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SHORT COMMUNICATION

REGULATION OF AROMATIC OXIDATION OF ESTRADIOL-17β IN MATERNAL HEPATIC, FETAL HEPATIC AND PLACENTAL TISSUES: COMPARATIVE EFFECTS OF A SERIES OF INDUCING AGENTS

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Summary—The effects of nine separate inducers of cytochrome P-450-dependent monooxygenases on the hydroxylation of estradiol-17β (E_2) were investigated in near-term pregnant rats. Isosafrole exhibited highly effective inducing properties in the maternal liver (20-fold and 5-fold increases in 4- and 2-hydroxylase activities respectively). Pregnenolone 16α-carbonitrile produced approx 20- and 30-fold increases in measured respective rates of 4- and 2-hydroxylase activities in fetal hepatic tissues; isosafrole produced only 2-fold increases in the same reaction. Only minor changes or slight increases in estrogen hydroxylation rates were observed in maternal hepatic, fetal hepatic or placental tissues following treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or other potent 3-methylcholanthrene (MC)-like inducing agents (β-naphthoflavone, MC, caffeine). Phenobarbital exhibited relatively weak inducing properties and exposure of pregnant rats to ethanol from days 3–19 of gestation was without statistically significant effects on the parameters investigated. Rat placentas exhibited extremely low estrogen hydroxylase activities irrespective of pre-exposure of pregnant rats to the inducers studied. The results suggested separate regulatory controls for estrogen 2- and 4-monooxygenase activities even though relatively high correlation between the two reaction were generally observed in all three tissues.

INTRODUCTION

Although peripheral plasma levels of circulating catechol estrogens are generally regarded as too low to exert neuroendocrine or other physiological effects, studies have shown [1, 2] that plasma levels are considerably higher during pregnancy. However, little is yet known concerning mechanisms of regulation of the generation of catechol estrogens during pregnancy. The liver is the most active organ in catechol estrogen biosynthesis, displaying estrogen 2- and 4-hydroxylase activities that are normally at least 1–2 orders of magnitude greater than those measured in extrahepatic tissues such as the brain and placenta [3–5]. This study was undertaken in order to gain further insights into regulatory aspects of catechol estrogen biosynthesis in maternal and fetal rat livers as well as in placentas. The latter organ was also chosen for study because previous investigations had demonstrated the responsiveness of human placental hydroxylation reactions to exogenous regulatory influences [6–8].

Enzyme systems which catalyze the biosynthesis of catechol estrogens include cytochrome P-450-dependent monooxygenases [9, 10] and their associated flavoproteins. Several P-450 isozymes that catalyze monooxygenation reactions display overlapping substrate specificities and many are highly inducible. Inducers, for the most part, are liposoluble, foreign organic chemicals with relatively long half-lives and have been categorized as either "3-methylcholanthrene (MC)-type" or "phenobarbital (PB)-type" inducing agents even though it is now recognized that such a classification is overly simplistic. Several "novel" or "unique" chemical inducing agents are reportedly capable of inducing specific isozymic forms of hepatic P-450. These include pregnenolon-16α-carbonitrile (PCN) [11], isosafrole [12], ethanol [13, 14] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [15]. Polyhalogenated biphenyls are established environmental contaminants that are also potent inducers of P-450-dependent monooxygenase systems. Commercial mixtures of these chemicals, such as Aroclor 1254 (hereafter referred to as PCB), contain combinations of both "MC-type" and "PB-type" inducing agents. This investigation was designed to compare the effects of several of the aforementioned inducers on catechol estrogen biosynthesis during pregnancy. Inducers chosen were PB and MC as standard reference inducing agents, PCN, isosafrole, TCDD, ethanol, caffeine, β-naphthoflavone (BNF) and PCB. The effects of inducers of pretreatment of near-term pregnant rats with these inducers were measured in terms of both estrogen 2- and 4-hydroxylase activities. For standardization purposes, the effects on these two activities also were compared with effects on aryl hydrocarbon hydroxylase (AHH, E.C. 1.14.19.1, formerly 1.14.14.2), a P-450-dependent monooxygenase which has been well-studied with respect to responses to induction.

