J. steroid Biochem. Vol. 26, No. 6, pp. 679–685, 1987
Printed in Great Britain.

0022-4731/87 $3.00 + 0.00

COOXIDATION OF STEROIDAL AND NON-STEROIDAL ESTROGENS BY PURIFIED PROSTAGLANDIN SYNTHASE RESULTS IN A STIMULATION OF PROSTAGLANDIN FORMATION

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(*Received* 13 *August* 1986)

Summary—Estrone (E_1), estradiol (E_2), the catechol estrogens 2-OHE_1 and 2-OHE_2, and diethylstilbestrol (DES) were incubated with purified prostaglandin synthase (PHS) *in vitro* in the presence of arachidonic acid and their PHS-catalyzed cooxidation was determined. 2-OHE_1, 2-OHE_2, and DES were extensively metabolized by PHS peroxidase activity, E_1 and E_2 to a lesser extent. The cooxidation of the estrogens is accompanied by an increased prostaglandin formation and an increase in cyclooxygenase activity *in vitro*; progesterone and nylestriol are without effect. Prostaglandins have been proposed to play a role in events related to early estrogen action in tissues such as the uterus. The cooxidation of estrogens and their metabolites by prostaglandin hydroperoxidase might represent one type of interaction between the hormones and the arachidonic acid cascade that could lead to changes in prostaglandins.

INTRODUCTION

Prostaglandins, prostacyclin, thromboxane, and leukotrienes are physiologically important compounds that are synthesized from arachidonic acid (AA). Ramwell *et al.* [1] highlighted data concerning the relationship of steroid hormones to prostaglandins which suggest that enzyme activities responsible for the synthesis and further metabolism of specific prostaglandins are under the control of gonadal hormones.

How estradiol (E_2) stimulates for example prostagladin production in the human uterus *in vivo* [2] or in human endometrium in organ culture [3] is unknown. It could involve the established estrogen receptor hypothesis in which action of estrogens at the genomic level lead to *de novo* synthesis of prostaglandin-synthesizing enzyme(s). However, a different mechanism appears to be required to explain data by Castracane and Jordan [4] who find that administration of an estrogen antagonist or of protein synthesis inhibitors do not block the estrogen-stimulated prostaglandin-synthesis in rat uterine tissue and plasma. The mechanism of *in vivo* stimulation of uterine prostaglandin biosynthesis in response to E_2 is difficult to delineate since various interactions of the steroid hormone with the arachidonic acid cascade can occur, for example modulation of phospholipase A_2 activity [5].

Few cell free *in vitro* studies of the direct effects of estrogens on prostaglandin formation have been reported. Naylor and Poyser [6] studied the effect of E_2 addition on the *in vitro* production of $PGF_{2\alpha}$ by guinea pig uterus homogenate incubations and found an almost 2-fold stimulation. Kelly and Abel have measured the prostaglandin production *in vitro* with rat uterine and with human endometrial tissue homogenates; addition of E_2 or various catechol estrogens stimulated prostaglandin formation [7, 8]. However, even the mechanism of the estrogen-induced *in vitro* rise in prostaglandin levels can be a complex one. Studies in crude tissue homogenates may not necessarily determine a direct effect of the estrogen upon the PHS complex: metabolism of the estrogen can take place in these incubations and endogenous factors may further modify the effect; additional effects on other prostaglandin metabolizing enzymes (e.g. isomerases, 15-hydroxy-prostaglandin-dehydrogenase; 9-ketoreductase) could also occur. It was therefore necessary to simplify the *in vitro* system even further and to investigate whether estradiol or its metabolites can stimulate prostaglandin formation by acting directly upon PHS.

Many compounds are oxidized as co-substrates during formation of prostaglandin from arachidonic acid; these compounds serve as reducing co-factors for the hydroperoxidase of PHS [9]. The converse reaction, formation of prostaglandin during oxidation of estrogens, has not been well studied. However, Ohki *et al.* [10] have reported that several endogenous or exogenous phenolic or catecholic compounds stimulate the conversion of AA to prostaglandin H_2. We recently observed that a non-steroidal estrogen, DES, is an excellent co-substrate

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for PHS [11], being oxidized to Z,Z-DIES; thus, we were interested in whether cooxidation of estrogens may contribute to a stimulation of prostaglandin synthesis.

