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# STIMULATION OF 12-LIPOXYGENASE ACTIVITY IN RAT PLATELETS BY 17β-ESTRADIOL

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**Abstract**—The effects of estradiol on the endogenous fatty acid (specifically, arachidonic acid) composition of cellular phospholipid fractions and the 12-lipoxygenase activity in rat platelets *in vivo* were studied. Estradiol had no significant effect on the endogenous fatty acid composition of cellular phospholipid fractions. However, estradiol significantly increased 12-lipoxygenase activity in platelets in a dose-dependent manner. The stimulatory effect of estradiol on platelet lipoxygenase was blocked by the anti-estrogen nafoxidine hydrochloride which was injected simultaneously together with estradiol *in vivo*, suggesting that the effect in target cells was due directly to estradiol.

In many tissues and cells, two distinct enzymatic pathways exist for the oxygenation of arachidonic acid. Prostaglandins, prostacyclin and thromboxanes are formed by the cyclooxygenase pathways, while the various polyunsaturated hydroxy fatty acids are formed by the lipoxygenase pathways. In blood vessel walls, arachidonic acid is converted to prostacyclin by the cyclooxygenase pathways [1]. In washed platelets, arachidonic acid is converted to 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT)‖ and thromboxane $B_2$ by the cyclooxygenase pathway and to 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) by the lipoxygenase pathways [2]. The "Ying–Yang" balance between prostacyclin and thromboxane $A_2$ in the blood-vessel interface and the possible roles of hydroperoxy fatty acids in the regulation of prostacyclin and thromboxane biosynthesis have attracted much attention in research on atherosclerotic heart disease.

We have been interested in the regulation of the arachidonate cascade in the vascular cells and platelets by estradiol, since epidemiologic studies indicate that females enjoy a lower incidence of coronary artery disease throughout their reproductive years than males of comparable age [3]. We have been able to culture homogeneous smooth muscle cells from rat aortas and have found that estradiol stimulates prostacyclin production in these cells by increasing fatty acid cyclooxygenase and prostacyclin synthetase activities in cells [4, 5]. Estradiol specific binding receptors are also detectable in the same cells [6].

In the present investigation, we first checked the effect of estradiol on the principal fatty acid composition of cellular phospholipid fractions in rat platelets and, then, studied the effect of estradiol on the lipoxygenase activity in platelets by using a cell-free assay. Evidence is presented showing that 12-lipoxygenase in platelets was increased following estradiol treatment and that the stimulatory effect of estradiol was blocked by simultaneous treatment with the anti-estrogen nafoxidine.

## MATERIALS AND METHODS

*Chemicals.* [1-$^{14}$C]Arachidonic acid (58 Ci/mole) was purchased from the Radiochemical Centre, Amersham, England, and [2,4,6,7-$^{3}$H]estradiol (115 Ci/mmole) from the New England Nuclear Corp., Boston, MA, U.S.A. Arachidonic acid and 17β-estradiol were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Depo-estradiol cypionate and depo-testosterone cypionate, dissolved in cotton seed oil, were supplied by Japan Upjohn Ltd., Tokyo, Japan. Nafoxidine HCl was provided by the Upjohn Co., Kalamazoo, MI, U.S.A. GSH (reduced glutathione) was purchased from Boehringer Mannheim GmbH, F.R.G. Bovine serum albumin (fraction V) was obtained from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. Prostaglandin and thromboxane standards were gifts of the Ono Pharmaceutical Co., Ltd., Osaka, Japan. Thin-layer chromatographic plates of silica gel 60 $F_{254}$, 0.25 mm in thickness, were purchased from E. Merck, Darmstadt, F.R.G. All reagents not specified above were of analytical grade.

*Animals.* Male Wistar rats aged 9–10 weeks, with body weights ranging from 220 to 260 g, were housed at the Shizuoka Experimental Animals Agricultural Corp., Hamamatsu, Japan, before initiation of the

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‖ Abbreviations: HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; 12-HPETE, 12-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; GSH, reduced glutathione; and GLC, gas–liquid chromatography.

experiments. Upon receipt, all animals were maintained on a commercial laboratory feed under a constant light–dark cycle (8:00 a.m. to 8:00 p.m.), and water was provided *ad lib*.

