Involvement of Prostaglandin Synthetase in the Peroxidative Metabolism of Diethylstilbestrol in Syrian Hamster Embryo Fibroblast Cell Cultures

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ABSTRACT

The mechanism for induction of tumors by estrogens is still unresolved. Neoplastic transformation of Syrian hamster embryo fibroblasts by diethylstilbestrol (DES) suggests that established principles of chemical carcinogenesis may be involved. The Syrian hamster embryo fibroblasts provide a system in which now the question can be asked whether the metabolic activation of DES is a prerequisite for its biological activity in this system. In this study, Syrian hamster embryo fibroblast cell cultures were shown to oxidatively metabolize DES to cis,cis-dienestrol (Z.Z-DIES) which is a DES metabolite commonly found in vivo. The only other metabolic conversion of DES detectable in these cell cultures was the formation of the glucuronides of DES and Z.Z-DIES. Z.Z-DIES is formed more efficiently in incubations with rapidly growing cells than in cultures approaching confluence. When arachidonic acid was added to the medium, Z.Z-DIES formation was enhanced, whereas indomethacin added to the cell cultures inhibited the formation of this metabolite. These data suggest the involvement of prostaglandin synthetase in the oxidative metabolism of DES by Syrian hamster embryo fibroblasts in culture and suggest that cooxidation may play a role for its biotransformation in whole cells. Moreover, since many competing metabolic pathways are available to DES in vivo, this present study adds important additional support to the hypothesis that metabolism of DES via a peroxidative route plays a role in its carcinogenicity.

INTRODUCTION

DES\textsuperscript{3,4} is a known carcinogen in humans and animals (11), and can induce neoplastic transformation of cells in culture (2, 13). However, its mechanism of action is still unclear. The observation that DES can neoplastically transform Syrian hamster embryo fibroblasts in culture in the absence of enhanced cell proliferation supports the idea that DES may act like other chemical carcinogens in directly inducing cell transformation rather than through its hormonal activity. The transformation frequency induced by DES or structural analogues in Syrian hamster embryo fibroblast cells suggested a role for metabolism in the observed cell transformation (13). Structural analogues of DES (such as TF-DES or Z.Z-DIES), which are good substrates for peroxidases, morphologically transformed Syrian hamster embryo cells, whereas analogues which are poor substrates for peroxidase (6) (dimethoxydiethylstilbestrol, hexestrol, or E.E-DIES) did not transform Syrian hamster embryo cells at a significant level (13). It has been proposed previously that peroxidative metabolism of DES plays an important role in DES toxicity (18, 19). Binding of DES to DNA and protein mediated by partially purified or pure peroxidases in vitro has been reported (5, 18, 19), indicating that DES can be metabolized to reactive intermediates via this pathway. Other metabolic products of DES the formation of which involves electrophilic intermediates have been studied in vivo and in vitro, and their possible role in the genotoxicity and tumorigenicity of DES has been reviewed recently (15, 16). The observed structure-activity relationship for DES and structural analogues in the Syrian hamster embryo cell transformation assay (13) raised the questions of whether and to what extent these fibroblasts are able to metabolize DES and what the products would be.

The present paper describes the metabolic profile for DES in the Syrian hamster embryo cell transformation assay; moreover, correlations between growth characteristics and oxidative metabolic capacity are made, and data are presented which link the formation of Z.Z-DIES to the peroxidative activity of prostaglandin synthetase. Thus, a model system is established in which modifications of metabolism of estrogens may be linked to biological responses.

MATERIALS AND METHODS

Chemicals. Monoethyl-[2-\textsuperscript{14}C]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; it was dissolved in DMSO (5 mg/ml). The chemical, radiochemical, and stereochemical purity of E-DES was >97% as tested by HPLC; impurities were <1.5% Z-DES and <0.7% Z.Z-DIES. Reference compound, Z.Z-DIES, was a gift of Dr. M. Metzler (Institute of Pharmacology and Toxicology, University of Würzburg, Federal Republic of Germany); Dr. P. Murphy (Eli Lilly and Co., Indianapolis, Ind.) generously supplied Z-DES.

Arachidonic acid, indomethacin, and \textbeta-glucuronidase were purchased from Sigma Chemical Co.; trypsin was from the Grand Island Biological Co. All other chemicals and solvents were of the highest purity commercially available.