The present study was designed to investigate whether DES and steroidal estrogens directly affect the formation of prostaglandins from AA *in vitro* and whether steroidal estrogens and their metabolites are oxidized by PHS. In order to study the effect of various estrogens on this particular step of prostaglandin biosynthesis directly, we have used purified reconstituted PHS. In this communication we report that the catechol estrogens 2-OHE_1 and 2-OHE_2, which are important *in vivo* metabolites of E_1 and E_2, can stimulate *in vitro* prostaglandin formation through cooxidation by prostaglandin hydroperoxidase; E_1 and E_2 also stimulate prostaglandin formation but are much less effective.

EXPERIMENTAL

Chemicals

Monoethyl-[2-^{14}C]diethylstilbestrol (Amersham-Searle Corp., Arlington Heights, IL) was recrystallized with unlabeled DES (Sigma Chem. Co., St Louis, MO) as described before [11] and proved to be of 98% purity as determined by HPLC (see below). Z,Z-DIES reference compound was a gift from Dr M. Metzler (Institute of Toxicology, University of Würzburg, F.R.G.). [6,7-^{3}H]E_2, [6,7-^{3}H]E_1, [6,7-^{3}H]2-OHE_1, were from New England Nuclear Co. (Boston, MA) and mixed with unlabeled steroids from Steraloids, Inc. (Wilton, NH) to give final specific activities between 0.7 and 1.0 μCi/μmol; radiochemical purity as tested by HPLC was at least 93%. [^{3}H] and [^{14}C]AA were obtained from New England Nuclear (Boston, MA) and unlabeled AA was purchased from NuChek (Elysian, MN); radiolabeled prostaglandin reference compounds ([^{3}H]$PGF_{2\alpha}$, [^{3}H]PGE_2, [^{3}H]PGD_2) were obtained from New England Nuclear. Indomethacin, hematin, and progesterone were from Sigma Chemical Co. (St Louis, MO). Nylestriol was from Eli Lilly, Co. (Indianopolis, IN). All other chemicals were of the highest purity commercially available and solvents were HPLC grade.

Enzyme preparation

Ram seminal vesicles were obtained from a local slaughterhouse, trimmed of excess fat and connective tissue, and stored at −70°C until use. Microsomes were prepared as previously described [12]. Microsomal PHS activity was determined by measuring the AA-dependent oxygen uptake using a Clark-type electrode; ram seminal vesicle microsomes with low enzyme activity were discarded. Purified PHS was purchased from Oxford Biochemicals (Oxford, MI; sp. act. = 30,000 U/mg).

Incubations

Incubations were carried out at 37°C in 0.1 M phosphate buffer, pH 7.4, 1.0 or 2.0 ml total volume: DES or the steroidal estrogen at the concentrations indicated was added in ethanol (not exceeding 1.5% final concentration): purified enzyme was reconstituted with hematin (1 μM) and the mixture was preincubated for 2–3 min. Reactions were initiated by addition of AA and stopped after 3 min by cooling on ice and immediate extraction; incubations carried out to measure the cooxidation of DES under various conditions were extracted as a whole with 3 times the 2-fold volume of ether. Incubations carried out to measure prostaglandin formation and conversion of the estrogenic co-substrate contained a combination of either [^{3}H]AA and [^{14}C]DES or of [^{14}C]AA and ^{3}H-labeled estrogen and were split in two 0.9 ml aliquots before extraction.

Estrogen analysis

Incubations were extracted with ether and the organic layers were evaporated under vacuum. The residues were dissolved in 200 μl methanol and subjected to HPLC analysis. Aliquots of the non-extractable aqueous phases were taken for LSC. Identification and quantitation of DES and its metabolites were carried out as published previously [11] with the following modifications: HPLC analysis was done using a Zorbax Sil ODS column (DuPont, Wilmington, DE) eluted with a linear water–methanol gradient (increasing from 50–88% methanol over 30 min at a flow rate of 1 ml/min). The effluent was monitored at 254 nm and collected in 0.3 min fractions in which radioactivity was determined by LSC. Radioactivity coeluting with E-DES, Z-DES and Z,Z-DIES was used to calculate the oxidative metabolism of DES.

