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DNA strand break and 8-hydroxyguanine formation induced by 2-hydroxyestradiol dispersed in liposomes

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Abstract

DNA damage induced by estrogens dispersed in liposomes was investigated. 2-Hydroxyestradiol ($2HOE_2$) and 4-hydroxyestradiol ($4HOE_2$), but not estrone, estradiol-17β or estriol, caused strand break of plasmid DNA damage in the presence of ADP-Fe^{3+}. The catechol structure may be necessary for DNA damage. When DNA was incubated with $2HOE_2$ for a long time (24 h), DNA damage was induced even at very low concentrations. Adding hydrogen peroxide markedly enhanced the sensitivity of DNA to the attack by $2HOE_2$. Hydroxyl radical ($HO^•$) scavengers strongly inhibited the $2HOE_2$-induced DNA damage, and EDTA partially inhibited DNA damage. However, $2HOE_2$ caused 8-hydroxyguanine formation from calf thymus DNA only in the presence of EDTA-Fe^{3+}, but not ADP-Fe^{3+}. In addition, deoxyribose, which is a detective molecule of $HO^•$, was not degraded by $2HOE_2$ in the presence of ADP-Fe^{3+}. Upon adding EDTA $2HOE_2$ rapidly degraded deoxyribose. These results suggest that DNA strand break caused by $2HOE_2$ in the presence of ADP-Fe^{3+} was due to ferryl ion rather than $HO^•$, whereas 8-hydroxyguanine (8HOG) induced by $2HOE_2$ in the presence of EDTA-Fe^{3+} was due to $HO^•$. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: DNA damage; Estrogen; Liposome; 2-Hydroxyestradiol; 4-Hydroxyestradiol

1. Introduction

It is widely recognized that estrogens induce human breast cancer [1], although, the carcinogenic mechanism of estrogens is not clearly understood. Han and Liehr [2] reported that estrogens induce carcinogenesis in the kidney as a result of DNA damage in the cells. Free radicals may be an important factor in the estrogen-caused damage of DNA [3]. However, estrogens are powerful inhibitors of lipid peroxidation of membranes [4–7]. We reported previously [8] that estrogens, including estradiol (E_2) and 2-hydroxyestradiol ($2HOE_2$), inhibit lipid peroxidation at the initial and propagation stages by scavenging lipid carbon-centred and/or peroxyl radicals. However, the relationship between the ability of estrogens to scavenge free radicals and carcinogenesis of estrogens is unclear.

Nutter et al. [9,10] showed that hydrogen peroxide is produced in 3,4-estrone quinone-treated cellular fractions, and that chromosomal DNA damage induced by 3,4-estrone quinone is prevented by catalase, suggesting that hydroxyl radicals ($HO^•$) play a crucial role in carcinogenic mechanisms. Generally, $HO^•$ is produced by the iron-catalyzed Fenton reaction [10]. However, the mechanism by which estrogens are involved in the generation of $HO^•$ is not completely understood. In the study on antioxidative action of $2HOE_2$, we found that when catecholestrogens were dispersed in liposomes, DNA damage was readily caused in the presence of iron. In this study, we have demonstrated that strand breaks of plasmid DNA and 8-hydroxyguanine (8HOG) formation from calf thymus DNA are due to ferryl ion and $HO^•$, respectively.

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2. Materials and methods

2.1. Materials

The following materials were used: estrone (E_1), E_2, and thiobarbituric acid (TBA) from Wako, Osaka, Japan; estriol (E_3), $2HOE_2$, 4-hydroxyestradiol ($4HOE_2$) and a liposome kit from Sigma, St. Louis, MO, USA; and plasmid PM2 from Boehringer-Mannheim-Yamanouchi, Tokyo, Japan. Other reagents were analytical grade products from commercial suppliers. ADP-Fe^{3+} was prepared by mixing ADP with Fe^{3+} (ADP:Fe^{3+} = 17:1).

