Oxidative Metabolism of Diethylstilbestrol by Prostaglandin Synthetase

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ABSTRACT

The cooxidative metabolism of the transplacental carcinogen, diethylstilbestrol (DES), was examined using ram seminal vesicle microsomes. The major extractable metabolite was β-diensethrol (Z,Z-DIES) and represented about 35% of the added DES in 3-min incubations supplemented with arachidonic acid. Its formation was dependent upon the presence of arachidonic acid, whereas reduced nicotinamide adenine dinucleotide phosphate failed to elicit Z,Z-DIES above background. Indomethacin and 1-phenyl-3-pyrazolidone, known inhibitors of prostaglandin synthetase, blocked Z,Z-DIES formation, probably by inhibiting the cyclooxygenase and the hydroperoxidase activities, respectively. Hydrogen peroxide and 15-hydroperoxyarachidonic acid (cofactors of the prostaglandin synthetase-hydroperoxidase), when replacing arachidonic acid in incubations, also supported oxidative metabolism of DES catalyzed by ram seminal vesicle microsomes. 1-Phenyl-3-pyrazolidone, but not indomethacin, inhibited the 15-hydroxyperoxyarachidonic acid-dependent formation of Z,Z-DIES. Incubation conditions which supported efficient Z,Z-DIES formation also resulted in the formation of 3,3-di( p-hydroxyphenyl)hexan-4-one and the cis-isomer of DES as well as nonextractable, protein-associated radioactivity indicating the presence of reactive intermediates. The implications of the peroxidative metabolism of DES for its toxic activity are obvious.

INTRODUCTION

The synthetic estrogen DES is a carcinogen in both animals and humans (18). The previous use of this compound as a medication during pregnancy has been associated with the rare occurrence of vaginal cancer in female offspring (17) and with genital tract anomalies in males (6, 12, 16). The use of DES as a growth-promoting substance with bioaccumulation potential (27), as a gynecological medication, and also as an extensively used treatment for prostatic cancer in humans requires a thorough understanding of its mode of toxic action. Previous work in this and other laboratories has shown that DES is metabolized extensively and that it can bind irreversibly to cellular macromolecules under conditions of bioactivation similar to other carcinogenic chemicals; the major oxidative metabolite formed in vivo in different species including humans is Z,Z-DIES. (For a recent review of DES metabolism, see the work of Metzler (28).) In vitro incubations of DES with a variety of tissues suggest that this major metabolite is not formed by a cytochrome P-450-mediated oxidation (9, 37). Additional in vitro studies have demonstrated that the same Z,Z-DIES isomer found in vivo is produced by oxidation via peroxidative systems, including HRP and MUP (19, 29).

In vitro incubations of [14C]DES with HRP or MUP in the presence of H2O2 yield Z,Z-DIES as a major metabolite, as well as yielding nonextractable radioactivity (29). The nature of the peroxidative activity involved in the in vivo metabolism of DES is as yet unknown. Our interest in this metabolic pathway led to an examination of enzymes associated with such activity in DES target tissues. Since genital tract tissue is known to contain high levels of PGS which is known to display potent peroxidative activity, PGS was considered for this enzyme system. This was reinforced by reports that other carcinogenic compounds are substrates for the PGS-mediated oxidation of AA. Recently, the PGS-dependent activation of 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene to mutagenic (21) and reactive intermediates capable of binding to tissue protein (36) has been demonstrated, and binding of benzidine to nucleic acids mediated by PGS preparations (from target tissues) has been reported (39). In this communication, we present evidence for the in vitro PGS-mediated oxidation of DES.

MATERIALS AND METHODS

Chemicals. Monoethyl[2-14C]DES (specific activity, 56 mCi/mmol; Amersham-Searle Corp., Arlington Heights, Ill.) was recrystallized with unlabeled DES (Sigma Chemical Co., St. Louis, Mo.) to give a final specific activity of 1.75 mCi/mmol. Chemical, radiochemical, and stereoechemical purity of E-DES was >97% as tested by HPLC; impurities were <1.5% Z-DES and <0.7% Z,Z-DIES. Reference compounds, Z,Z-DIES, and DES ketone were a gift of Dr. M. Metzler (Institute for Pharmacology and Toxicology, University of Würzburg, Federal Republic of Germany); Dr. P. Murphy, (Eli Lilly Co., Indianapolis, Ind.) generously supplied Z-DES. AA (>99% pure), BHA, indomethacin, and NADPH were purchased from Sigma. Metyrapone and SKF 525A were kindly provided by Dr. J. Fouts (National Institute of Environmental Health Sciences, Research Triangle Park, N.C.). 15-HPAA was prepared by the method of Funk (11). All other chemicals and solvents were of the highest purity commercially available.