Extracts from incubations with estrogen and catechol estrogens were chromatographed on the same reverse phase column eluted with a linear methanol–water gradient (increasing from 55–100% methanol over 30 min, flow rate 1 ml/min). The U.V. absorption of the column eluates was monitored at 280 nm and radioactivity in fractions measured by LSC. Radioactivity in fractions coeluting with the parent compound was used to calculate the amount of unchanged parent compound.

Analysis of arachidonic acid metabolism

Incubation aliquots (0.9 ml) were acidified to pH 3.0–3.5 and extracted twice with ethylacetate (4.0 ml). The organic layer was removed, dried over anhydrous Na_2SO_4, and evaporated. The residues were redissolved in 0.1 ml acetone and aliquots were analyzed. Prostaglandins were separated by HPLC as described previously and quantitated by measurement of radioactivity in eluted peaks by liquid scintillation techniques [13]. The peaks were identified by coelution with authentic standards.

RESULTS

Stimulation of arachidonic acid metabolism by estrogens

The biosynthesis of prostaglandins from AA requires molecular oxygen for the cyclooxygenase-catalyzed formation of prostaglandin endoperoxide-hydroperoxide (PGG_2) and the presence of a reducing co-factor for the hydroperoxidase activity for PHS for the conversion of PGG_2 to PGH_2. Since previous studies [10, 14] suggested that phenolic or catechol compounds would serve as reducing co-factor for the hydroperoxidase and thus stimulate overall AA metabolism, we measured the oxygen incorporation into AA catalyzed by PHS from ram seminal vesicle microsomes. DES, the phenolic estrogen E_2 and the catechol estrogen 2-OHE_2 all produced a concentration dependent stimulation of oxygen uptake. DES appeared to be the best reducing co-factor in that maximum stimulation was observed at lower concentrations (data not shown). The addition of different estrogens to the enzyme preparation produced a stimulation in oxygen uptake (Fig. 1a). In the absence of a reducing co-factor, only negligible incorporation of oxygen was observed. The phenolic estrogens, E_1 and E_2, produce a small but significant increase in oxygen uptake while the catechol estrogens, 2-OHE_1, 2-OHE_1, and DES, were very potent stimulators of PHS-cyclooxygenase catalyzed oxygen incorporation.

On the other hand, nylestriol, an estrogen compound with no free hydroxyl group in the A ring was unable to stimulate PHS cyclooxygenase activity (Fig. 1b) and also progesterone, a steroid hormone which has been implicated in the hormonal regulation of prostaglandin biosynthesis, did not show any direct effect on PHS *in vitro* between 10 and 250 μM (Degen and Schlatterbeck, unpublished data).

Metabolism of estrogens by PHS

In acting as a reducing co-factor for the hydroperoxidase activity of PHS, compounds are themselves oxidized [9]. We have previously shown that DES is oxidized by PHS from ram seminal vesicle microsomes to its metabolite Z,Z-DIES [11].

Here we studied the conversion of ^{14}C-labeled DES at various concentrations of AA and purified enzyme. HPLC analysis revealed that Z,Z-DIES was the only extractable oxidative metabolite formed from [^{14}C]DES in the presence of purified reconstituted PHS and AA. Formation of Z,Z-DIES was dependent upon the amount of enzyme (Fig. 2). Controls without enzyme, or hematin, produced only background levels of DES-metabolite. AA concentrations above 50 μM did not further increase the cooxidation of DES to Z,Z-DIES (Fig. 3). DES oxidation also depended upon the DES concentration, and Z,Z-DIES formation was found to increase up to 100 μM DES (Fig. 4). Higher DES concentrations inhibited its cooxidation as observed

