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CONVERSION OF ESTRADIOL-17β TO REACTIVE EMBRYOTOXIC INTERMEDIATES BY CYTOCHROME P-450-DEPENDENT BIOACTIVATING SYSTEMS

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P-450-dependent enzyme systems added to media of cultured rat embryos markedly increased the embryotoxicity of estradiol-17β. Increases were markedly attenuated by a) omission of NADPH, b) omission of enzyme, c) substitution of female for male rat liver as enzyme source, d) replacement of N_2 with CO or e) replacement of estradiol-17β with diethylstilbestrol. Embryotoxicity correlated well ($r=0.84$) with catecholestrogen generating activities. Addition of a catechol-methylating system failed to modify embryotoxicity even though large quantities of methoxyestrogens were formed. The results document that endogenous estrogen can be converted by P-450 to embryotoxic intermediates and suggest that reactive proximate metabolites are precatechols, perhaps epoxyenones.

Recent investigations have established that various foreign organic chemicals will exhibit embryotoxicity only after metabolic conversion to reactive intermediates (1-3). However, it is known that various endogenous chemicals can also be converted to toxic intermediary metabolites (4-6). Perhaps the most intensively investigated of the latter category are the steroidal estrogens. Conversion of these chemicals to catecholestrogens by cytochrome P-450-dependent systems in hepatic as well as extrahepatic tissues represents a major metabolic route for steroidal and nonsteroidal estrogens and is also considered a potential bioactivating pathway in carginogenic, mutagenic and other genotoxic/cytotoxic effects of these chemicals (7-10). It has been considered possible that an epoxide intermediate formed immediately prior to catecholestrogen generation (11) or an o-semiquinone radical species produced subsequently via oxidation of the catecholestrogen (12) could represent proximate reactive/toxic metabolites.

The relatively large quantities of estrogens produced by the pregnant mammal raise concerns that, under certain specified conditions, bioconversion of estrogens to catecholestrogens could lead to undesired embryotoxicity. Indeed, estrogenic chemicals do exhibit transplacental carcinogenic as well as teratogenic properties (13,14) in humans and experimental animals. In this communication, we present results demonstrating for the first time that P-450-dependent oxidation of an endogenous hormone, estradiol-17β, markedly increases the embryotoxic potential of this steroid and that catecholestrogen formation is closely correlated with incidence and severity of embryotoxic manifestations.

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MATERIALS AND METHODS

Materials Estradiol-17β, NADPH, catechol-O-methyltransferase, S-adenosylmethionine, diethylstilbestrol and glucose 6-phosphate were obtained from Sigma Chemical Co., St. Louis, Missouri. [^{3}H]-S-adenosylmethionine (99% pure, 10.5 Ci/nmole) was obtained from New England Nuclear Corp. Boston, Massachusetts. Hepatic postmitochondrial supernatant (S9) fractions were prepared by homogenizing the livers of adult (200-250g) Sprague-Dawley rats previously treated with polychlorinated biphenyls (Aroclor 1254, 500 mg/kg 5 days prior to euthanasia), with phenobarbital (80 mg/kg/day for 3 days with the last injection given 24 hrs prior to euthanasia), with 3-methylcholanthrene (50 mg/kg 48 hrs prior to euthanasia), or with the corresponding vehicles. All substances were injected intraperitoneally. Fresh livers were homogenized in 2 volumes of 1.15% cold KCl solution and centrifuged at 9,000g for 20 min. The supernatant fractions were utilized as enzyme sources. Carbon monoxide was obtained from the Matheson Co., St. Louis, Missouri.

Embryo Culture System The postimplantation system originally developed by New (15) was utilized as described by Faustman-Watts et al. (16). Conceptuses were explanted on Day 10 (10 ± 2 somites) and were exposed to varying concentrations of estradiol-17β added to the medium at the beginning of the 24 hr culture period. Estrogens were dissolved in dimethyl sulfoxide (DMSO) and added such that final DMSO concentrations did not exceed 25 mM, previously shown (17) to have no detectable effects on cultured whole embryos. Cultures were gassed with either O_2:N_2:CO_2 (20:75:5) or O_2:CO:N_2:CO_2 (20:20:45:5) for the initial 20 hrs of culturing after which the mixture was changed to O_2:N_2:CO_2 (75:20:5) or O_2:CO:CO_2 (75:20:5). It was previously (17) demonstrated that gassing with CO produces no detectable effects on cultured whole embryos. After 24 hrs, embryos were removed from culture flasks and evaluated, without knowledge of treatment, for several indices of embryotoxicity.