Incubations. RSV M were prepared as described previously (34), stored at −70° until use, and tested for PGS activity by measuring their AA-dependent oxygen uptake, using a Clark-type electrode. RSV M (2 mg protein) were added to a mixture containing E-[14C]DES (5 mg/ml of dimethyl sulfoxide) in 0.05 M sodium phosphate buffer (pH 7.6) to a final substrate concentration of 0.06 mM. In some experiments with inhibitor, all tubes were preincubated with the test compounds (or solvent) for 2 min. The reactions were initiated by addition of AA or the other cofactors, incubated for 3 min at 25° under air (shaken), and stopped by adding 8 ml absolute ethanol.

Compounds tested for their effect on RSV M-mediated metabolism of DES were dissolved in ethanol; the same amounts of solvent (10 to 20 μl) were added to all incubations to eliminate solvent effects. Controls were run using heat-denatured microsomes or by omitting cofactor or

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Separate experiments were performed where the incubation time or the substrate concentration was varied.

After addition of ethanol, the precipitates were removed by filtering over Whatman No. 1 filter paper; repeatedly washed with ethanol, ethanol:ether (1:3), and ether; wetted with water; and digested in NCS tissue solubilizer (Amersham-Searle). Radioactivity of aliquots was determined using Ultrafluor (National Diagnostics, Inc., Somerville, N. J.) scintillation cocktail, samples were counted in a Beckman LS 9000 scintillation counter (Beckman Instruments, Norcross, Ga.) with external standardization.

The filtrate and the wash fluid from each incubation tube were combined, evaporated under vacuum, and dissolved in methanol, and radioactivity of aliquots was determined. The soluble radioactive material was subjected to further analysis by HPLC and GC/MS. Overall recovery of radioactivity exceeded 97% in all incubations.

Identification and Quantitation of DES and Metabolites. HPLC analysis was performed using a Waters Associates, Inc. (Milford, Mass.) liquid chromatograph consisting of 2 pumps (M 6000 A), a gradient programmer (M 660), an injector (WISP 710 A), an absorbance detector (M 440), a data module, and a C18-Bondapak reverse phase column eluted with a linear methanol:water gradient. Compounds in the column eluate were detected by absorbance at 254 nm, the effluent was collected in 0.45-ml fractions, and radioactivity was counted.

Fractions from semipreparative HPLC separations were pooled, derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, Ill.), and further analyzed by GC/MS. The vast majority of soluble radioactive compounds could be identified based on their mass spectra and comparison of their retention times with those of reference compounds (Table 1).

All experiments were carried out at least in duplicate, and the data presented are mean values. Radioactivity in the precipitate and in the soluble phase were measured in triplicate aliquots. Radioactivity in HPLC fractions (pertaining to Peaks I to IV in Chart 2) was calculated as percentage of total radioactivity eluted from the column. Since total recovery usually exceeded 98% of the injected radioactivity, radioactivity/peak was further used to quantify the amount of metabolites in percentage of dose or in nmol of metabolite, based on the specific radioactivity.

RESULTS

In Vitro Metabolism of E-DES. Incubation of E-[14C]DES with AA and RSVM at 25°C resulted in a very rapid conversion of the parent compound to lipophilic, extractable metabolites and the formation of nonextractable radioactivity (Chart 1). This insoluble radioactivity is associated with protein; comparable amounts are found in incubations in which protein is precipitated with trichloroacetic acid after extraction of soluble metabolites (data not shown) and therefore considered for its majority to be covalently bound.

HPLC analysis of the soluble phase from these incubations yielded 4 radioactive peaks (Chart 2, Peaks I to IV) which cochromatographed with E-DES (parent compound), Z-DES, Z,Z-DIES, and DES ketone reference compounds, respectively. Pooled fractions from HPLC separations were used for further GC/MS analysis, and their identity was confirmed by GC/MS of their trimethylsilyl derivatives (Table 1). Methoxy or catechol metabolites were not detected (detection limit, 0.2 to 0.5% of the dose).

Radioactivity identified in Peaks I to IV accounted for at least 85% of the extractable radioactivity in all incubations. Z,Z-DIES is the major oxidative metabolite; under our conditions, up to 35% of DES was converted to this metabolite.