EXPERIMENTAL

Chemicals

Estradiol-17β (E_2), MC, caffeine, benzo(a)pyrene, NADPH, glucose 6-phosphate (G6P), catechol-*O*-methyl transferase and *S*-adenosylmethionine were obtained from Sigma Chemical Co., St Louis, MO. TCDD was obtained from Cambridge Isotopes Laboratories, Inc., Woburn, MA. Isosafrole was purchased from ICN Pharmaceuticals, Plain-

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Table 1. Dosing schedule for the investigation of the regulation by inducers of catechol estrogen formation in tissues of the fetal-placental unit of the rat

Inducers	Dose	Vehicle	Schedule[a]
Phenobarbital	40 mg/kg	Normal saline	Twice daily, days 16–19
3-Methylcholanthrene	40 mg/kg	Corn oil	Once daily, days 17–20
β-Naphthoflavone	30 mg/kg	Corn oil	Once daily, days 17–20
Aroclor 1254	500 mg/kg	Corn oil	Single dose, day 14
Ethanol	10% in drinking water	5% Fructose in H_2O	10% in H_2O, days 3–20
Caffeine	75 mg/kg	1.15% KCl	Twice daily, days 16–19
Pregnenolone-16α-carbonitrile	40 mg/kg	Corn oil	Twice daily, days 16–19
Isosafrole	150 mg/kg	Corn oil	Once daily, days 16–19
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	6 μg/kg	Corn oil	Single dose, day 17

[a]Primigravada Sprague–Dawley (Wistar-derived) rats weighing 250–300 g were mated by and obtained from Tyler Laboratories, Bellevue, WA. Conception was checked by the presence of copulatory plugs on the morning after mating and the first day following mating was designated as day 0 of gestation. Except as noted, the pregnant animals were received on day 15 of gestation and housed 2–3 animals/cage on hardwood bedding and given regular Purina rat chow and water *ad libitum*. All animals were sacrificed on day 21 of gestation. All drugs except ethanol were given by intraperitoneal injection. Ethanol was taken in the drinking water with 5% fructose.

view, NY. BNF was supplied by Aldrich Chemical Company, Milwaukee, WI. PCB was purchased from Analabs Inc., North Haven, CT. PB was obtained as the pure powdered sodium salt from the University Hospital Pharmacy, Seattle, WA and PCN was received as a generous gift from Searle Laboratories, Inc., Chicago, IL. [4-^{14}C]E_2 (58 mCi/mmol, 98% purity) was purchased from Amersham Corp., Arlington Heights, IL. Spectroquality solvents were obtained from Matheson, Coleman and Bell, Norwood, OH. Specifically, labelled 2- and [4-^{3}H]E_2 were synthesized according to methods described earlier [16].

