹ prior to their hydrolysis, because E₁ from E₂ was not found in the same reaction. The enzyme 17 β -hydroxysteroid dehydrogenase is known to react with estrogen-3-sulfates with retention of the C₃-conjugate group.²²

On the Antioxidant Effect of Estrogens: In conclusion, the antioxidant activity of estrogens might be explained as follows. (1) For expression of a strong inhibitory effect, conversion of phenolic estrogens to catechols is required. In this case, the polarity of the substituent group on the ring-D affects the activity (E₁ > E₂ > E₃ > E₂-17-S). (2) A difference in the antioxidant activity among free phenolic estrogens (E₁ > E₂ > E₃) was found. As was reported by Barbieri *et al.*,²³ the affinity of phenolic steroids for the enzyme estrogen 2-hydroxylase was in the order of E₂ > E₁ > E₃, while the order of antioxidant activity of their catechols was 2-OH-E₁ > 2-OH-E₂ > 2-OH-E₃.¹¹

After all, the order of the inhibitory activity may be the result of the sum total of these factors. (3) Significant activity was found in the C₁₇-sulfoconjugated catechols, 2-OH-E₂-17-S and 4-OH-E₂-17-S. (4) The weak inhibitory effect of E₂-17-S may be due to the low conversion to the catechol because of its low affinity for cytochrome P-450.²⁴⁾ (5) Inhibitory activities observed with free guaiacol estrogens (2- and 4-OMe-E₂) are considered to be caused probably by rather guaiacol itself than their *O*-demethylated products. The occurrence of *O*-demethylation was influenced by the presence of the C₁₇-sulfoconjugate group, this may be explained by the difference in the binding affinity between guaiacols and cytochrome P-450.²⁵⁾ (6) The antioxidant effect of E₁-S and E₂-3-S seems to be based on the catechols derived from the hydrolyzates once produced. (7) Because of the low affinity of ring-B hydroxylated estrogens for cytochrome P-450,²⁰⁾ no conversion to their catechols occurred.

Measurement of the Inhibitory Effect by Hb-Mb Method: The TBA method used for measuring the antioxidant effect is reported to be affected by contaminants.²⁶⁾ Accordingly, we calculated the inhibitory effects under the NADPH system by the Hb-Mb method¹⁶⁾ as well, which is known to be simpler and more sensitive than the TBA method. The results (Table 2) designated by the IC₅₀ value show the same tendency in Table 1. It is suggested, therefore, that the catechol structure in the molecule of estrogen antioxidants is required for inhibitory activity.

On the Mechanism of the Inhibition To investigate the molecular species acting as antioxidant, the decrease of absorbance of DPPH at 517 nm was measured on phenolic, guaiacol and catechol estrogens. The results obtained are compared in Table IV, where the reducing activities were observed when catechol guaiacol estrogens were used. Because antioxidants are known to reduce the stable free radical DPPH,²⁷⁾ the above result means that catechol or guaiacol estrogens, the estrogenic antioxidant, act as radical scavenger. This may be supported by the recent report,²⁸⁾ where antioxidant effect of estrogen on lipid peroxidation of rat liver microsomes is attributed to not the phenolic estrogens but its catechol product. No further evidences are known on the exact mechanism of antioxidant effect of these estrogens.

Physiological Role of C₁₇-Sulfoconjugated Catechol Estrogens Yoshioka *et al.*²⁹⁾ have assumed superoxide dismutase, catalase, glutathione peroxidase and α -Toc to be endogenous antioxidant factors during gestation, and they have measured their placental activities. Judging from the behavior of these substances during pregnancy, however, they cannot be placental antioxidants during pregnancy. On the other hand, Nakagawa *et al.*⁴⁾ have proposed free catechol estrogens as the most likely antioxidants, this was also found in the present paper. However, plasma free catechol estrogen levels were extremely low because they are rapidly *O*-methylated by erythrocytic catechol *O*-methyltransferase (COMT).⁵⁾ Thus, in contrast to catechols, the plasma concentration of free guaiacol estrogens becomes higher.³⁰⁾ In spite of the high concentration, however, it cannot be considered that free guaiacol estrogens act as effective antioxidants

because of their tight binding activity with testosterone-estradiol binding globulin.³¹⁾ The possibility that free catechol estrogens act as endogenous antioxidants is thus unlikely.

In sharp contrast to free catechol estrogens, 2- and 4-OH-E₂-17-S, though their activity is inferior to that of their free steroids, suffer no *O*-methylation by erythrocytic COMT.⁶⁾ As the result, their biological half-times become longer than those of free catechol estrogens.⁶⁾ Moreover, E₂-17-S, the precursor of these catechols, is a more suitable substrate for microsomal 2-hydroxylase in the placenta than E₂.⁹⁾ It appears from these results that 2-OH-E₂-17-S and also 4-OH-E₂-17-S act as placental antioxidants to maintain a healthy pregnancy. This may be proved by confirming these plasma catechols estrogens to be derived from placenta.