*Treatment with sex steroids.* Depo-estradiol cypionate or depo-testosterone cypionate, in cotton seed oil, was administered subcutaneously. Controls received the same volume of cotton seed oil (1 ml/kg body weight) only. Estradiol and testosterone were given at doses of 0.5 and 10 mg/kg body weight, respectively, which were repeated at intervals of 3–4 days, unless otherwise stated. Rats were killed on day 3 after the last injection. In experiments with anti-estrogen, nafoxidine HCl, dissolved in 0.15 M NaCl at a dose of 5 mg/kg body weight, was injected subcutaneously together with estradiol treatment. Controls received an equal volume of saline vehicle (1 ml/kg body weight).

*Preparation of platelets.* Each rat was anesthetized with diethyl ether and 5 ml of blood was withdrawn from the abdominal aorta into a one-tenth volume of 3.8% solution of sodium citrate. Platelet-rich plasma was prepared by centrifugation of citrated blood at 200 *g* for 10 min at room temperature. Platelet pellets were obtained by centrifuging the platelet-rich plasma at 1000 *g* for 10 min at room temperature. Platelet number in platelet-rich plasma was determined with a model MEK-1200 Nikon Kohden automatic blood cell counter.

*Lipid extraction and fractionation.* Platelets were sonicated in 1 ml of 1% methanol containing 0.1% EDTA by a model W185 Branson Sonifier. A whole lipid extraction was then carried out by the method of Bligh and Dyer [7]. Separation of neutral lipids, free fatty acids and phospholipids was carried out by thin-layer chromatography in a solvent system of *n*-hexane–diethyl ether–acetic acid (90:10:1, by vol.).

*Fatty acid analysis by gas–liquid chromatography.* Analysis of the fatty acyl chains of phospholipids was carried out by gas–liquid chromatography (GLC) of their corresponding methyl esters. These were obtained by taking the sample to dryness under $N_2$ and transesterifying with 1 ml of 5% methanolic hydrochloric acid solution at 80° for 2 hr. Internal standard heptadecanoic acid was added before the esterification. The methyl esters were extracted in *n*-hexane and dried in $N_2$ and then dissolved in a small volume of chloroform for GLC analysis. The fatty acid methyl esters were separated on a column of 16% polyethylene glycol succinate coated on Celite 545 at 185° with an $N_2$ flow rate of 40 ml/min in a Shimazu model 7A gas chromatograph, and the data were computerized by a Shimazu Chromatopac-ElA.

*Preparation of cytoplasmic supernatant fractions.* Platelet pellets were resuspended in 25 mM Tris–HCl buffer (pH 7.7) and freeze-thawed three times in liquid nitrogen and in a water bath at 37° respectively. These preparations were centrifuged at 105,000 *g* for 60 min, and the resulting supernatant fractions were designated the cytoplasmic supernatant fraction (cytosol).

*Enzyme assay.* Platelet 12-lipoxygenase activity was assayed with [1-$^{14}$C]arachidonic acid as substrate. The assay mixture contained 12 μg [1-$^{14}$C]arachidonic acid (0.2 μCi/assay) and cytosol fraction in a final volume of 1 ml of 25 mM Tris–HCl buffer. For the routine assays of enzyme activity, 1 mM GSH was included in the incubation mixtures. Incubations were performed in air at 37° for 2 min with shaking. The incubation was terminated by acidification to pH 3.0 with 1 N HCl. The reaction mixture was then extracted with 10 ml of ethyl acetate. The resulting organic phase was evaporated to dryness under reduced pressure. Residues were dissolved in a small amount of ethanol and applied to thin-layer chromatographic plates. The plates were developed in a solvent system of ligroin–diethyl ether–acetic acid (50:50:1, by vol.). The radioactive products were detected by a Dünnschicht scanner. Reference standards of prostaglandins and arachidonic acid were visualized by spraying with 10% phosphomolybdic acid in ethanol. Details of the enzyme assay and the identification of metabolites have been described in our previous paper [8].