Enzyme assays

All animals were sacrificed after treatment (Table 1) by cervical dislocation on the morning of day 21 of gestation. Tissues were surgically excised and pooled such that 3–4 maternal livers represented in each pool of inducer-treated animals, 2–3 maternal livers were represented in each pool of vehicle-treated animals, 18–40 placentas or fetal livers were represented in each pool of inducer-treated animals and 9–32 placentas or fetal livers were represented in each pool of vehicle-treated animals. Placentas, fetal livers and maternal livers were each blotted, weighed, pooled and homogenized in a glass homogenizing vessel (Potter) with a teflon pestle in 2 volumes of ice-cold, 1.15% KCl. The homogenates from each tissue pool were then centrifuged at 9000 *g* for 20 min (maternal livers) and for 10 min (fetal livers and placentas). The supernatant fractions (S-9) were decanted and used immediately (without storage) as the enzyme source. Maternal hepatic S-9's were diluted (in 1.15% KCl) 1:4 for assays of estrogen biotransformation and 1:9 for assays of AHH activity. Placental and fetal hepatic S-9's were not diluted. Measurements of estrogen hydroxylation with tritium release have been utilized by several previous investigators (e.g. see reference 17) and, in these experiments, hydroxylase activities were routinely assessed by measurements of released tritium present in the aqueous phase following incubations and extractions (4X) of the respective labelled estrogens into chloroform–acetone (4:1, v/v). For tissues with low activities (fetal liver and placenta) a final extraction of the aqueous phase with activated charcoal was performed on a routine basis. Incubation flasks typically contained [2-^{3}H]E_2 (32 mCi/mmol) or [4-^{3}H]E_2 (34 mCi/mmol), (final substrate concentrations were 500 μM), 0.2–5.0 mg protein, G6P (8.77 mM), NADPH (0.35 mM), and sufficient potassium phosphate buffer (0.1 M, pH 7.4) to bring the total volume to 1.0 ml. Reaction flasks were normally incubated for 120 min (10 min for maternal hepatic tissues) at 37°C in a Dubnoff Shaking incubator with 100% O_2 as the gas phase. The reaction was stopped by adding an equal volume of ice-cold acetone followed by 4.0 ml of cold chloroform. The mixture was then vortexed at high speed for 1 min and centrifuged. The organic layer was removed and 4.0 ml of fresh chloroform–acetone were added to the incubation flasks. The mixture again was vortexed, centrifuged, and the organic phase removed. This procedure was repeated once more after which a final extraction with 4.0 ml of chloroform–acetone and a large excess of activated charcoal was carried out. Fifth and sixth extractions with chloroform–acetone (80:20, v/v) or with chloroform–methanol–water (8:4:3, by vol) indicated that significant additional radioactivity did not appear in the organic layer following the fourth chloroform–acetone extraction. A 0.1 ml aliquot of the aqueous phase was added to scintillation vials containing 10.0 ml Aquasol (New England Nuclear). Counting efficiency was consistently greater than 33% and samples were counted for a sufficient length of time to provide for less than a 5% error with 95% confidence limits. To verify that the radioactivity measured in the aqueous phase was actually due to the presence of ^3H_2O, 1.0 ml samples were obtained from incubation flasks containing S-9 preparations that had been incubated according to the procedures described above. The aqueous phase was extracted four times with chloroform–acetone according to the described assay. A 100 μl aliquot of the original, undistilled aqueous phase contained 63,799 dpm; after one distillation of the aqueous phase, a 100 μl aliquot contained 60,991 dpm. A second distillation resulted in water containing 61,567 dpm/100 μl.

Incubation flasks to which ice-cold acetone and chloroform were added at time zero were utilized as controls. Corrections for spontaneous tritium release were made by subtracting radioactivity released by the same concentration of labeled E_2 into buffer at 37°C in a 100% O_2 atmosphere during a 2 h incubation period (placenta and fetal liver) or a 10 min period (maternal liver). Reaction rates were linear with time for at least 15 min with maternal hepatic preparations (0.1–0.4 mg protein) and for at least 120 min with placental and fetal hepatic preparations (0.5–5.0 mg protein). Reaction rates also increased linearly with increasing protein concentrations [18] within the ranges used in these experiments. Detection limits (41 pmol) for the assay procedures were calculated as defined by IUPAC (k = 3) using the Propagation of Errors method as described by Long and Winefordner [19]. AHH was assayed according to methods described by Nebert and Gelboin [20].