2.2. Preparation of liposomes

The estrogens used in this study are water insoluble. Organic solvents, such as methanol, ethanol and acetone, completely prevent $2HOE_2$-induced DNA damage. To avoid the effect of organic solvents on DNA damage, estrogens were dispersed in liposomes. A liposome kit contained l-α-phosphatidylcholine (63 μmol), dicetylphosphate (18 μmol) and cholesterol (9.0 μmol) in 5.0 ml of chloroform. Estrogens were added to the liposome kit, which was then evaporated to dryness. The lipid layer in test tubes was dispersed using a vortex mixer and then was sonicated in 10 mM phosphate buffer at pH 7.4 containing 0.15 M NaCl. The stock suspension of liposomes contained 100 μM of estrogens

2.3. Gel electrophoresis

The reaction mixture in polyethylene tubes (1.5 ml) contained PM2 (0.5 μg/ml), 10 μM ADP-Fe^{3+} (as Fe^{3+}) and 0.15 M NaCl in 10 mM phosphate buffer at pH 7.4. Various amounts of estrogens were added to the reaction mixture. The reaction was started by adding liposomes. After incubating at 37°C, a loading buffer (0.25% bromophenol blue, 10% glycerol) was added and then the DNA samples were applied to the agarose gel (1.0%). The gel was run for about 2 h (10 mV/cm) and was submerged in TAE buffer (pH 8.0) consisting of Tris bases (40 mM), ethylenediaminetetraacetate (EDTA) (2.0 mM) and acetic acid. DNA was visualized by staining with 1.3 μM ethidium bromide after washing with the TAE buffer (30 min). The decrease in closed circular (cc) DNA was calculated by integration of the area with Epi-Light (Aisin Cosmos EU-1150).

2.4. Deoxyribose degradation

The deoxyribose was measured by the method of Halliwell and Gutteridge [11] with a minor modification. The reaction mixture contained 2.0 mM deoxyribose, 100 μM ADP-Fe^{3+} (as Fe^{3+}) and $2HOE_2$ in 3.0 ml of 10 mM phosphate buffer at pH 7.4 containing 0.15 M NaCl. After incubating for 30 min, 30 μl of 30% trichloroacetic acid (TCA) and 1.0 ml of 0.6% TBA were added to the reaction mixture. After heating for 8 min at 100°C, thiobarbituric acid-reactive substances (TBARS) were determined by measuring the absorption at 532 nm.

2.5. Preparation of ferritin and release of iron

Commercial horse spleen ferritin was incubated in 10 mM EDTA for 1 h at 4°C and then the EDTA was removed by dialysis against 0.15 M NaCl. The iron content of the prepared ferritin was 1600 Fe/molecule. The release of iron from ferritin was assayed in terms of the formation of bathophenanthroline sulfonate (BPS)-Fe^{2+} by measuring the absorbance at 530 nm ($\varepsilon = 22.14$ mM^{-1}) [12].

2.6. Formation of 8HOG

The reaction mixture contained calf thymus DNA (0.5 mg) and 100 μM of EDTA-Fe^{3+} or ADP-Fe^{3+} in 2.0 ml of 10 mM phosphate buffer containing 0.15 M NaCl at pH 7.4. The reaction was ended by adding 100 μl of 30% TCA and 3.0 ml of ethanol. After keeping at room temperature for 15 min, the sample was centrifuged at 3000 rpm for 10 min. The supernatant was discarded and then 0.5 ml of 60% (v/v) formic acid was added. The sample was hydrolyzed for 45 min at 150°C, and then the formic acid was removed by freeze drying. The residue was solubilized in distilled water and then analyzed for 8HOG by using the high-pressure liquid chromatography (HPLC) method as described by Kaur and Halliwell [13] with a minor modification. The HPLC analysis was performed on a nucleocil C18 column (4.6×250 mm) with a mobile phase containing 20 mM sodium citrate, 17 mM acetate buffer (pH 3.01) and methanol (100:1) at a flow rate of 1.0 ml/min. 8HOG was detected by electrochemical detector at a potential of 0.85 V.