Cell Culture. Primary cultures of Syrian hamster embryo fibroblasts were established from 13-day fetuses as described previously (8, 13, 21). Secondary cultures were initiated from frozen stocks in IBR-modified Dulbecco's Eagle's reinforced medium (Grand Island Biological Co., Grand Island, N. Y.) containing sodium bicarbonate (3.7 g/liter), 10% (v/v) Hy-Clone fetal bovine serum (Sterile Systems, Inc., Logan, Utah), penicillin (100 units/ml), and streptomycin (100 \mu g/ml) (Grand Island Biological Co.).
Incubations. The growth of the cells during the experiment is presented in Chart 1. Tertiary passage fibroblasts were seeded at a density of 10^5 cells onto 100-mm dishes (No. 3003; Falcon Plastics, Oxnard, Calif.) in IBR-modified Dulbecco's Eagle's reinforced medium (containing 10% fetal calf serum) using 10 ml/dish. Cultures were refed every 2 days with this medium or incubated with medium containing DES (at concentrations of 5 or 0.5 µg/ml). Cells were treated with [14C]JEG-DES either during growth in preconfluent cultures (Chart 1, closed circles) or when they were reaching confluency (Chart 1, open circles) for the indicated 48-hr period (-----).

The chart also pictures cell number per dish and changes thereof as they occur during incubation with DES. This is taken into account for a comparison of data from growing and confluent cultures metabolizing DES in Tables 1 and 2. Here, the nmol of product measured per dish was corrected for the number of cells per dish catalyzing the conversion.

In experiments where modulation of DES metabolism was investigated, arachidonic acid (100 mg/ml ethanol) or indomethacin (36 mg/ml DMSO) was added to yield final concentrations of 0.2 mM or 0.1 mM in the medium, respectively. To prevent toxic effects of solvents, the total concentration of DMSO was ≤0.1%, and that of ethanol was ≤0.06% in the medium. One of the treatment groups contained the same amounts of solvent without arachidonic acid or indomethacin. DES concentration was 5 µg/ml medium. Nine dishes with 5 ml medium each were incubated per treatment group. Cell number per dish was as shown in Table 2 and did not change significantly throughout the 4 hr of incubation.

Isolation of DES and Metabolites from Incubations. After incubation in a 37° humidified incubator with 10% CO2, the media from each treatment group were aspirated and collected. The cells on the dishes were then washed with phosphate-buffered saline, and this buffer was pooled with the collected media. Cells on the dishes were trypsinized (0.1%, 30 min, 37°), collected and sonicated, and pooled with the respective media from each treatment group.

Absolute ethanol was added to the cellular fractions and media to yield a final concentration of 80% ethanol. The ethanol precipitates were removed by filtration through Whatman No. 1 filter paper; then they were washed with ethanol, ethanol:ether (1:3), and ether, wetted with water; and digested with NCS tissue solubilizer (Amersham/ Searle). Radioactivity of aliquots was determined using Ultrafluor (National Diagnostics, Inc., Somerville, N. J.) scintillation cocktail for dilution of the samples, which were then counted in a Beckman Model LS 9000 scintillation counter (Beckman Instruments, Inc., Norcross, Ga.) with external standardization.

The aqueous-ethanolic filtrate and the wash fluid from each incubation group (control or experimental) were combined. Evaporated under vacuum, and dissolved in methanol, and radioactivity of aliquots was determined. The soluble radioactive material was further analyzed by HPLC as described below.

Dishes with DES in fetal bovine serum-containing medium but without Syrian hamster embryo fibroblasts were incubated as controls to reveal any modification of DES under our incubation conditions and work-up procedures which were not due to cell-mediated metabolic activities. Values obtained for cell-mediated metabolic conversion are corrected for these background values.

