NON-ESTROGENIC METABOLITES OF DIETHYLSTILBESTROL PRODUCED BY PROSTAGLANDIN SYNTHASE MEDIATED METABOLISM

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ABSTRACT

Incubation of trans-diethylstilbestrol (E-DES) with prostaglandin synthase (PGS) in vitro leads to the formation of the metabolites cis, cis-dienestrol (Z,Z-DIES) and cis-diethylstilbestrol (Z-DES) which have considerably decreased estrogenic activity compared to their parent compound. Incubations of (^{14}C)-E-DES with PGS in the presence of arachidonic acid (AA) predominantly catalyze formation of the oxidative metabolite Z,Z-DIES, accompanied by the formation of protein bound radioactivity. Inhibition of peroxidative metabolism through addition of indomethacin or absence of AA favors isomerization of E-DES to Z-DES without concomitant formation of protein bound radioactivity. Isomerization is inhibited by phenidone (1-phenyl-3-pyrazolidone). Since PGS activity is present in uterine tissue, these pathways may play a role in the metabolism of DES in its target tissue.

INTRODUCTION

Diethylstilbestrol, a synthetic estrogen, is a suspected carcinogen in humans (1) and has been found to induce tumors of the genital tract in female mice after exposure in utero (2,3). It is not yet known whether this transplacental carcinogenicity is related to the estrogen's hormonal activity or to its metabolic activation, in analogy with classical chemical carcinogens. DES metabolism has been studied in vivo and in vitro (4) and the hormonal and estrogen receptor binding activities of the metabolites have been investigated (5,6). It is known that DES itself can exist in two stereoisomeric forms: E-DES possesses estrogenic activity comparable to estradiol; Z-DES is only weakly active, based on

its interaction with the estrogen receptor in vitro (6). Nonenzymatic isomerization of E-DES to Z-DES has been reported previously (7). Here we wish to report data showing that a mammalian peroxidase, prostaglandin synthase (PGS), is involved in catalyzing the formation of Z-DES in vitro. Recently, this enzyme has been shown to catalyze the cooxidation of DES producing Z,Z-DIES (8), another metabolite with no or very weak estrogenic activity as measured both by uterotropic and estrogen receptor assays (5). PGS activity has been found in the rodent uterus (9) and in human endometrium (10) and it may well play a role for the metabolism of DES in the target tissue.

RESULTS AND DISCUSSION

Ram seminal vesicle microsomes (RSVM) are a rich source of PGS. PGS possesses two enzyme activities: a cyclooxygenase activity catalyzing the formation of prostaglandin endoperoxide PGG_2 from arachidonic acid (AA), a step which is specifically inhibited by indomethacin; and a peroxidase activity catalyzing the formation of prostaglandin precursor prostaglandin H_2. During the reduction of PGG_2 to PGH_2, many different xenobiotics can be oxidized (11).

Metabolism of (^{14}C)-DES (>97% E-DES) mediated by PGS was studied under various conditions by inhibiting the cyclooxygenase and/or the peroxidase activity of the enzyme and then comparing the pattern of metabolites to those from incubations with inactivated enzyme or buffer controls. Figure 1 depicts the extractable radioactivity identified as parent compound E-DES, its weakly estrogenic isomer Z-DES, the peroxidative metabolite Z,Z-DIES, and non-extractable radioactivity bound to

microsomal protein in incubations of (^{14}C)-DES with PGS from RSVM in the presence of AA. The data show that the formation of Z,Z-DIES is dependent upon the presence of active enzyme and arachidonic acid and is inhibited by indomethacin. Formation of both Z,Z-DIES and bound radioactivity in these incubations (Figure 1) are inhibited by indomethacin in a 'dose dependent' manner, indicating that the formation of Z,Z-DIES can give rise to reactive intermediates capable of binding to protein. Peroxidative metabolism of DES catalyzed by horseradish perox-