3. Results

3.1. Strand break of DNA by $2HOE_2$ and $4HOE_2$

We have tested estrogens in the induction of DNA damage in the presence of ADP-Fe^{3+}. When plasmid DNA was incubated with estrogens in the presence of ADP-Fe^{3+}, $2HOE_2$ and $4HOE_2$, induced extensive strand break of plasmid DNA, as indicated by the decrease of ccDNA (Table 1). In contrast, E_1, E_2 and E_3 did not cause the DNA damage. $4HOE_2$ was less effective regarding DNA damage than $2HOE_2$. Either

2HOE$_2$, 4HOE$_2$ or ADP-Fe^{3+} alone did not damage DNA.

The time course of the DNA strand breakage was studied (Fig. 1). At 4 h, ccDNA almost disappeared by converting to open circular (oc) DNA. The dose responses of the DNA strand break caused by 2HOE$_2$ and 4HOE$_2$ are shown in Fig. 2. Almost all ccDNA was converted to ocDNA at 20 μM of 2HOE$_2$ and 40 μM of 4HOE$_2$. With 1.0 μM 2HOE$_2$, only about 20% of DNA was damaged after incubating for 4 h. However, as shown in Fig. 3, by 24-h incubation, 1.0 μM of 2HOE$_2$ caused complete DNA strand break. Furthermore, when 10.0 μM of H_2O_2 was added, only 4-h incubation was needed to induce complete DNA strand break with 1.0 μM of 2HOE$_2$. At 4-h incubation, 10 μM H_2O_2 alone had no effect on DNA. These results indicated that the amount of 2HOE$_2$ necessary to cause the same DNA damage is strongly dependent on incubation time and that DNA is highly sensitive to 2HOE$_2$ in the presence of H_2O_2, even at low concentrations.

Table 1
DNA strand break induced by estrogens dispersed in liposomes[a]

Additions	Residual ccDNA (%)
None	100
E_1	100.9 ± 18.0
E_2	102.1 ± 10.7
2HOE$_2$	0
4HOE$_2$	27.0 ± 15.2
E_3	100.8 ± 13.9

[a] The reaction mixture contained plasmid DNA (0.2 μg/ml), 10 μM ADP-Fe^{3+} and 0.15 M NaCl in 10 mM phosphate buffer at pH 7.4. A suspension of estrogens was added to the reaction mixture, in which estrogens (40 μM) were contained. After incubating for 4 h at 37°C, DNA was applied to agarose gel. The disappearance of ccDNA was measured as described in Section 2. Each value represents the mean of three experiments.

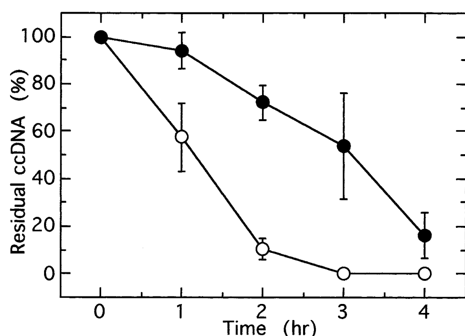

Fig. 1. Time course of DNA strand breaks induced by 2HOE$_2$ and 4HOE$_2$. Conditions were the same as described in Table 1. The suspension of estrogens was added to the reaction mixture. Each point represents the mean ± S.D. of three experiments. (○), 2HOE$_2$ and (●), 4HOE$_2$.