A series of experiments with DES, RSVM, different cofactors, and inhibitors was carried out to determine whether the formation of Z,Z-DIES is catalyzed by PGS. Table 2 shows that AA supports the oxidation of E-DES to Z,Z-DIES and the formation of nonextractable, protein-associated radioactivity. The conversion is expressed in both nmol/mg/3 mm and in relative activities for a comparison. (This does not reflect possible nonlinearities in the rates over 3 min.) The data in Table 2 further show that omission of AA decreased the oxidation of DES to Z,Z-DIES; also, the formation of nonextractable radioactivity was 12 and 15%, respectively, of the levels obtained in AA-fortified incubations. Likewise, NADPH-supplemented RSVM under identical conditions yield only 9 and 15% of these
Oxidative Metabolism of DES

The data presented in this communication clearly establish DES as a substrate for cooxidation. The present concept of cooxidation of xenobiotics (7, 23, 38) is outlined in Chart 3; PGS, which displays 2 enzyme activities, catalyzes, by means of its cyclooxygenase, the dioxygenation of AA to the cyclic endoperoxide prostaglandin G2, a step which can be inhibited by indomethacin (or lack of oxygen). The reduction of prostaglandin G2 to prostaglandin H2 is catalyzed by PGS-hydroperoxidase, which has a relatively broad specificity for perox-
p-chloro-N-methylaniline PGS preparations and AA was not this discrepancy is not fully understood.

With these data, metyrapone was ineffective as an inhibitor of 

However, impaired by metyrapone or SKF 525A (35, 40). In accordance with this, residual activity, observed in NADPH-fortified incubations, was not affected by metyrapone or SKF 525A (Table 3).

Indomethacin inhibits the AA-dependent formation of Z,Z-DIES and, as shown in parallel incubations, it blocks AA-dependent oxygen uptake at concentrations which inhibit cooxidation of various substrates. Because indomethacin inhibits the cyclooxygenase but not the peroxidase activity of PGS, it should not affect cooxidation when prostaglandin G₃ or a hydroperoxide (instead of AA) is provided in the incubation (23, 32, 38). The slight inhibitory effect of indomethacin on Z,Z-DIES formation in 15-HPAA-supplemented incubations may be explained by low levels of endogenous AA present in the incubations (RSVM); this fraction of Z,Z-DIES formation would be indomethacin sensitive.

1-P-3-P, another inhibitor of PGS (2), suppressed DES-oxidation very efficiently in both AA- and 15-HPAA-supplemented incubations, whereas AA-dependent oxygen uptake in parallel incubations was not impaired (Table 3). These data suggest that 1-P-3-P inhibits prostaglandin synthesis and cooxidation by acting upon the peroxidase rather than upon the cyclooxygenase activity. Similar conclusions have been reached by Marnett.³

BHA (5 x 10⁻⁴ M) was found to be an effective inhibitor of Z,Z-DIES formation; it impaired AA-dependent oxygen uptake at this concentration, indicating that it affects the cyclooxygenase activity in addition to its radical scavenging properties. Free radicals have been demonstrated during prostaglandin synthesis and are associated with the oxidation of foreign chemicals (22) and thought to be responsible for the self-inactivation of PGS (8). The free radical-scavenging properties of BHA may effectively compete for the oxidation of DES.

Metyrapone and SKF 525A are well-known inhibitors of cytochrome P-450-mediated drug metabolism and have not been reported to inhibit PGS-mediated cooxidation at the concentrations used in our assay (0.5/1 mM concentration). Cooxidation of [¹⁴C]DES with rabbit kidney microsomes in the presence of AA or other cofactors of PGS like 15-HPAA or H₂O₂ is Z,Z-DIES. This oxidative metabolite of DES has also been found in humans and in many adult animal species (for review, see Ref. 28) and also in newborn mice (30), hamster fetuses (14), and cultures of fetal mouse genital tissue (25). It is formed in vitro with HRP in the presence of H₂O₂ probably via a semiquinone-quinone pathway. Also, under these conditions, formation of macromolecule-bound radioactive product(s) was reported (29).

These data and the finding that PGS-mediated oxidation of DES leads to protein-bound metabolite(s) demonstrate clearly the potential for DES to function as an activated chemical. What role its metabolic activation plays in the well-known transplantal carcinogenesis in the mouse model (26) or in humans (18) remains to be established. However, recent work from our laboratory has demonstrated that DES is capable of inducing neoplastic transformation in Syrian hamster embryo fibroblast cells (1), a system in which conversion of DES to Z,Z-DIES occurs (5). The peroxidative metabolism of DES may play an important role in the genotoxic effects of DES such as induction of sister chromatid exchange (33), unscheduled DNA synthesis (24), mutation in lymphoma cells (3), and in an epidermal carcinogenicity test (10).

The relationship of PGS-hydroperoxidase to the previously described uterine peroxidase or MUP (20) in tissues susceptible to DES raises the question of whether MUP (which oxidizes...
DES to Z,Z-DIES in the presence of H$_2$O$_2$) and PGS-hydroperoxidase might be the same enzyme. This question is especially pertinent since PGS levels are reported to be high in the uterus of cycling rats (15). Preliminary attempts at association of these 2 enzyme activities in the mouse uterus have not been successful. Further studies to elucidate the role of metabolism in DES target tissue toxicity are in progress.

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REFERENCES

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