Identification and Quantitation of DES and Metabolites. HPLC analysis of the soluble radioactive material was performed using a Waters Associates, Inc. (Milford, Mass.) liquid chromatograph consisting of 2 pumps (M 6000 A), a gradient programmer (M 660), an injection unit (WISP 710 A), an absorbance detector (M440), a data module, and a C18-bondapak reverse-phase column. Separation was carried out with a linear methanol:water gradient increasing from 38 to 82% methanol in 30 min, operated at a flow rate of 1.5 ml/min. 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. Identity of DES and metabolites was confirmed in some samples through further gas chromatography-mass spectrometry analysis of pooled HPLC fractions as described earlier (5). In some cases, HPLC fractions which had retention times identical to DES:glucuronides were incubated overnight with β-glucuronidase in 0.2 M acetate buffer at pH 5.0; the fraction of aglycones extractable after enzymatic hydrolysis was then chromatographed on HPLC.

RESULTS

We studied DES metabolism in Syrian hamster embryo fibroblast cultures at confluence and during growth. Cells were treated with [14C]DES either as they approached confluence or during growth in preconfluent cultures and incubated for 48 hr. For studies of DES metabolism in the presence of arachidonic acid or indomethacin, incubations with Syrian hamster embryo cells in growing or stationary culture were carried out for 4 hr, respectively, as described in "Materials and Methods." At the end of the incubation period, media and cells from each treatment group were collected and worked up as described in "Materials and Methods."

The overall recovery of radiolabel exceeded 90% in both cell cultures and control incubations. Distribution of radioactivity between ethanol-insoluble and -soluble fractions was in the range of 0.3 to 1.1 and 98.9 to 99.7% of the overall recovered radioactivity, respectively, which was not significantly different between treatment groups and controls.

A representative metabolite profile in the pooled ethanol-extractable fraction from cells and media is presented in Chart 2. Identifiable radioactivity is associated with parent compound E-DES, its isomer Z-DES, and the oxidative metabolite, Z,Z-DIES. Significant levels of di- and monoglucuronides were also identified. Following β-glucuronidase cleavage, the distribution of aglycones resembled closely the distribution of E-DES, Z-DES, and Z,Z-DIES seen in the unconjugated fraction. In experiments where the ethanol-soluble fractions of cells and culture medium were worked up separately, a relatively higher concentration of glucuronides in the extracellular medium was noted as compared to their concentration in the cells (data not shown).
Table 1 presents data comparing DES metabolism by Syrian hamster embryo cell cultures at confluence or during exponential growth. At either a high (5 μg/ml) or low (0.5 μg/ml) concentration, DES was metabolized more extensively by growing than confluent cultures. In growing cultures treated with a high concentration of DES (186 nmol/dish), approximately 6 times as much of the conjugates (in pmol/10^5 cells) and 14.5 times as much oxidative metabolite (Z,Z-DIES) were formed than in corresponding confluent cultures. Similarly, in growing cultures treated with a lower amount of DES (18.6 nmol/dish), almost 5 times as much of the conjugates and 4 times as much Z,Z-DIES were formed than in the corresponding confluent cultures.

The influence of the DES concentration on the metabolic profile of confluent Syrian hamster embryo cells should be noted, since a 10-fold reduction in the administered "dose" in vitro led to a 10-fold reduction in conjugate but only a 2-fold reduction in Z,Z-DIES production. However, a reduction in the DES dose added to growing cultures results in more parallel reductions of Z,Z-DIES (8-fold) and conjugate formation (12-fold).

For a further investigation concerning the nature of the enzyme activity involved in the oxidative DES metabolism, another series of incubations was carried out. The influence of arachidonic acid and indomethacin (a cofactor and an inhibitor of prostaglandin hydroperoxidase-mediated cooxidation reactions, respectively) was studied in short-term incubations (4 hr). The results are presented in Table 2. Addition of arachidonic acid to the incubation medium increased the amount of Z,Z-DIES formed, about 2-fold in confluent cultures and about 4-fold in growing cell cultures. Again, there was a pronounced difference in the extent of oxidative metabolism between confluent and growing cells, the latter metabolizing DES to Z,Z-DIES more extensively in both incubations with and without arachidonic acid. However, arachidonic acid supplementation of the medium did not alter the quantity of the conjugates formed in cultures with either growing or confluent cells. Indomethacin (at 0.1 mM) completely inhibited the formation of free Z,Z-DIES. No Z,Z-DIES was formed in the hydrolyzed conjugate fraction either. Total conjugate formation in these cultures was impaired, although the cells appeared morphologically normal throughout the incubation. Additional experiments with different primaries of Syrian hamster embryo cells yielded similar results.