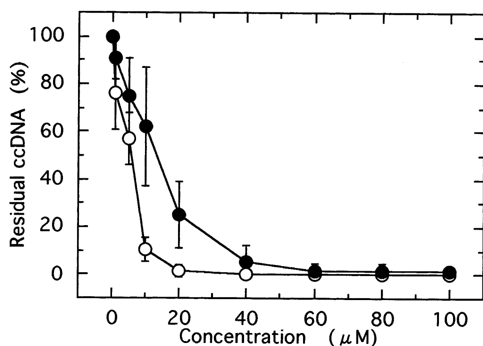

Fig. 2. Effect of 2HOE$_2$ and 4HOE$_2$ concentrations on DNA strand breaks. Conditions were the same as described in Fig. 1, except for the concentrations of 2HOE$_2$ (○) and 4HOE$_2$ (●). After incubating for 4 h, plasmid DNA was applied to an agarose gel.

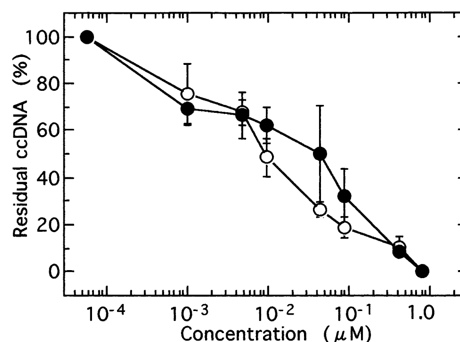

Fig. 3. Effect of H_2O_2 on 2HOE$_2$-induced DNA strand breaks. Plasmid DNA was incubated with 2HOE$_2$ for 24 h in the absence of H_2O_2 and for 4 h in the presence of H_2O_2 (10.0 μM). Other conditions were the same as described in Table 1. Each point represents the mean ± S.D. of three experiments. (●), incubation for 24 h in the absence of H_2O_2 and (○), incubation for 4 h in the presence of H_2O_2.

3.2. Involvement of reactive oxygens

To demonstrate the involvement of reactive oxygens in DNA damage, we tested the effect of various scavengers and iron chelator on the DNA damage (Table 2). Catalase very strongly inhibited the DNA damage induced by 2HOE$_2$, indicating involvement of H_2O_2 in the DNA damage. Superoxide dismutase did not affect 2HOE$_2$-induced DNA damage, suggesting no superoxide was involved. Mannitol and dimethylsulfoxide, typical $HO^\bullet$ scavengers, strongly inhibited the strand break of DNA, indicating that the strand break of DNA was due to $HO^\bullet$ or $HO^\bullet$-like species generated through H_2O_2 dependent reaction. Methanol and ethanol completely prevented the strand break of DNA. Glutathione and trolox had no effect. Interesting, the iron chelator, EDTA, partially protected the DNA from the attack of $HO^\bullet$ by about 40%.

Table 2
Effect of radical scavengers and the iron chelator on $2HOE_2$ induced DNA strand breaks[a]

Additions	Concentration (mM)	Residual ccDNA (%)
Complete system	–	0
+SOD	0.3×10^{-3}	0
+Catalase	45.5×10^{-6}	98.1 ± 28.2
+Mannitol	10.0	84.9 ± 18.1
+DMSO	10.0	108.0 ± 32.1
+EDTA	0.1	39.0 ± 7.6
+Glutathione	1.0	0
+Trolox	0.1	0
+Methanol	10.0	106.1 ± 4.1
+Ethanol	10.0	119.5 ± 7.1

[a] Conditions were the same as described in Fig. 1, except for adding scavengers and EDTA. After incubating for 4 h, the decrease in ccDNA was measured. Each value represents the mean of three experiments.