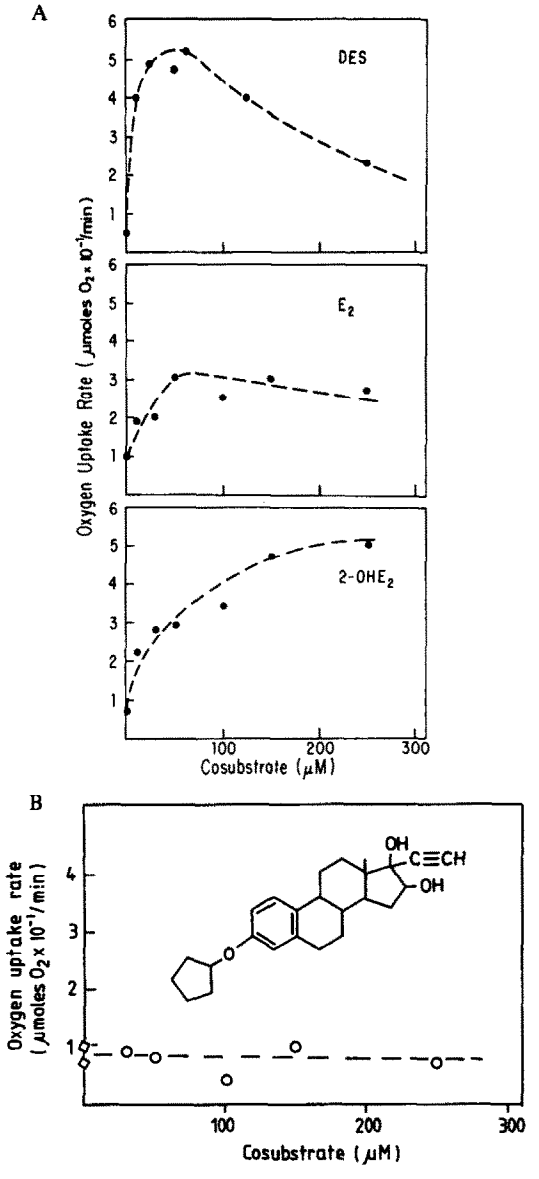

Fig. 1. Stimulation of PHS-catalyzed oxygen incorporation into AA by estrogen. Oxygraph tracings are shown from 2.0 ml incubations containing ram seminal vesicle microsomes (0.2 mg/ml) estrogens (0–250 μM) or solvent only (20 μl ethanol) in phosphate buffer (0.1 M, pH 7.4). The mixtures were stirred for 2 min at 37°C before addition of AA (100 μM) and were incubated for another 3 min. A. Incubations with DES, estradiol (E_2), or 2-hydroxy estradiol (2-OHE_2). B. Incubation with nylestriol.

in incubations with ram seminal vesicle microsomes, where DES concentrations above 100 μM did not further increase oxygen incorporation into AA.

The other estrogens shown to stimulate arachidonic acid metabolism (Fig. 1) should also be oxidized by PHS. Since peroxidative metabolism of E_2 or catechol estrogens as studied by others [15, 16] does not lead to an easily identifiable product as it is the case for DES, metabolism of the steroidal estrogens was estimated either based on the amount of recovered parent compound or on the amount of

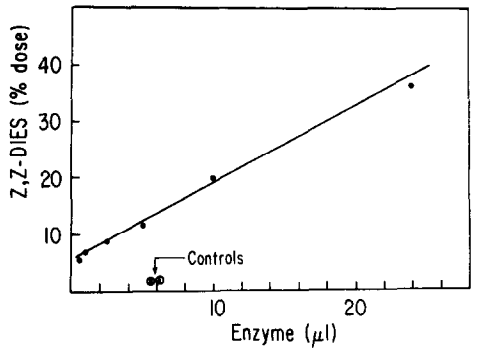

Fig. 2. DES-cooxidation in the presence of varying amounts of PHS. Purified enzyme (10 μl ≙ 300 U) was incubated in 1.0 ml buffer (Hepes, pH 7.8) containing hematin (1 μM), [^{14}C]DES (62 μM), and AA (200 μM) at RT for 3 min. Reactions were stopped by cooling on ice and immediate extraction with ether. Controls with no hematin or no enzyme were treated identically. Extracts were analyzed by HPLC for metabolism of DES as described in the Experimental section. The amount of Z,Z-DIES formed is shown to depend on the amount of PHS added to individual incubations and is negligible in the controls.

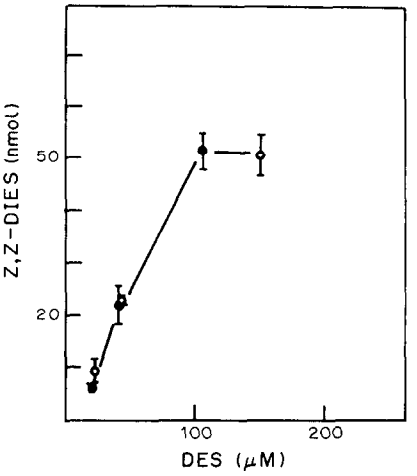

Fig. 4. DES-cooxidation catalyzed by PHS. Incubations as described in Fig. 2 containing purified PHS (300 U), AA (100 μM), and varying concentrations of DES (20–150 μM) were analyzed as described in the Experimental section. Z,Z-DIES formation was found to increase with co-substrate (DES) concentration up to 100 μM.