*Estradiol binding receptor assay.* Studies of estadiol binding in intact platelets and platelet cytosol fraction were performed according to the methods described by Nakao *et al.* [6] and Roth and Livingston [9] respectively.

*Protein determinations.* Protein contents were determined by the method of Lowry *et al.* [10] with bovine serum albumin (fraction V) as a standard.

## RESULTS

*Effect of estradiol on the principal fatty acid composition of cellular phospholipid fractions in rat platelets.* Total lipid was extracted from platelets of control and estradiol-treated rats, respectively, and separated into phospholipids, neutral lipids and free fatty acids. The composition of fatty acids in phospholipid fractions was estimated quantitatively by GLC. The principal fatty acid composition is indicated in Table 1. The fatty acids, e.g. linoleic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid, which composed less than 1% of the total were neglected. The saturated acids e.g. palmitic (16:0) and stearic acid (18:0), and the unsaturated acids, oleic (18:1) and linolenic acid (18:3), made up about 75% of the total. The unsaturated precursor of prostaglandins (arachidonic acid, 20:4) made up

Table 1. Effect of estradiol on the principal fatty acid composition of platelet phospholipid fractions*

| | Fatty acid (%) | |
|---|---|---|
| | Control | Estradiol |
| 16:0 | 43 ± 0.3 | 39 ± 0.8 |
| 18:0 | 16 ± 0.2 | 18 ± 0.1 |
| 18:1 | 9 ± 0.3 | 8 ± 0.1 |
| 18:3 | 10 ± 0.1 | 9 ± 0.2 |
| 20:4 | 23 ± 1.0 | 25 ± 0.7 |

* Rats were injected with 0.5 mg/kg body wt estradiol three times. After treatment, the fatty acid composition in phospholipid fractions of cells was analyzed. Values are the percentages of total fatty acids in phospholipid fractions, and they represent the mean ±S.E.M. from four individual animals.