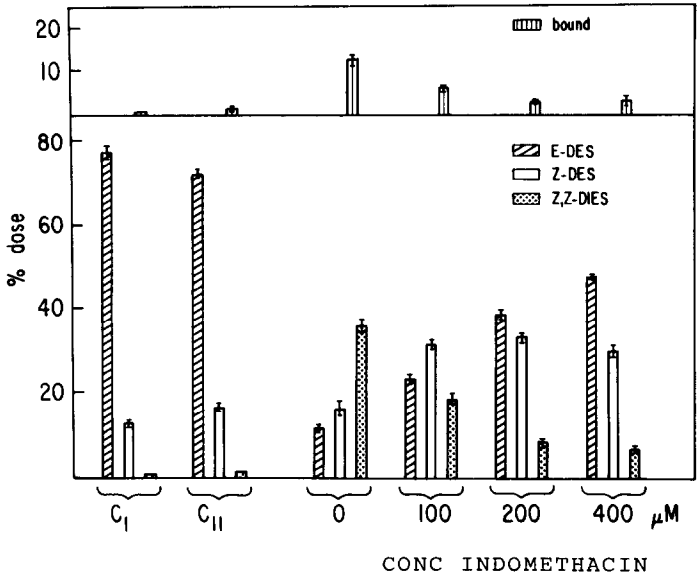

Figure 1: Profile of metabolites from incubations of DES with RSVM (1 mg protein/ml) and AA (0.6 mM). (^{14}C)-E-DES (65 μM) was incubated in 2.0 ml total volume of phosphate buffer (0.05 M, pH 7.6) containing indomethacin (final conc. 0, 100, 200, or 400 μM) and AA for 5 min at RT (2 min preincubation, then 3 min incubation with AA). Controls containing no enzyme (C I) or boiled microsomes (C II) were treated identically. Incubations were terminated by addition of 8.0 ml ethanol, and precipitated protein removed by filtration, washed with ethanol and ether, and digested with tissue solubilizer for LSC as published previously (8); protein bound radioactivity is shown in the top panel. Soluble radioactivity was further analyzed by HPLC as described in the experimental section, and metabolites appear in the bottom panel.

idase or mouse uterine peroxidase also leads to macromolecularly bound material, as observed by others (12).

Moreover, the absolute amounts of Z-DES in our incubations containing indomethacin are about doubled when compared to controls or non-inhibited incubations. These levels of Z-DES exceed those expected for chemical isomerization or those measured in control incubations without or with denatured enzyme. Furthermore, if we compare the ratios of Z-DES to E-DES found in incubations with no or inactivated enzyme, with the ratios from incubations with active or partially inhibited PGS, the changes are even more pronounced; the ratios for (Z-DES/E-DES) in Figure 1 are: 0.16 and 0.23 for controls but 1.41, 1.35, 0.86 and 0.63 for incubations with 0, 100, 200, and 400 μM indomethacin, respectively. In a non-enzymatic chemical isomerization, the ratio between trans- and cis- isomers would be expected to remain stable, independent of the amount of E-DES utilized for cooxidation. The elevated levels of Z-DES, together with the dramatic changes in the ratio of the isomers in incubations containing PGS compared to controls, is indicative for an enzyme mediated formation of the Z-isomer of DES. However, this does not give conclusive evidence about the nature of the enzyme involved in the isomerization reaction. The following experiments were designed to clarify this point.

Figure 2 A shows the rapid formation of Z,Z-DIES in an incubation of (^{14}C)-E-DES with RSVM in the presence of AA. As previously reported, the formation of this oxidative metabolite is catalyzed through the peroxidase activity of the enzyme PGS, is dependent upon AA or peroxides, can be inhibited by indomethacin or phenidone (1-phenyl-3-pyra-