water soluble radioactivity formed in the incubations. As seen in Table 1, both the phenolic and catechol estrogens were extensively oxidized. Approximately 20% of the added phenol estrogens were metabolized while the catechol estrogens were essentially completely degraded. The recovery of radiolabeled E_1 and of 2-OHE_1 from control incubations in the absence of enzyme was 95 and 86%, respectively, indicating some non-enzymatic degradation occurred, particularly with the catechol estrogens. In complete incubations with PHS, no catechol estrogens were recovered unchanged, and about half of the radioactivity from [^{3}H]2OHE_1 was found as water soluble, non-extractable material. The more extensive metabolism of the catechol estrogens compared to E_1 and E_2 is in agreement with a more efficient stimulation of prostaglandin synthesis and their ability to serve as a reducing co-factor for the peroxidase.

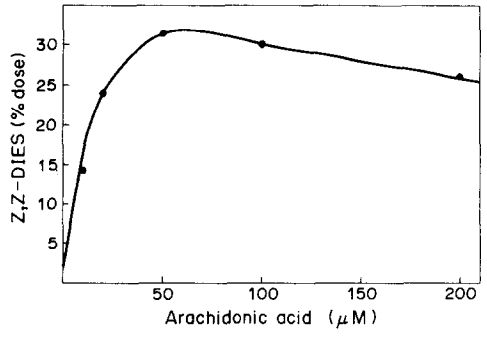

Fig. 3. DES-cooxidation catalyzed by PHS in the presence of varying amounts of co-factor AA. Incubations containing radiolabeled DES, purified PHS (300 units), and AA (10–200 μM) as in Fig. 2 were analyzed as described there. DES oxidation leading to Z,Z-DIES was found to be optimal between 50–100 μM AA.

Effect of estrogens on prostaglandin formation

The phenolic and catechol estrogens stimulated PHS-cyclooxygenase activity. Since oxygen incorporation into AA can be a measure of the formation of the endoperoxide PGH_2, this should also be reflected in an increased production of PGH_2. PGH_2 was estimated by HPLC analysis of the decomposition products, which were PGE_2, PGD_2, and HHT with smaller amounts of $PGF_{2\alpha}$ found (Table 2). The

Table 1. PHS-catalyzed metabolism of steroidal estrogens

Estrogens added to incubation (150 μM)	Water soluble radioactivity (% of dose)	Remaining parent compound (% of dose)	Estrogen conversion (nmol/mg/3 min) min–max*
E_1	8.2 ± 1.1	79.2 ± 10.1	28.9–62.4
20H-E_1	49.1 ± 2.4	0	148–300
E_2	9.4 ± 2.1	78.6 ± 15.6	32.1–64.3
20H-E_2	N.A.†	0	N.A.–300

Incubations were carried out at 37°C in 2.0 ml phosphate buffer (0.1 M, pH 7.4) with 300 U purified PHS, hematin (1 μM), ^{3}H-labeled estrogen, initiated by addition of [^{14}C]AA (100 μM), and analyzed as described in the Experimental section.

Incubations were done in triplicate and data given are mean values ± SD.

*Estimated conversion was calculated as a range: max from (100% − x% parent compound recovered), min from water soluble radioactivity formed; 1% conversion is equivalent to 3 nmol estrogen converted in a 2.0 ml incubation with 150 μM estrogen.

†N.A., not analyzed because ^{3}H-labeled 20H-E_2 was not available. In extracts, no unchanged parent compound was detectable by HPLC/u.v. analysis.