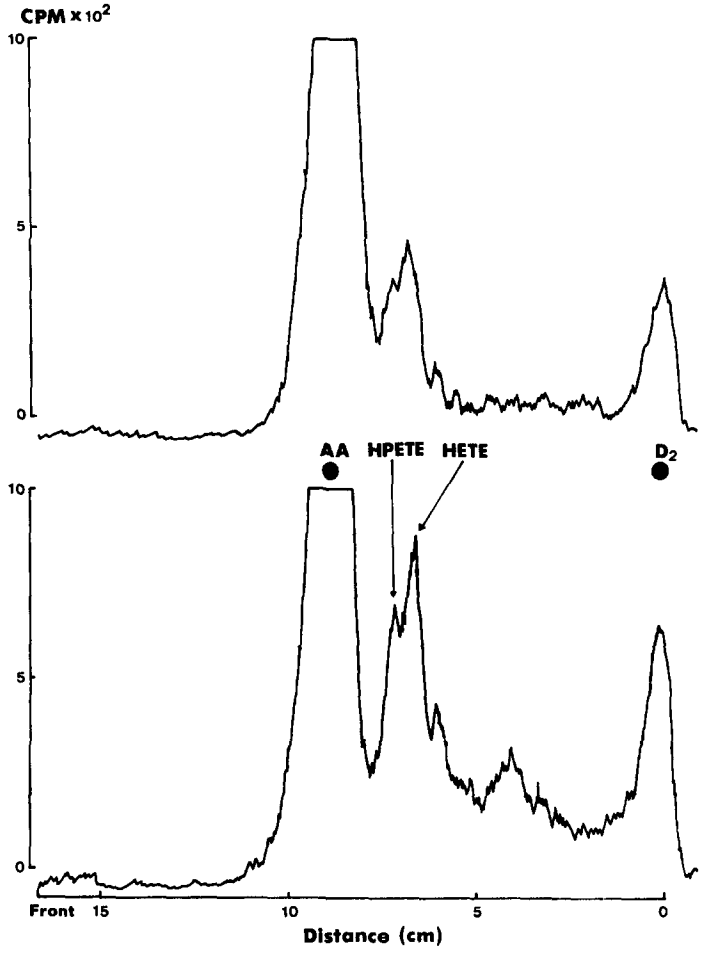


Fig. 1. Scanning profiles showing the effect of estradiol on the transformation of [1-$^{14}$C]arachidonic acid by rat platelet cytosol. Rats were injected five times at 3 to 4 day intervals with 0.5 mg/kg body wt estradiol. Cytosol fractions prepared from $1.5 \times 10^8$ platelets of control and estradiol-treated animals were incubated with 12 μg [1-$^{14}$C]arachidonic acid (0.2 μCi/assay) in a final volume of 1 ml of 25 mM Tris–HCl buffer at 37° for 3 min. Abbreviations: $D_2$ and AA are the standards of prostaglandin $D_2$ and arachidonic acid. Upper panel: control. Lower panel: estradiol-treated.

about 25% of the total. The main fatty acid composition of cellular phospholipid fractions was not affected significantly by estradiol.

*Effect of estradiol on 12-lipoxygenase activity in rat platelets.* When platelet cytosol fraction was incubated with [1-$^{14}$C]arachidonic acid, the patterns of formation of arachidonate metabolites in both control and estradiol-treated groups were the same, as shown in Fig. 1. Peaks which were more polar than 12-HPETE and 12-HETE, including the one at the origin, were designated the "more polar products". The more polar products constituted about 60% of total products transformed from arachidonic acid by platelet cytosols. They are formed via the lipoxygenase pathways the same as 12-HPETE and 12-HETE, since their formation is not inhibited by indomethacin but by ETYA (5,8,11,14-eicosatetraynoic acid), and they may be composed of a mixture of 8,9,12-trihydroxyeicosa-5,10,14-trienoic acid (8,9,12-THETA), 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid (8,11,12-THETA), 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid, and some unidentified products [8].

Table 2 indicates the effect of estradiol treatment on the biosynthetic activities of 12-HPETE, 12-HETE and the more polar products in platelet cytosols. Over 93% of the 12-lipoxygenase activity in cells was recovered in the cytoplasmic fraction of both vehicle- and estradiol-treated platelets (results not shown). Estradiol significantly stimulated the biosynthesis of 12-HPETE, 12-HETE and the more polar products.

We have reported that the presence of 0.5 to 1.5 mM GSH in the reaction mixture prevents the formation of the more polar products and produces 12-HETE as the only metabolite from arachidonic acid by the 12-lipoxygenase pathway [8]. Thus, the routine assays of 12-lipoxygenase were carried out in the presence of 1 mM GSH in the reaction mixture in the following experiments, and the 12-lipoxygenase activity was expressed by the formation of 12-HETE. Table 3 demonstrates the stimulation of 12-HETE production in rat platelets by estradiol as a function of frequency of treatment. When rats were treated with 0.5 mg estradiol/kg body weight, a significant stimulation of 12-lipoxygenase activity

Table 2. Effects of estradiol on the transformation of arachidonic acid by the platelet lipoxygenase pathway*

| Groups | No. of rats | 12-HETE | 12-HPETE | More polar products | 12-Lipoxygenase activity |
|---|---|---|---|---|---|
| | | [nmoles · min$^{-1}$ · mg$^{-1}$] | | | |
| Control | 4 | 0.48 ± 0.04 (100%) | 0.26 ± 0.01 (100%) | 1.47 ± 0.19 (100%) | 2.21 ± 0.20 (100%) |
| Estradiol | 3 | 1.08 ± 0.07 (225%)† | 0.64 ± 0.08 (246%)† | 2.84 ± 0.20 (193%)† | 4.56 ± 0.17 (206%)† |

* Estradiol administration and the enzyme assay were performed as described in Fig. 1. 12-Lipoxygenase activity represents the sum of 12-HETE, 12-HPETE and the more polar products. Values are means ±S.E.M. and those in parentheses are the percentages compared to their parallel controls. P values were determined by Student's *t*-test.
† $P < 0.01$.