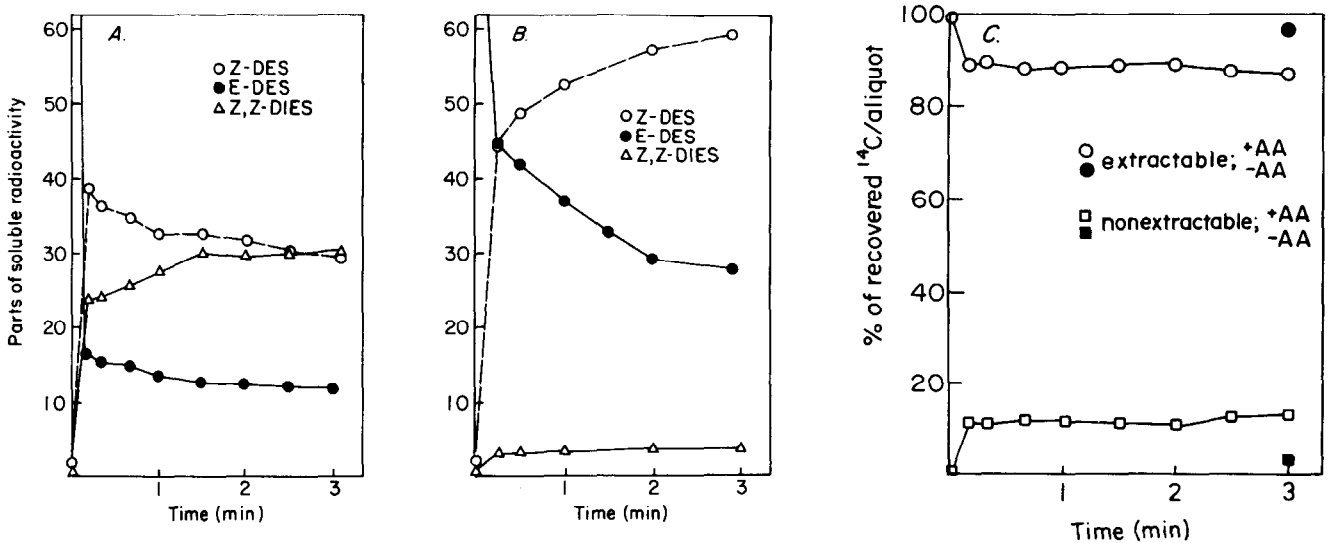

Figure 2 : Time course of the AA-dependent (A) and independent (B) metabolism of (^{14}C)-DES with RSVM. Incubations were carried out at RT in a total volume of 10 ml phosphate buffer (0.05 M, pH 7.5) containing (^{14}C)-E-DES (56 µM) and RSVM (1 mg protein/ml). The timed reaction was started by addition of AA (final conc. 0.2 mM, in 7 µl ethanol) (A) or ethanol (B). 1.0 ml aliquots were withdrawn at specific times, and immediately mixed with 4.0 ml ice-cold ethanol to stop the reaction. The distribution of radioactivity in the extracts and in the protein precipitates from the incubations with or without AA is shown in (C).

zolidone) (8), and therefore fulfills the criteria established for other xenobiotics undergoing cooxidation (11). Figure 2 B shows the fate of (^{14}C)-E-DES incubated with RSVM without the addition of AA: Z-DES becomes the major component isolated at various times during the incubation.

Z-DES formation is very rapid within the first minute and continues for the next two minutes. Only small amounts of Z,Z-DIES are formed, probably due to some endogenous AA. Figure 2 C depicts differences in the formation of non-extractable and extractable radioactive material as they relate to the metabolite patterns found in incubations with or without exogenous AA (Fig. 2 A and B). Efficient cooxidation leading to Z,Z-DIES is accompanied by formation of non-extractable, protein associated radioactivity whereas incubations without AA, in which isomerization of E-DES to Z-DES dominates, do not contain significant amounts of bound radioactive material.

Enhanced levels of Z-DES are found in incubations where cooxidation leading to Z,Z-DIES formation is inhibited by indomethacin or lack of AA. This could be explained by two different mechanisms: (a) under conditions where PGS is not carrying out efficient cooxidation of DES, it may preferentially catalyze the isomerization of trans-DES (E-DES) to the cis-isomer (Z-DES), possibly through the peroxidase activity which is not inhibited by indomethacin; (b) if DES is not utilized as a substrate for cooxidation by PGS, other enzyme activities present in RSVM may utilize more effectively E-DES as a substrate for enzyme-catalyzed isomerization.

In order to study these possibilities, we carried out incubations of (^{14}C)-E-DES with purified reconstituted PGS. If enhanced formation of Z-DES and changes in the ratio of Z- to E-isomer compared to controls with no enzyme are observed, then the first hypothesis (a) suggesting that isomerization is not catalyzed by enzyme activities other than those of PGS would be supported. Tables 1 and 2 contain the results of these experiments.

Data from two independent experiments using incubations of DES with purified enzyme show that in the absence of enzyme, both the absolute levels of Z-DES and the ratio of Z- to E-DES are lowest compared to incubations containing purified PGS. Table 1 shows, moreover, that addition of indomethacin inhibited cooxidation by 30 %, and increased Z-DES formation by 200 % compared to a complete incubation with no inhibitor, an effect seen before with RSVM preparations as enzyme source. Also, in incomplete incubations, the amounts of both Z,Z-DIES and Z-DES range above control values. This is seen in Table 2, where cooxidation and isomerization were measured at different DES concentrations and compared to a control without enzyme: the ratio of Z-DES to unmetabolized parent compound is higher in all incubations compared to the control; cooxidation is less efficient at the highest substrate concentration (150 μM) suggesting substrate-inhibition. The data demonstrate that cooxidation and isomerization of DES are catalyzed by purified PGS and exclude possibility (b).