RESULTS

Basal rates of estrogen hydroxylation, as measured in maternal and fetal livers and in placentas, are presented in Table 2. For purposes of comparison, rates of the same reactions in adult rat livers of non-pregnant females and males and of the 3-hydroxylation of BaP are presented in the same table. As indicated, profound differences in rates were noted among these various tissues. Adult male rat livers exhibited estrogen monooxygenase activities that were ap-

Table 2. Measured mean rates of hydroxylation of estradiol-17β (E_2) and benzo(a)pyrene (BaP) in tissues of rats treated with vehicle only

Enzyme source	Reaction		
	E_2-2-hydroxylation	E_2-4-hydroxylation	BaP-3-hydroxylation
Maternal liver	61.20 ± 18.30 (22)	39.30 ± 14.20 (22)	24.90 ± 6.21 (22)
Fetal liver	1.86 ± 0.49 (22)	1.52 ± 0.71 (22)	0.23 ± 0.11 (22)
Placenta	0.80 ± 0.38 (24)	0.66 ± 0.38 (24)	0.03 ± 0.02 (24)
Nonpregnant female liver (adult)	185.00 ± 39.40 (3)	125.00 ± 33.9 (3)	33.60 ± 7.35 (3)
Male liver (adult)	891.00 ± 161.00 (3)	513.00 ± 102.00 (3)	94.60 ± 16.30 (3)

Rates are expressed as pmol/mg protein/min with standard deviations. Numbers in parentheses are the number of pooled (minimum of 2 [usually 3] organs per pool) tissue homogenate subfractions (see Experimental) used to calculate the means.

prox 4–5-fold higher than those measured in livers of non-pregnant, adult females, 13–15-fold higher than those in maternal livers, 300–500-fold higher than in fetal livers, and 800–1100-fold higher than activities observed in rat placentas.

The effects of inducing agents on estrogen and BaP hydroxylase activities in the tissues investigated are presented in Table 3. In maternal livers, PB produced 2–3-fold increases, PCB produced 7–8-fold increases and PCN elicited approx 9- and 14-fold increases in 2- and 4-hydroxylation respectively. The most profound increases produced in the maternal liver were those elicited by isosafrole (approx 19-fold) on 4-hydroxylation. The same inducer produced only about 5-fold increases in 2-hydroxylation. All other agents tested produced only minimal or negligible effects on estrogen hydroxylation, even though MC, BNF and TCDD produced profound effects on AHH activities.

In the fetal liver, PCN was a remarkably effective inducer, producing 20 and 30-fold increases in estrogen 4- and 2-hydroxylation rates respectively. By comparison, the next best response was obtained with PB on rates of 2-hydroxylation but the increase (as compared with vehicle-treated control) was only approx 3-fold. All other inducers produced less than 2-fold increases even though extremely large increases in AHH activity were produced by MC, BNF, PCB, PCN, isosafrole and TCDD.

In the placenta, inducers were strikingly ineffective in terms of estrogen hydroxylation reactions. The largest increase was produced by PB on 2-hydroxylation, but this was less than 2-fold. In the same placentas, more than 10-fold increases in AHH activity were observed following induction with MC, BNF, PCB and TCDD. More than 3-fold increases were also observed with ethanol, PCN and isosafrole. Only caffeine and PB produced less than 2-fold increases in placental AHH activity.

DISCUSSION

Critical in the reported assay procedure is an accurate assessment of blank values. This is complicated by the spontaneous release of tritium which occurs during incubations, even when incubated only with a buffer solution or distilled water. In fact, it was found that a very slow, spontaneous release occurs even when either 2- or 4-tritiated estrogen is stored in absolute (>99.5%) ethanol at liquid nitrogen temperatures. Over time, this results in increased blank values which are potentially troublesome. However, this can be corrected by repurifying the estrogens on HPLC. Rates of spontaneous release during a 2 h incubation at 37°C in a 100% O_2 atmosphere are sufficiently rapid that zero-time blanks cannot be utilized unless the spontaneous release is corrected for. With washed microsomal preparations, the recommended blank is an incubated flask in which only the requisite cofactors for monooxygenation are omitted. For S-9 fractions, cofactors are present in the cytosol, rendering a spontaneous release correction for zero-time blanks necessary. Heat- or protease-inactivated tissue blanks were not satisfactory in our experience because such blanks yielded slightly higher values which were also more variable. Some tissues (e.g. uterus) also catalyze peroxidase-mediated tritium release [21, 22] in preference to P-450-mediated, hydroxylation-dependent release. None of the three tissues under study appeared to catalyze significant peroxidase-mediated tritium release as judged from studies of inhibition with catalase and carbon monoxide. Nevertheless, Fishman and Norton [23] have pointed out that the nucleophilic nature of the 2 and 4 carbons render these carbons subject to attack by activated oxygen which could be generated by a variety of chemical reactions. Thus, we cannot be entirely positive that the basal reaction rates reported in Table 2 resulted wholly from P-450-dependent monooxygenations. The *increased* reaction rates resulting from treatment with P-450 inducers, however, are very likely to be P-450-dependent.