Evaluation of Embryos Microscopic examinations of cultured embryos were utilized to evaluate viability, axial rotation (flexure), neural tube closure, maximal embryonic length, somite numbers, prosencephalic index, limb bud size, and other microscopically detectable indices of normal development. Conceptuses were scored as viable only if circulation in the vitelline yolk sac and active heartbeat were both detectable. Only viable embryos were further evaluated. Complete axial rotation (ventral) was scored as 0, partial rotation was scored as 1.0 and embryos exhibiting dorsal flexure were scored as 2.0. Neural tube closure was scored strictly on a quantal basis as either normally closed or abnormally open. Maximal embryonic length, maximal distance from the tip of the prosencephalon to the outer edge of the mesencephalon (prosencephalic index) and limb bud size were measured with a calibrated optical micrometer attached to the dissecting microscope. Following evaluations by light microscopy, embryos were sonicated and assessed for content of protein and DNA by methods described by Bradford (18) and Labarca and Paigen (19) respectively. Representative embryos ere photographed and fixed in glutaraldehyde (2.5% in 0.1 M sodium phosphate buffer, pH 7.4) for histological examinations. They were then dehydrated, embedded in hydroxyethyl methacrylate, sectioned with glass knives at 2 to 3 μM, and stained with toluidine blue.

Assays of Monooxygenase Activity. Conversion of estrogens to corresponding catechol metabolites was assessed as described by Hoffman et al. (20). Monooxygenase activities were measured under conditions described previously (21) as well as under conditions existing in the culture system. Reactions were linear with respect to time and protein concentration under the reaction conditions utilized. For certain experiments, tritium displacement analyses of estrogen hydroxylase activity (22) were employed to provide corroborative data for the routinely utilized assay.

Statistics. Student's t test was used to analyze differences in parameters measured on a continuous (graded) scale. Included were embryonic protein and DNA, measurements of length, limb bud size, prosencephalic index, somite numbers, and enzymatic activities. The χ^2 test was utilized for analyses of differences in parameters measured on a quantal scale (viability, abnormally open neural tubes, malformation incidence). Both the t test and Wilcoxon's Signed-Rank test were employed for analyses of differences in axial rotation. Correlations between embryonic abnormalities and catecholestrogen formation were analyzed by calculating Pearson's product-moment or "common" correlation coefficients and Spearman's coefficient of rank correlation. All statistical procedures are described by Steele and Torrie (23).

RESULTS

Concentrations of estrogen utilized were chosen from initial, preliminary range-finding experiments. Analyses of control data gathered during the course of all investigations revealed that none of the various estrogen-independent culture conditions utilized produced a statistically significant effect on the embryonic growth/development parameters measured. Also, additions of estradiol-17β (alone or in combination with CO) to the culture medium (Table 1) did not produce statistically significant changes in

Table 1. Estradiol-17β-dependent embryotoxicity to cultured whole rat embryos: Effect of cytochrome P-450-dependent biotransformation

Parameter[a]	Addition to culture medium			
	Estradiol-17β (0.1mM)		Estradiol-17β + S9/Cof[b]	
	O_2(32)[b]	CO(18)	O_2(44)	CO(19)
% Viable	95.8	100.0	84.1	94.7
% Abnormalities	9.4	11.1	100.0[c]	68.4[d]
flexure	6.3	5.6	52.3	26.63
neural tube	3.1	0.0	4.6	5.3
prosencephalon	6.3	11.1	100.0[c]	57.9[d]
other	6.3	5.6	86.4[c]	47.4[d]
Somite number	20.8 ± 0.64	20.3 ± 1.2	15.0 ± 1.1[c]	17.8 ± 1.3[d]
Embryo length (mm)	2.91 ± 0.2	2.84 ± 0.4	2.42 ± 0.2[c]	2.63 ± 0.4
Limb index	2.0 ± 0.3	1.8 ± 0.6	0.8 ± 0.5[c]	1.4 ± 0.4[d]
Prosencephalic index	0.84 ± 0.14	0.79 ± 0.18	0.48 ± 0.17[c]	0.67 ± 0.19[d]
Protein (μg/embryo)	190.0 ± 34.0	220.0 ± 61.0	91.0 ± 27.0[c]	162.0 ± 39.[d]
DNA (μg/embryo)	14.8 ± 2.9	14.7 ± 3.2	4.6 ± 2.4[c]	9.8 ± 3.1[d]

[a] Incidences of viability and flexure, neural tube, prosencephalic and other abnormalities are provided as quantal (all-or-none) data. All other indices of embryotoxicity are provided as quantitative measurements (See methods) on a continuous (graded) scale. These data represent the accumulation of all control values acquired during the course of these investigations.