To learn more about the PGS enzyme activities mediating the isomerization of DES, we incubated DES with RSVM in the presence and absence of AA and the inhibitor phenidone. Figure 3 shows data from

TABLE 1 : DES METABOLISM WITH PURIFIED PGS

Incubation [a]	Metabolites analyzed (nmol) [c] E-DES	Z-DES	Z,Z-DIES	Ratio Z-DES / E-DES
complete	36.6	6.26	7.17	0.17
" + indomethacin 20µM [b]	36.92	12.09	4.95	0.33
without enzyme	49.95	4.59	1.34	0.09

a) Incubations were carried out in 1.0 ml total volume of Hepes buffer (0.05 M, pH 7.8) containing (^{14}C)-E-DES (62.5 µM), hematin (1 µM), purified PGS (5µl) and AA (200 µM) for 5 min at RT.
b) Indomethacin in ethanol (5µl) was preincubated with the mixture for 1 min before AA addition.
c) DES isomers and Z,Z-DIES were analyzed and quantitated by HPLC and liquid scintillation counting (LSC) of the fractionated effluent as described in the experimental section.

TABLE 2 : DES METABOLISM WITH PURIFIED PGS

Incubation [a]	Metabolites analyzed (nmol) E-DES	Z-DES	Z,Z-DIES	Ratio Z-DES / E-DES
DES (21 µM) complete	6.24±1.56	0.94±0.71	9.59±1.58	0.15
DES (42 µM) complete	8.88±3.55	2.97±0.12	22.94±0.99	0.33
DES (42 µM) - enzyme	36.98±0.62	1.60±0.18	0.75±0.09	0.04
DES (150 µM) complete	36.00±3.12	25.76±1.91	50.93±4.39	0.72

a) Incubations were carried out in a total volume of 1.0 ml phosphate buffer (0.1 M, pH 7.4) containing (^{14}C)-E-DES as indicated, purified PGS (5 µl) and AA (100 µM) for 3 min at 37° and were analyzed as described in the experimental section.

this experiment. Formation of Z,Z-DIES and of protein bound radioactivity require both enzyme and AA; the amount of Z-isomer found is more than twice that of the remaining E-DES (Fig. 3, A). Omission of AA from the incubation considerably decreases the oxidative metabolism and the formation of bound radioactivity; Z-DES now constitutes

more than 60 % of extractable metabolites, and levels are more than twice those of E-isomer (Fig. 3, B). Interestingly, addition of phenidone inhibits the isomerization of DES in these AA-deficient incubations (Fig. 3, C) completely. Both amounts and ratio of Z- to E-DES are virtually indistinguishable from control incubations with denatured enzyme (Fig. 3, D).

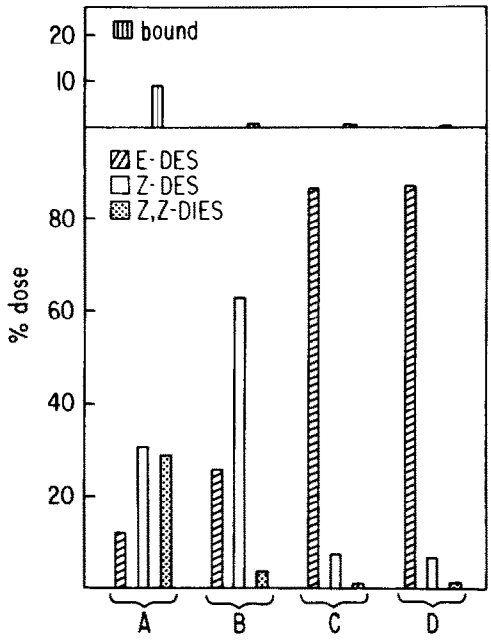

Figure 3: (^{14}C)-DES (67µM) was incubated with PGS from RSVM (1 mg protein/ml) in phosphate buffer (0.05 M, pH 7.5), (A) in the presence of AA (200 µM); or (B) without exogenous cofactor; or (C) same as (B) but plus phenidone (0.5 mM); or with denatured RSVM (D). Incubations were carried out at RT for 5 min, extracted, and analyzed as described in the experimental section. Bottom panel shows extractable metabolites analyzed by HPLC, top panel represents protein bound radioactivity.

We found previously that phenidone (0.5 mM) inhibits the AA-dependent and the 15-HPETE (15-hydroperoxy-5,8,11,13-eicosatetraenoic acid) dependent oxidation of E-DES to Z,Z-DIES without affecting the AA-dependent oxygen uptake (8). The inhibition of peroxide (15-HPETE) dependent cooxidation of DES and the finding that phenidone itself can be oxidized by PGS (11) is consistent with the idea that phenidone inhibits DES metabolism competitively at the level of the peroxidase.