Acknowledgement This work was supported in part by a Grant-in-Aid for General Scientific Research (1991, Yoshizawa), a Grant-in-Aid for Encouragement of Young Scientists (1992, Takanashi) from the Ministry of Education and the Sasakawa Scientific Research Grant from The Japan Science Society (1993, Takanashi), to whom our thanks are due.

REFERENCES AND NOTES

- 1) A part of this work was presented preliminarily in the following: Yoshizawa I., Takanashi K., Watanabe K., Satoh T., Honjo H., Tanaka K., Sakuragi N., Fujimoto S., *J. Steroid Biochem. Molec. Biol.*, **41**, 567 (1992).
- 2) The following review and references cited therein: Hubel C. A., Roberts J. M., Taylor R. N., Musci T. J., Rogers G. M., McLaughlin M. K., *Am. J. Obstet. Gynecol.*, **161**, 1025 (1989).
- 3) Yoshioka T., Ando M., Taniguchi K., Yamasaki F., Motoyama H., *Acta Obstet. Gynaecol. Jpn.*, **42**, 1630 (1990).
- 4) Nakagawa Y., Yoshino K., Komura S., Ishihara M., Yagi K., *J. Clin. Biochem. Nutr.*, **6**, 87 (1989).
- 5) Kono S., Merriam G. H., Brandon D. D., Loriaux D. L., Lipsett M. B., *J. Clin. Endocrinol. Metab.*, **54**, 150 (1982).
- 6) Watanabe K., Takanashi K., Yoshizawa I., *J. Steroid Biochem.*, **32**, 823 (1989).
- 7) Kashiwagi T., Tanaka K., Honjo H., Okada K., Yoshizawa I., Takanashi K., *Nippon Naibunpi Gakkai Zasshi*, **69**, 764 (1993).
- 8) Honma T., Takanashi K., Yoshizawa I., The 114th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, March 1994.
- 9) Takanashi K., Watanabe K., Yoshizawa I., *Biol. Pharm. Bull.*, **16**, 217 (1993).
- 10) Honjo H., Tanaka K., Yasuda J., Ohno Y., Kitawaki J., Naitoh K., Ogino Y., Yamamoto T., Okada H., Watanabe K., Yoshizawa I., *Acta Endocrinol.*, **126**, 303 (1992).
- 11) Nakano M., Sugioka K., Naito I., Takekoshi S., Niki E., *Biochem. Biophys. Res. Commun.*, **142**, 919 (1987).
- 12) Watanabe K., Yoshizawa I., *J. Pharmacobio-Dyn.*, **10**, 302 (1987).
- 13) Watanabe K., Kimura R., Yoshizawa I., *Chem. Pharm. Bull.*, **34**, 2231 (1986).
- 14) Watanabe K., Yoshizawa I., *J. Pharmacobio-Dyn.*, **5**, 340 (1982).
- 15) Wills E. D., *Biochem. J.*, **113**, 315 (1969).
- 16) Buege J. A., Aust S. D., *Methods Enzymol.*, **52**, 302 (1978).
- 17) Ohishi N., Ohkawa H., Miike A., Tatano T., Yagi K., *Biochem. Int.*, **10**, 205 (1985).
- 18) Uchiyama M., Suzuki Y., Fukuzawa K., *Yakugaku Zasshi*, **88**, 678 (1968).
- 19) Watanabe K., Yoshizawa I., *Chem. Pharm. Bull.*, **32**, 2745 (1984).
- 20) Marrian G. F., Sneddon A., *Biochem. J.*, **74**, 430 (1960).
- 21) Aoshima Y., Kochakian C. D., *Endocrinology*, **72**, 106 (1963).
- 22) Hobkirk R., Nilsen M., Jennings B., *Can. J. Biochem.*, **53**, 1333 (1975).

- 23) Barbieri R. L., Canick J. A., Ryan K. J., *Steroids*, **32**, 529 (1978).
- 24) Watanabe K., Yoshizawa I., *J. Chromatogr.*, **277**, 71 (1983).
- 25) Hoffman A. R., Paul S. M., Axelrod J., *Endocrinology*, **107**, 1192 (1980).
- 26) Ohkawa H., Ohishi N., Yagi K., *J. Lipid Res.*, **19**, 1053 (1978).
- 27) Mellors A., Tappel A. L., *J. Biol. Chem.*, **241**, 4353 (1966).
- 28) Ruiz-Larrea M. B., Leal A. M., Liza M., Lacort M., de Groot H., *Steroids*, **59**, 383 (1994).
- 29) Yoshioka T., Ando M., Taniguchi K., Yamasaki F., Motoyama H., *Acta Obstet. Gynaecol. Jpn.*, **42**, 1634 (1990).
- 30) Emons G., Ball P., Postel G. V., Knuppen R., *Acta Endocrinol.*, **91**, 158 (1979).
- 31) Dunn J. F., Merriam G. R., Eil C., Kono S., Loriaux D. L., Nisula B. C., *J. Clin. Endocrinol. Metab.*, **51**, 404 (1980).