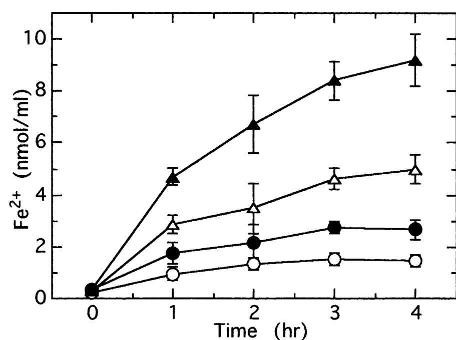

Fig. 4. Iron release from ferritin induced by $2HOE_2$ dispersed in liposomes. Iron released from ferritin was measured in the reaction mixture containing 100 μM of $2HOE_2$, 1.0 mM BPS, ferritin and 0.15 M NaCl in 10 mM phosphate buffer at pH 7.4. Each point represents the mean ± S.D. of three experiments. (○), 5.0 μg/ml; (●), 10 μg/ml; (△), 20 μg/ml; and (▲), 40 μg/ml ferritin.

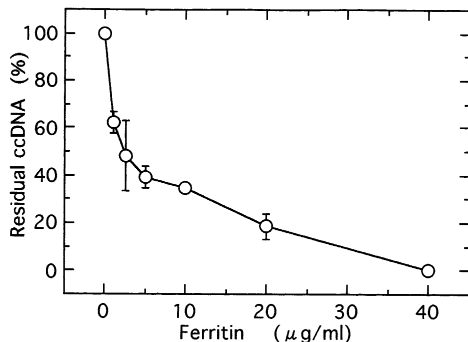

Fig. 5. DNA strand break caused by the interaction between $2HOE_2$ and ferritin. Plasmid DNA was incubated with $2HOE_2$ (5.0 μM) in the presence of ferritin (40 μg/ml). After incubating for 4 h, DNA was applied to an agarose gel. Other conditions were the same as described in Fig. 1.

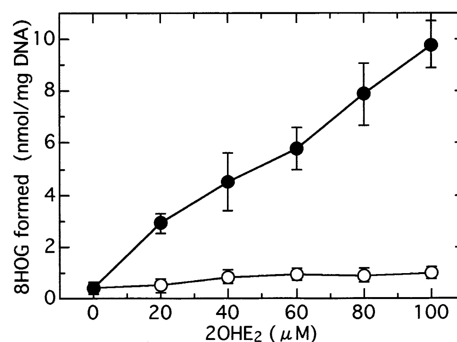

Fig. 6. 8HOG formation from calf thymus DNA by the interaction between $2HOE_2$ and EDTA-Fe^{3+} or ADP-Fe^{3+}. Conditions were described in Section 2. Each point represents the mean ± S.D. of three experiments. (○), ADP-Fe^{3+} and (●), + EDTA-Fe^{3+}.

3.3. Effect of ferritin

In cells, iron is stored mainly in a biologically inactive form bound to ferritin. Many phenolic compounds are capable of reducing Fe^{3+} to Fe^{2+} and releasing iron from ferritin [13]. We therefore examined if $2HOE_2$ is able to mobilize iron from ferritin and then cause DNA damage. Fig. 4 shows that $2HOE_2$ released iron from ferritin concentration dependently. In addition, the DNA damage was also caused during the interaction of $2HOE_2$ with ferritin as shown in Fig. 5. During the 4-h incubation of $2HOE_2$ with 3.0 μg/ml of ferritin, about 50% of DNA strand break occurred. Complete disappearance of ccDNA was caused by 4-h incubation with 40 μg/ml of ferritin.

3.4. Formation of 8HOG induced by $2HOE_2$

8-Hydroxylation of the guanine base of DNA can be caused by the attack of $HO^•$, a one electron oxidation and exposure to singlet oxygen [14]. It has been used widely as an indicator of oxidative damage in DNA and as a general marker of oxidative stress. We therefore examined if 8HOG can be produced by $2HOE_2$. Fig. 6 shows that the formation of 8HOG from calf thymus DNA was readily induced by $2HOE_2$ in the presence of EDTA-Fe^{3+}, but not ADP-Fe^{3+}. In addition, 8HOG formation depended on the concentration of $2HOE_2$ (Fig. 7). After incubating for 60 min, the formation of 8HOG induced by $2HOE_2$ reached about 10 nmol. These results obviously conflicted with the electrophoretic data that EDTA partially inhibits DNA strand break induced by $2HOE_2$ in the presence of ADP-Fe^{3+}. Hydroxyl radicals can be detected by their ability to degrade deoxyribose into fragments that generate pink chromogen on heating with TBA at low pH. We therefore examined the deoxyribose degradation induced during the interaction between $2HOE_2$ and ADP-Fe^{3+} or EDTA-Fe^{3+}. Fig. 8 shows that pink