As shown here (Figure 3), phenidone also blocks the enzyme mediated formation of Z-DES. That leads us to believe that the peroxidase activity of PGS is responsible for the observed isomerization. This is consistent with the idea that isomerization of DES is favored under conditions where cooxidation is decreased. Both cooxidation and isomerization will lead to the formation of non-estrogenic metabolites of E-DES. However, as seen in Figures 1, 2 and 3, cooxidation is accompanied by the formation of reactive intermediates capable of binding to protein. However, the levels of protein bound radioactivity are not significantly different from controls in incubations in which efficient isomerization occurs. This places different toxicological significance on the two pathways mediated by the same enzyme and leads us to speculate about the mechanism of enzyme catalyzed isomerization of E-DES to Z-DES: A bimolecular mechanism involving a proton shift from the phenolic groups to the stilbene double bond or a monomolecular ionic mechanism have been proposed by Winkler et al.(7) for the chemical isomerization of DES in organic solvents depending on the pH. An alternative mechanism would involve a transient radical intermediate formed by one electron oxidation which could either disproportionate to Z,Z-DIES and Z-DES or after reduction form Z-DES. However, assuming that such a radical is a reactive intermediate, one would expect increased levels of protein bound radioactivity in incubations with efficient isomerization.

Peroxidative metabolism of DES leading to Z,Z-DIES, as catalyzed by horseradish peroxidase or mouse uterine peroxidase in vitro, has been found to give rise to product(s) bound to protein or calf thymus DNA

(12). Similarly, we found that PGS from RSVM catalyzes the formation to Z,Z-DIES as well as binding to protein (this report, 8), and recently Benett et al. (13) reported AA-dependent binding of (^{14}C)-DES to exogenous DNA catalyzed by a uterine microsomal preparation in vitro. These data and the presence of PGS in uterine tissue (9, 10) raise the possibility that it is mediating oxidative metabolism of DES in vivo in a target tissue for estrogen action, and that this leads to some as yet unidentified biochemical lesion(s) when cooxidation is operative. On the other hand, isomerization may be dominating when cooxidation is impaired, for example due to a limited pool of free arachidonic acid.

Either way, the PGS-peroxidase mediated metabolism of E-DES in vitro leads to essentially non-estrogenic metabolites; the role of these enzyme-mediated pathways in vivo is presently unknown. Z,Z-DIES has been reported as a major metabolite in vivo in many species including humans, and has been found in target tissue preparations and the isolated fetal genital tract in organ culture (14). Z-DES has also been found under these conditions, but has usually been accounted for together with parent compound E-DES. Therefore, there is the possibility that DES is metabolized by target tissue enzymes such as mouse uterine peroxidase in a way similar to that observed in our in vitro study. We are currently studying this point and also the relationship of prostaglandin synthase and mouse uterine peroxidase.

EXPERIMENTAL

Chemicals: Monoethyl-(2-^{14}C)-DES (Amersham-Searle Corp., Arlington Heights, Ill.) was recrystallized with unlabeled DES (Sigma Chemical Corp., St. Louis, Mo), specific activity 1.75 mCi/mmol; radiolabeled and unlabeled DES used thoughout our studies were found to contain

less than 2 % Z-DES. All other chemicals and solvents were from the same sources described previously (8) and were of the highest purity available.

Incubations: The incubations with PGS from ram seminal vesicle microsomes were carried out as published (8) and details are given in Fig. 1 - 3. Incubations with purified reconstituted PGS (kindly provided by Dr. L.J. Marnett, Wayne State University, Detroit, Mich.) were carried out in 1.0 ml total volume; details are given in Tables 1 and 2; preincubation of (^{14}C)-DES, hematin, indomethacin and enzyme were used; reactions were initiated by addition of AA, stopped after 3 min by extraction with ether and cooling on ice. Organic solvent layers were evaporated under vacuum, residues dissolved in methanol, and aliquots used for liquid scintillation counting and further analysis by HPLC.

Identification and Quantitation of DES and Metabolites: HPLC analysis of the extractable fraction was carried out using a Waters Associates, Inc. (Milford, Ma) liquid chromatograph with a reverse phase column (Zorbax Sil ODS; Dupont, Wilmington, Del) eluted with a linear methanol : water gradient, increasing from 56 to 88 % methanol in 30 min at 42° at 1.0 ml/min flow rate. Eluting compounds were monitored by absorbance at 254 nm; the effluent was collected in 0.3 min fractions and radioactivity determined after mixing with scintillant (Ultrafluor, National Diagnostics). The samples were measured in a Beckman LS 9000 scintillation counter with external standardization (Beckman Instr., Norcross, Ga). Identity of E-DES and metabolites was confirmed by cochromatography with reference compounds and GC/MS as published (8).

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