**DISCUSSION**

The results presented in this paper show that Syrian hamster embryo cells in culture can metabolize DES. The only oxidative metabolite observed in the Syrian hamster embryo cells in culture was Z,Z-DIES; in addition, glucuronides of Z,Z-DIES and parent compound were formed. Glucuronidation of benzo(a)pyrene in this cell system has been reported before as well as formation of oxidative metabolites of benzo(a)pyrene which are catalyzed by arylhydrocarbon hydroxylase (14).

The formation of the major oxidative metabolite described in this paper, Z,Z-DIES, has been shown previously to be catalyzed by a variety of peroxidases (5, 12, 18). The presence of peroxidative activity in Syrian hamster embryo cells as concluded from the presence of Z,Z-DIES is further supported by our finding that cell extracts assayed with guajacol and H₂O₂ for peroxidase (10) gave a positive reaction (about 2 milliunits/mg protein/min). The metabolic pathways observed for DES in Syrian hamster embryo fibroblasts are outlined in Chart 3.

The concentrations of DES used in this study induce neoplastic transformation of these cells (2, 13). Our previous reports on cell transformation by DES did not produce a typical dose response over the concentration range of 0.01 to 10 μg/ml medium (13). This may be explained in part by the apparent nonproportional oxidative metabolism as shown in Table 1 of...
this report. We think that the 10-fold reduction in conjugate formation but only 2-fold reduction in Z,Z-DIES formation in the confluent cultures seen after a 10-fold reduction in the DES concentration (5 μg/ml medium compared with 0.5 μg/ml) reflects differences in the Km values for the oxidative and the conjugation enzymes. This difference, while present, is not so striking in rapidly growing cultures. Studies by Neumann in rats have shown that the ratio of glucurononlated metabolites of DES to the corresponding unconjugated forms increased with dose (20). Thus, disproportionate increases in oxidative metabolism, as well as increased levels of conjugation at higher doses, may partially account for the dose-response relationship observed in the transformation assay (13). The metabolic relationships described in Chart 3 for the cells in culture will therefore be determined by the dose of DES and by the growth characteristics of the cells.

In addition, the present study clearly demonstrates that oxidative metabolism of DES via the peroxidase pathway (Chart 3) is enhanced in cell cultures in log growth phase when compared to stationary phase cultures. Previous reports have demonstrated increased levels of prostaglandin synthesis in growing Syrian hamster embryo cells when compared to confluent cells (1). We have recently described the formation of the peroxidative metabolite Z,Z-DIES during arachidonic acid metabolism with prostaglandin synthetase and shown that DES oxidation is arachidonic acid dependent and can be inhibited by indomethacin (5). The influence of prostaglandin synthesis inducers and inhibitors on peroxidative metabolism of DES in Syrian hamster embryo cell cultures (Table 2) and the increased production levels of Z,Z-DIES in rapidly growing cultures compared to those at confluence (Tables 1 and 2) strongly support the idea that Z,Z-DIES formation in Syrian hamster cell cultures is mediated, at least in part, by prostaglandin hydroperoxidase. Very recently in another fibroblast transformation system, prostaglandin synthetase-mediated metabolism of benzo(a)pyrene 7,8-diol has been demonstrated (3).

Our previous study demonstrated that cooxidation of DES by prostaglandin synthetase and arachidonic acid in vitro occurs very rapidly (5). When comparing the separate experiments contained in Table 1 (48-hr culture) and Table 2 (4-hr culture), it is apparent that most oxidative metabolism occurs within the first hr of incubation, while conjugation appears to continue over time. If Z,Z-DIES formation is mediated by prostaglandin synthetase, it may cease after a few hr due to self-inactivation of the enzyme (9) or a limit in the supply of free available arachidonic acid.

These results are further significant, since most studies on carcinogen metabolism done in cell cultures are carried out at confluency (3, 4, 14). Further studies on metabolism of xenobiotics in rapidly growing cultures are important, since these are the conditions in which cell transformation assays in Syrian hamster embryo cells are done.

These data, together with data on transformation of Syrian hamster embryo cells by DES analogues (13), support the idea that DES metabolism through peroxidase(s) may be associated with cell transformation in vitro and provide a basis for further experiments to measure Syrian hamster embryo cell transformation by DES in combination with arachidonic acid or indomethacin.

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