[b] Numbers in parentheses are the numbers of embryos in each category. S9 is the hepatic postmitochondrial (9000g x 20min) supernatant fraction; 16 ± 3 mg protein/culture flask; Cof refers to NADPH (0.5 mM) and glucose 6-phosphate (5.0 mM) added as final concentrations to the culture medium at the beginning of the 24 hr culture period.

[c] Significantly different ($p<0.05$) from corresponding values appearing in columns 1 and 2 of this table and from corresponding values from 111 control embryos not exposed to estrogen.

[d] Significantly different ($p<0.05$) from corresponding values appearing in column 3 of this table.

growth parameters. However, addition of a hepatic microsomal (S9) fraction together with NADPH, glucose 6-phosphate and the same concentrations of estradiol-17β elicited profound changes in all parameters except viability and incidence of neural tube abnormalites. Partial replacement of nitrogen with carbon monoxide in the gas mixture resulted in statistically significant attenuations of estrogen-elicited, S9/cofactor-dependent embryotoxic effects for all parameters except embryonic length. For this

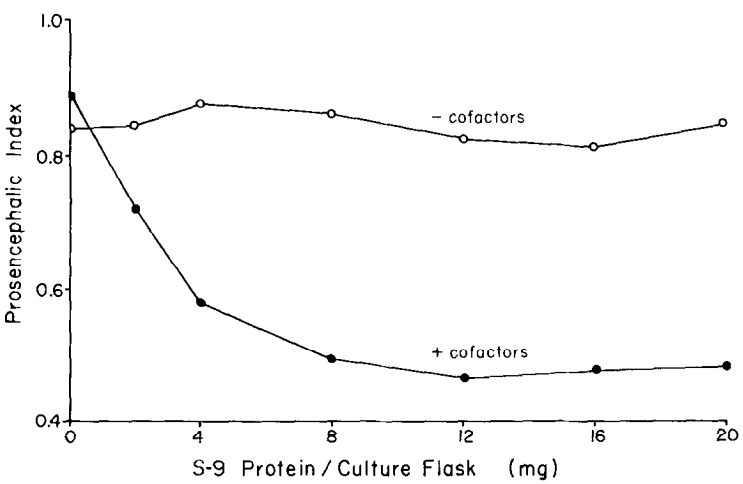

Figure 1. Effects of varying quantities of enzyme on estradiol-17β-mediated embryotoxicity in cultured whole embryos. Cofactors were NADPH and glucose 6-phosphate at 0.5 and 5.0 mM final concentrations, respectively. S9 was from livers of adult, male rats pretreated with a mixture of polychlorinated biphenyls (Aroclor 1254; see Methods).

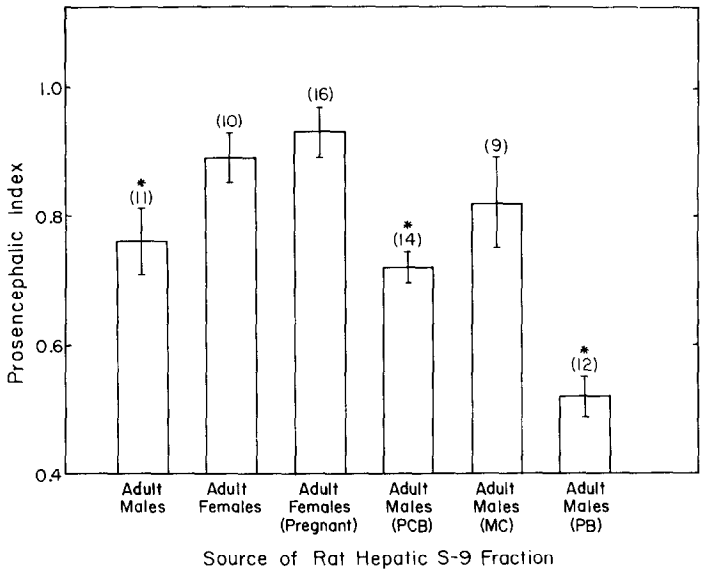

Figure 2. Effects of varying sources of enzyme on estradiol-17β-mediated embryotoxicity in cultured whole embryos. Cofactors (see legend to Fig. 1) were added in all cases. Culture flasks contained 2 mg S9 protein/flask. Numbers in parentheses are numbers of embryos evaluated. Error bars represent standard deviations. Asterisks indicate statistically significant ($p<0.05$) differences from controls to which enzyme was not added.

parameter, the trend was in the same direction but did not achieve statistical significance ($p>0.05$).