Table 2. Effect of various estrogens on arachidonic acid metabolism by prostaglandin synthase *in vitro*

Addition	HHT (nmol/mg/3 min)	Total PGs (nmol/mg/3 min)	Prostaglandin pattern (% of total prostaglandins) $PGF_{2\alpha}$	PGE_2	PGD_2
None	4.9 ± 0.54	7.3 ± 1.9	—	51.2 ± 3.3	48.8 ± 3.3
E_2	8.0 ± 1.08	15.8 ± 2.8	14.8 ± 0.8	61.6 ± 1.0	23.6 ± 0.3
E_1	8.2 ± 0.6	12.4 ± 2.5	15.6 ± 3.7	58.2 ± 2.6	26.2 ± 5.3
2-OH-E_2	9.6 ± 0.6	46.2 ± 4.8	29.2 ± 0.9	53.9 ± 1.7	16.9 ± 1.3
2-OH-E_1	8.8 ± 0.48	35.6 ± 3.0	26.1 ± 0.7	61.7 ± 4.4	12.3 ± 4.9

Incubations with estrogens (150 μM) were carried out in triplicate and analyzed for prostaglandins as described in the Experimental section.

products were formed nonenzymatically since the enzymes that convert PGH_2 to prostaglandins were not present in the purified enzyme preparation. These results (Tables 2 and 3) are in agreement with previous studies on PGH_2 decomposition [17]. Addition of phenolic and catechol estrogens at equimolar concentrations increased total PGH_2 formation as measured by HPLC analysis of the products; E_2 and E_1 about 2-fold, the catechol estrogens 4–5-fold (Table 2). Moreover, analysis of the prostaglandins found indicated that the presence of reducing cofactor also altered the decomposition of PGH_2. An addition of an estrogen increases $PGF_{2\alpha}$ and decreases PGD_2 formation. This effect was most notable with the catechol estrogens (Table 2). These results indicated a non-enzymatic reduction of PGH_2 to $PGF_{2\alpha}$, a finding previously seen with lipoic acid [18].

Likewise, the addition of DES to the purified PHS increased AA-dependent O_2-consumption rates and this appeared to correlate with PGH_2 formation as measured by HPLC. Addition of DES yielded a concentration dependent increase in oxygen uptake and in total PG formation (Table 3) and was accompanied by the cooxidation of DES (Fig. 4).

The relationship between net increase in prostaglandin H_2 biosynthesis in the presence of DES and DES-oxidation can be examined further. DES oxidation to the metabolite Z,Z-DIES was used to estimate DES-oxidation and was compared with the net increase in PGH_2 biosynthesis as measured by HPLC analysis of the nonenzymatic decomposition products and corrected for background production found in the absence of DES. The presence of DES in an incubation mixture produced an increased formation of PGH_2, with the concomitant formation of Z,Z-DIES.

Taken together, the data in Tables 2 and 3 as well as the stimulation of the cyclooxygenase activity (Fig. 1) show that all the tested estrogens stimulate prostaglandin formation *in vitro*. These compounds while being metabolized act directly on the PGS, however, with different potencies: 2-OHE_2 and 2-OHE_1 stimulate prostaglandin formation as well as if not better than DES; E_2 and E_1 stimulate also but less efficiently.

DISCUSSION

In the present study catechol estrogens and DES were metabolized very rapidly by PHS *in vitro* in the presence of AA; the estrogens themselves showed much less conversion. PHS-dependent oxidation is catalyzed by the hydroperoxidase activity, and, thus, PHS present in the uterus may be involved in the peroxidative metabolism of catechol estrogens in this tissue. Indeed, Kelly and Abel [7, 8] observed rapid "degradation" of catechols in their *in vitro* incubations with uterine tissue homogenates, but no product analysis was reported.

Our incubations with radiolabelled estrogens showed that the catechol estrogen 2-OHE_1 was metabolized by PHS probably involving the oxidation of catechol estrogens to free radical intermediates with formation of o-semiquinones and quinones as demonstrated recently by electron spin resonance techniques in incubations of 2- and 4-hydroxyestradiol with horseradish peroxidase or during autooxidation

Table 3. Effect of DES on the *in vitro* metabolism of AA by PHS

Incubation addition of DES (μM)	Initial O_2 uptake rates (nmol/min)	HHT	Prostaglandins total (nmol/mg/3 min)	Total PGH_2 estimation
Non	75.6	12.2 ± 3.0	4.88 ± 1.6	17.08
DES 3	270.0	11.4 ± 1.4	19.08 ± 3.1	30.48
DE[illegible]21	288.0	14.4 ± 1.6	22.14 ± 3.3	36.54
[illegible]S-54	615.6	19.1 ± 0.64	23.04 ± 3.4	42.14

Incubations were carried out with stirring in an oxygraph at 37°C under air in 2.0 ml phosphate buffer (0.1 M, pH 7.4) with 210 U purified PHS, hematin (1 μM) and with [^{14}C]DES (0–54 μM). After 2 min preincubation, the reaction was initiated by addition of [^{3}H]AA (100 μM), and after 3 min terminated by withdrawing two 0.9 ml aliquots which were immediately cooled and extracted for further analysis for prostaglandins as described in the Experimental section. The data presented are from triplicate incubations and represent mean values ± SD.