Table 3. Effect of estradiol on 12-lipoxygenase activity*

| Groups | 12-HETE [nmoles · min$^{-1}$ · mg$^{-1}$] | | |
|---|---|---|---|
| | 1 Injection | 3 Injections | 5 Injections |
| Control | 2.26 ± 0.06 (100%) | 2.20 ± 0.19 (100%) | 2.04 ± 0.04 (100%) |
| Estradiol | 3.10 ± 0.06 (137%) | 4.55 ± 0.12 (207%) | 3.91 ± 0.21 (192%) |

* Rats were injected at 3- to 4-day intervals with 0.5 mg estradiol/kg body weight as described in Materials and Methods. Cytosol protein prepared from $1.5 \times 10^8$ cells was used for each assay, and 1 mM GSH was included in the assay mixture. Each value is the mean ±S.E.M. from four individual animals. Values in parentheses are the percentages compared with their parallel controls.

in rat platelets was first observed on day 3 after the first injection of estradiol. The maximum stimulation was observed after three injections of estradiol. The stimulation of 12-lipoxygenase activity was found to be dose dependent, as shown in Fig. 2. The maximum stimulation was observed at a dose of 0.5 mg/kg body weight of estradiol. Direct addition of $10^{-8}$–$10^{-4}$ M estradiol to either cytosol fraction prepared from

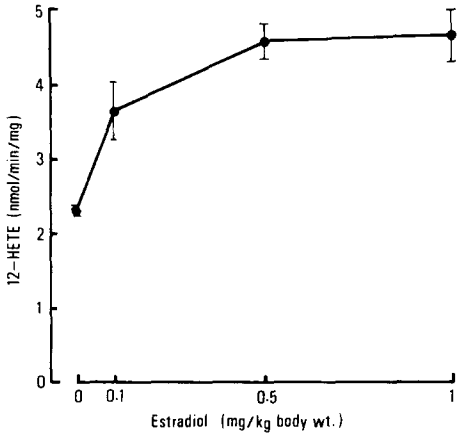


Fig. 2. Effect of estradiol concentration on the stimulation of 12-lipoxygenase activity. Rats were injected three times at 3 to 4-day intervals with various concentrations of estradiol. Cytosol protein prepared from $1.5 \times 10^8$ cells was used for each assay, and 1 mM GSH was included in the assay mixture. Each point is the mean of three individual animals.

control platelets or to platelet-rich plasma at 37° for 3 hr *in vitro* had no significant effect on 12-lipoxygenase activity. In contrast to estradiol, treatment of animals with testosterone did not have any significant effect on 12-lipoxygenase activity.

*Blockade of the stimulatory effect of estradiol on 12-lipoxygenase activity by nafoxidine HCl.* Table 4 shows that the simultaneous injection of the antiestrogen, nafoxidine HCl, together with estradiol blocked the increase in 12-lipoxygenase activity. Nafoxidine HCl had no significant effect on 12-lipoxygenase activity by itself; however, 67% of the 12-lipoxygenase activity induced by estradiol was blocked by it.

*Estradiol binding studies in platelets.* Using receptor binding assay, we could not demonstrate any significant binding of estradiol by either intact platelets or platelet cytosol fractions.

## DISCUSSION

We are interested in systematically studying the effects of estradiol on the arachidonate cascade at the blood-vessel interface. We previously reported that estradiol has no significant effect on the endogenous fatty acid (specifically, arachidonic acid) composition of cellular phospholipid fractions in cultured rat aortic smooth muscle cells [5]. Similar results were observed in rat platelets (Table 1). These results indicate that the endogenous pool of arachidonic acid in blood vessels is not affected by estradiol administration.

Table 4. Effect of the anti-estrogen, nafoxidine HCl, on 12-lipoxygenase activity*

| Groups | 12-HETE [nmoles·min$^{-1}$·mg$^{-1}$] | % of control |
|---|---|---|
| Control | 2.16 ± 0.08 | 100 |
| Nafoxidine | 2.29 ± 0.15 | 106 |
| Estradiol | 3.54 ± 0.38 | 164 |
| Estradiol + nafoxidine | 2.65 ± 0.01 | 122 |

* Nafoxidine HCl (5 mg/kg body wt) was injected simultaneously with estradiol (0.5 mg/kg body wt). Three treatments were performed; see Materials and Methods for detail. Cytosol protein prepared from $1.5 \times 10^8$ cells was used for each assay, and 1 mM GSH was included in the assay mixture. Each value is the mean ± S.E.M. from three individual animals.