Because the data indicated that P-450-dependent conversion of estradiol-17β to reactive intermediates was responsible for the effects observed, we hypothesized that conversion to catecholestrogens, a major, P-450-dependent pathway for estrogen biotransformation, mediated the embryotoxic response. Experiments to examine this hypothesis showed that embryotoxicity varied as a function of enzyme concentration (Fig. 1), reaching a maximal effect at approximately 12 mg protein/vessel. Catecholestrogen generating activity in the medium varied linearly over the same range. Comparisons of S9 fractions from various sources (Fig. 2) showed marked differences in capacities of various enzyme sources to elicit changes in developmental parameters. At the protein concentrations utilized (2mg/culture flask), S9 from pregnant rats produced effects that did not differ from those observed in the absence of enzyme source, whereas S9 from phenobarbital-treated, adult male rats elicited the most profound effects (Fig. 2). Fractions from other sources produced intermediate effects. The capacity of these fractions to produce embryotoxicity likewise varied as a function of their catechol-estrogen-generating activities (Fig. 3). Regression analyses yielded a correlation coefficient (r) of 0.84 ($p<0.05$). In each of these analyses (Figs. 1-3) we utilized the prosencephalic index as the marker of embryotoxicity. This was found to provide the most consistent index and was also the most noticeable effect observable. A series of correlation analyses, however, showed that other measurements of embryotoxicity correlated fairly well with the prosencephalic index ($r = 0.63$-0.83; $p<0.05$ in all cases).

Substitution of equimolar diethylstilbestrol for estradiol-17β produced no statistically significant embryotoxic effects, even in the presence of the most active bioactivating system. Attempts to ameliorate the embryotoxic effects of estradiol-17β by adding a catechol-methylating system to the culture medium were unsuccessful. Separate experiments with radiolabelled S-adenosylmethionine and catechol-O-

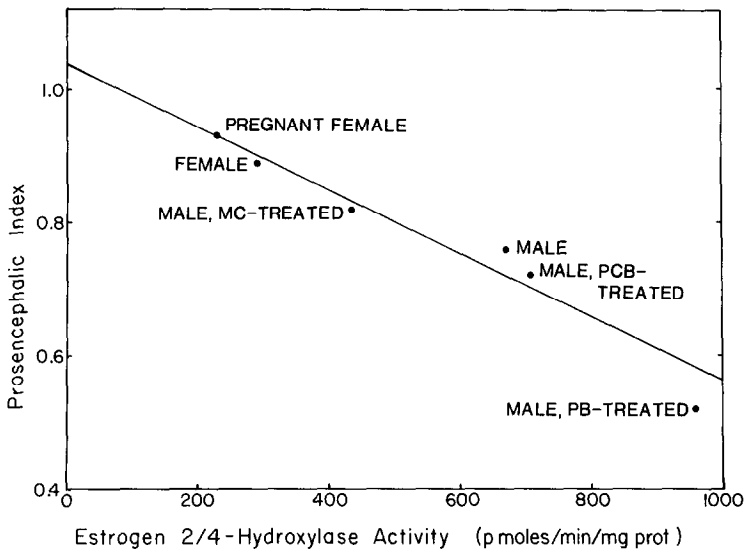

Figure 3. Relationship between estrogen 2/4-hydroxylase activity and capacity to convert estradiol-17β to embryotoxic intermediary metabolites as assessed with the prosencephalic index. Regression analysis yielded a correlation coefficient (r) of 0.84 ($p<0.05$).

methyl transferase indicated that relatively large quantities of methoxy estrogens were formed in the culture medium under those conditions (data not shown).

DISCUSSION

The results of these experiments show that an endogenous hormone estradiol-17β can be converted by P-450-dependent enzyme systems to reactive intermediates that elicit a variety of embryotoxic effects. The data also strongly suggest that the generation of catecholestrogens is involved in the embryotoxicity. However, the exact nature of putative reactive intermediates immediately responsible remains conjectural at this point. Inability to modulate the response through additions of an active catechol methylating system is compatible with the idea that a precatechol, perhaps an epoxyenone (11) may be a proximate embryotoxin. Nevertheless, the degree to which the generated catechols would be inactivated by this procedure cannot be accurately determined and further research will be required to fully resolve this issue.

It seems worthy of emphasis that, whatever reactive intermediates may have been directly responsible for the estrogen-elicited embryotoxicity, they caused a specific defect spectrum rather than merely a generalized growth retardation. This is best evidenced from comparisons of effects on neural tube closure vs. axial rotation illustrated in Table 2. No significant closure defects were manifest after any of the various treatments whereas both other parameters were drastically affected. This stands in direct contrast to the effects of bioactivated 2-acetylaminofluorene as assessed in the same system in which neural tube abnormalities were the primary defect, with no significant flexure changes observed (16). Thus, it seems clear that different reactive intermediates are capable of eliciting dramatically different malformations and it will be highly interesting to determine which intermediates are involved in the causation of specific defects.

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