[19]. The PHS-catalyzed formation of reactive intermediates from steroidal estrogens has been recently demonstrated *in vitro* [20] and may be of toxicological significance for the metabolic activation of these compounds in estrogen target tissues.

During their metabolism by PHS, catechol estrogens and DES are effective in stimulating *in vitro* prostaglandin formation in purified enzyme preparations as measured by oxygen incorporation and HPLC analysis. E_1 and E_2 at the same concentration as used for the catechol estrogens are less efficient in stimulating the total prostaglandin formation. They are converted in part by the peroxidase activity of PHS to unidentified water-soluble radioactive metabolites. Likewise, other peroxidases convert E_2 to water-soluble products and the amounts of these products correlated with enzyme activity [16].

As observed in our experiments with purified PHS, a difference in the extent of prostaglandin stimulation by catechol estrogen and by estradiol has been observed in incubations of uterine tissue homogenates [6, 7]. Moreover, in one study, the pattern of prostaglandins produced by the uterine tissue preparations (especially $PGF_{2\alpha}$ and PGI_2) was influenced by the compound tested [7]. In our present study, a shift in the pattern of prostaglandins was observed with an increased proportion of $PGF_{2\alpha}$ in incubations with 2-OHE_2 or 2-OHE_1 compared to those with E_2 or E_1. This suggests that the non-enzymatic decomposition of PGH_2 is altered in the presence of reducing compounds like the catechol estrogens. Thus, estrogens not only increase total prostaglandin formation *in vitro* but can preferentially increase a particular prostaglandin ($PGF_{2\alpha}$).

Stimulation of prostaglandin synthesis by estrogens and catechol estrogens *in vitro* in homogenates of different tissues has been reported [6–8, 21], and our *in vitro* experiments support the concept that the catechol estrogens are more efficient than the parent estrogen in stimulating prostaglandin biosynthesis through cooxidation [22].

It is attractive to speculate that increased prostaglandin-formation observed as early as 1 h after a single E_2 injection *in vivo* [23] could be mediated, in part, by a direct interaction of the estrogen or its metabolites with the prostaglandin-synthesizing enzymes. Conflicting experimental data obtained with inhibitors of prostaglandin synthesis (e.g. indomethacin) for E_2 induced early wet weight gain both support [24, 25] and challenge [26–28] the view that prostaglandins may mediate early estrogenic effects. In this context it is interesting that nylestriol did not undergo PHS-catalyzed cooxidation as indicated by its lack of stimulating PHS cyclooxygenase *in vitro*. Nylestriol is a potent estrogen with delayed tissue response *in vivo* but fails to induce the early (1 h) increase in wet weight as observed with E_2 in the rat or mouse uterus [29; Korach, pers. commun.]. The delayed *in vivo* response to nylestriol is most likely due to the slow hydrolysis of the cyclopentyl ether function of the molecule; in the present *in vitro* study, the lack of comparable hydrolysis results in a compound which does not participate in cooxidation.

The observation that PHS-mediated metabolism of estrogens is accompanied by increased prostaglandin formation *in vitro* was made at pharmacological concentrations in our experiments. Yet, one should keep in mind that breast tumor tissue can contain E_2 levels 50–300-fold higher than circulate in plasma [30], and local production via sulfatase and aromatase [31] could provide high cellular concentrations of hormone. Whether the principle that estrogens and their metabolites can have a direct effect on PHS leading to increased prostaglandin formation *in vitro* may be relevant for the *in vivo* situation clearly requires further experiments which are underway.

Acknowledgements—We thank Jill Shirley for technical asistance and Sandy Sandberg and Anna Lee Howard for manuscript typing; Drs Valerie Quarmby and Gregory Reed made helpful comments on the manuscript; the Deutsche Forschungsgemeinschaft gave partial support to Dr G. H. Degen.

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