Estradiol treatment of rats induces an increase in prostacyclin production by aortas, with no change in thromboxane and HHT production, and a significant increase in 12-HETE production by washed intact platelets [11]. The latter observation can be explained by a stimulation of 12-lipoxygenase activity in platelet cytosol fractions as shown in the present study. The increase in platelet lipoxygenase activity was blocked by the anti-estrogen, nafoxidine HCl (Table 4). The anti-estrogenic activity of nafoxidine HCl is thought to be due to its ability to reduce the number of estradiol–receptor complexes entering target nuclei [12]. The present results with nafoxidine HCl thus suggest that the stimulation of 12-lipoxygenase activity is due to the effect of estradiol.

At least two different enzymes are involved in the transformation of arachidonic acid into 12-HETE in platelets. The first enzyme is 12-lipoxygenase, which transforms arachidonic acid into 12-HPETE. 12-HPETE is then converted to 12-HETE by the second enzyme, 12-HPETE peroxidase. We have reported that 12-HPETE peroxidase is a GSH-dependent peroxidase. The more polar products might be formed from the non-enzymatic breakdown of 12-HPETE due to insufficient GSH-like reducing agent(s) in the subsequent peroxidase system to completely reduce 12-HPETE to 12-HETE [8]. Siegel *et al.* [13] recently reported that aspirin-like anti-inflammatory drugs inhibit 12-HPETE peroxidase activity and cause an accumulation of 12-HPETE, which results in stimulation of 12-lipoxygenase activity, using an *in vitro* preparation. In the present study, estradiol administration *in vivo* almost equally stimulated the formation of 12-HPETE, 12-HETE and more polar products by rat cytosol (Table 2), but estradiol had no significant effect on 12-lipoxygenase activity when it was added directly to the platelet cytosol *in vitro*. The results suggest that the mechanism of action of estradiol is different from that of aspirin-like anti-inflammatory drugs. Estradiol causes a direct induction of 12-lipoxygenase activity and also may possibly induce peroxidase activity in platelets.

The mechanism of steroid hormone action has now been fairly well elucidated (for reviews, see Refs. 14 and 15). Steroids generally diffuse passively into cells and bind to soluble receptors in the cytoplasm. The receptor–hormone complexes then become active and translocate into the nucleus where they bind to the chromatin, affecting gene expression. It is known that the platelet is an anucleate cell. It contains no DNA. Direct effects of steroid hormones on platelets would, therefore not be expected, and we did not detect any specific binding of estradiol to platelets in this study. Thus, stimulation of 12-lipoxygenase activity cannot be explained by a direct effect of estradiol on platelets. However, estradiol might cause induction of 12-lipoxygenase in megakaryocytes, the parent cells of platelets, in the bone marrow. Investigation along this line is under way.

Various fatty acid hydroperoxides that are analogs of HPETE have been shown to inhibit the formation of prostacyclin synthetase in porcine aorta [16] and other tissues [17]. However, Turk *et al.* [18] studied the possibility of 12-HPETE being liberated from platelets and inactivating vascular prostacyclin synthetase; they concluded that vascular prostacyclin is not likely to be influenced by 12-HPETE released from platelets. Thus, the possible physiological role of 12-HPETE, produced by platelets in the vascular system, may be primarily in platelet functions. Estradiol administration inhibits the aggregatory response of platelets in rabbits to ADP [19] and in pigeons to arachidonic acid [20]. Siegel *et al* [21] recently reported that 12-HPETE is able to block collagen- and arachidonate-induced platelet aggregation, which supports the idea that the increase in 12-lipoxygenase activity in platelets by estradiol may contribute to the anti-thrombogenic effect of estradiol.

Uzunova *et al* [22] have reported that male rats have approximately twice the thrombus size and death rate and a lower obstruction time compared to females, and estradiol treatment significantly decreases the thrombus weight and increases the obstruction time in male rats. However, no relevant biochemical evidence has been presented to account for this observation. The present result, showing the increase in 12-lipoxygenase activity in platelets, together with our previous report showing the selective increase in prostacyclin production by aortas after estradiol administration [11], may account, in part, for the explanation of the anti-thrombogenic effect of estradiol.

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