chromogen did not develop during the interaction between 2HOE$_2$ and ADP-Fe^{3+}. However, addition of EDTA to the reaction mixture developed pink chromogen. A more rapid formation of TBARS was caused by adding EDTA-Fe^{3+} before the start of the reaction. These results strongly suggest that there was not much $HO^{\bullet}$ formed during the interaction of 2HOE$_2$ and ADP-Fe^{3+}, whereas the interaction of 2HOE$_2$ and EDTA-Fe^{3+} produced a significant amount of $HO^{\bullet}$. When being added to the mixture, EDTA might partially remove iron from ADP-Fe^{3+} and caused deoxyribose degradation. Therefore, calf thymus DNA was presumably damaged, forming 8HOG, by $HO^{\bullet}$ generated during the interaction between 2HOE$_2$ and EDTA-Fe^{3+}.

4. Discussion

This study has demonstrated that 2HOE$_2$ dispersed in liposomes in the presence of ADP-Fe^{3+} or ferritin was capable of causing DNA strand break. Among estrogens, including E_1, E_2, E_3, damage to DNA was caused only by 2HOE$_2$ and 4HOE$_2$, indicating that the catechol structure of estrogens is necessary in the induction of DNA damage. The doses of 2HOE$_2$ needed to induce DNA strand break, within several hours, were 10–40 μM. These concentrations of 2HOE$_2$ were much higher than the level in cells [11]. However, when DNA was incubated with 2HOE$_2$ for a long time (24 h), DNA strand break was caused at about 10^{-3} μM of 2HOE$_2$. Ruiz-Larrea et al. [15] showed that 2HOE$_2$ readily reduces Fe^{3+} to Fe^{2+} in aqueous solution. We confirmed this, when 2HOE$_2$ dispersed in liposomes rapidly reduced Fe^{3+} to Fe^{2+} (data not shown). 2HOE$_2$ by itself may convert to the semiquinone radical and quinone. Adding a small amount of H_2O_2 markedly facilitated the DNA strand break caused by 2HOE$_2$. Hydrogen peroxide is produced in various biological processes [16,17]. Our results suggest the possibility that DNA damage could be readily caused by catecholestrogen. Hydrogen peroxide may have a crucial role in DNA damage because catalase completely protects DNA from oxidative attack. A long time may be required to form H_2O_2 during autoxidation of Fe^{2+}. The DNA damage induced by 2HOE$_2$ dispersed in liposomes is inhibited by catalase and hydroxyl radical scavengers, indicating that DNA damage is due to $HO^{\bullet}$ generated by the Fenton reaction. Although EDTA is a good catalyst for the Fenton reaction, it partially prevented strand break of plasmid DNA induced by 2HOE$_2$ in the presence of ADP-Fe^{3+}. Deoxyribose is a detector molecule of $HO^{\bullet}$ [11]. The development of pink chromogen from deoxyribose degradation was only slightly observed during the interaction between 2HOE$_2$ and ADP-Fe^{3+}. However, adding EDTA greatly enhanced the degradation of deoxyribose. With EDTA-Fe^{3+}, 2HOE$_2$ caused deoxyribose damage forming TBARS. This damage can be related to the formation of $HO^{\bullet}$ [18]. These results indicated that EDTA removes iron from ADP-Fe^{3+} to produce free $HO^{\bullet}$ that contributes to degradation of deoxyribose. Mello-Filho and Meneghini [19] observed that strong iron chelators protected DNA from oxidative damage. In their study, EDTA protected DNA from strand break, whereas the EDTA-iron complex formed is capable of reacting with H_2O_2. They proposed that $HO^{\bullet}$ generated under their conditions is not close enough to affect DNA. Halliwell and Gutteridge [11] observed essentially the same reactions. However, intermediates formed in the Fenton reaction consist of $HO^{\bullet}$ and iron species [20]. The efficiency of $HO^{\bullet}$ generation varies with the nature of the iron chelators used. The oxidizing species of iron, which are classified as ferryl, FeO_2^{+} or Fe(IV)=O, are generated effectively in the presence of ADP. Yamazaki and Piette [21] showed that the typical $HO^{\bullet}$ scavengers, including mannitol, reacted not only with $HO^{\bullet}$, but also with ferryl ion. As another possibility, therefore, ferryl ion, which is generated during the interaction between 2HOE$_2$ and ADP-

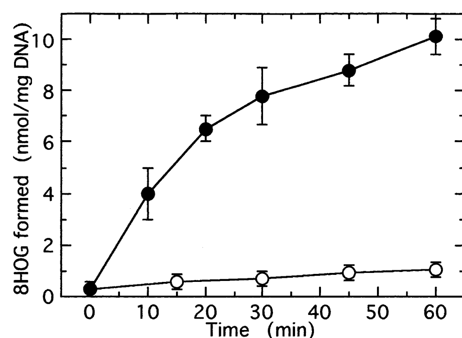

Fig. 7. Effect of 2HOE$_2$ concentrations on 8HOG formation. Conditions were the same as described in Fig. 6, except for 2HOE$_2$ concentrations. After incubation of 1 h, 8HOG was measured by HPLC. (○), ADP-Fe^{3+} and (●), + EDTA-Fe^{3+}.

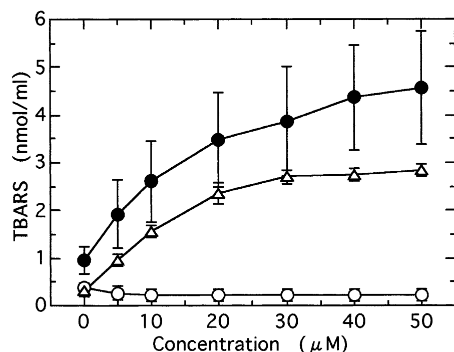

Fig. 8. Deoxyribose degradation induced by 2HOE$_2$ in the presence of EDTA-Fe^{3+} or ADP-Fe^{3+}. Conditions were described Section 2. Each point represents the mean ± S.D. of three experiments. (○), ADP-Fe^{3+}; (△), ADP-Fe^{3+} + EDTA (1.0 mM); and (●), + EDTA-Fe^{3+}.

Fe^{3+}, may participate in sugar damage of DNA to cause strand break.

8HOG was efficiently induced by $2HOE_2$ in the presence of EDTA-Fe^{3+}, but ADP-Fe^{3+} was a poor catalyst of 8HOG formation. Iron ions are not efficient catalysts of 8HOG formation [22]. In addition, Aruoma et al. [23] studied damage to the DNA bases by hypoxanthine-xanthine oxidase in the presence of EDTA-Fe^{3+}, and showed that 8HOG is formed as a major product induced by $HO^{\bullet}$. These findings suggest that 8HOG formation induced by $2HOE_2$ in the presence of EDTA-Fe^{3+} is due to $HO^{\bullet}$, not to ferryl ion. The intracellular molecules to which iron ions attach include organic acid, phosphate ion and phosphate ester, such as ATP and ADP, but not EDTA [24,25]. Therefore, catecholestradiols in the presence of iron may contribute to the damage of DNA, rather than to 8HOG formation in cells.

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