Using the IUPAC (International Union of Pure and Applied Chemistry) approach [17] with a k value of 3, a standard deviation of 382 dpm per flask and a slope of 28.16 dpm/pmol, the limit of detection of tritium for the described assay was calculated to be 41 pmol. With 120 min incubations and 5.0 mg of protein, this would correspond to a sp. act. of 0.07 pmol/mg protein/min. (Placental and fetal hepatic preparations could be incubated for 120 min without significant deviation from linearity.) The use of $k = 3$ allows a confidence level of 99.86%, assuming that the blank signal follows a normal distribution, or 89% for a non-normal distribution. Thus the sensitivity of the assay is more than adequate for measurements in tissues with extremely low specific activities. The Propagation of Errors approach yielded only very slightly higher values, indicating, as expected, that the major source of error was in the blank.

The data demonstrate the effectiveness of several different inducing agents in terms of their capacity to increase rates of catechol estrogen formation in rats during the latter part of gestation. The most striking results were obtained with PCN which was extremely effective in both maternal and (particularly) fetal hepatic tissues. PCB and isosafrole were highly effective in the maternal liver, but neither exhibited pronounced effects in fetal liver or placenta. This pattern of response is unexplained at present, but is probably a function of the presence of different isozymic species of P-450 in each tissue. Presumably, different P-450 species would vary, not only in terms of their relative specificities for hydroxylation at the 2 vs 4 positions, but also in regulatory response to various inducing agents. The placental estrogen-hydroxylating enzymes appeared remarkably resistant to the effects of inducers, particularly when compared with placental response to induction of AHH. This also may reflect the functional and regulatory heterogeneity of tissue-specific P-450 isozymes.

In general, a strong correlation between estrogen 2- and 4-hydroxylase activities was observed. Utilization of the data in Table 2 yielded a correlation coefficient of 0.999 for 2- vs 4-hydroxylase activities and utilization of the data in Table 3 yielded a correlation coefficient of 0.810. This would suggest that the two estrogen hydroxylation reactions could

Table 3. Effects[a] of treatment with inducing agents on estrogen-2-hydroxylase, estrogen-4-hydroxylase and aryl hydrocarbon hydroxylase activities in pregnant rats near term

Inducing agent	Reaction								
	E_2-2-hydroxylation			E_2-4-hydroxylation			BaP-3-hydroxylation		
	Maternal hepatic	Fetal hepatic	Placental	Maternal hepatic	Fetal hepatic	Placental	Maternal hepatic	Fetal hepatic	Placental
Phenobarbital	2.8	3.1	2.0	2.8	1.8	1.0	2.1	2.4	2.0
3-Methylcholanthrene	1.6	1.9	1.1	1.4	1.7	1.8	13.4	69.1	52.2
β-Naphthoflavone	1.2	1.3	0.9	1.1	1.8	0.9	13.5	51.4	10.9
Aroclor 1254	8.2	1.3	1.1	7.2	1.3	1.7	22.4	41.4	16.3
Ethanol	1.3	1.2	1.0	1.2	1.3	1.3	1.1	0.7	5.1
Caffeine	1.0	1.1	0.9	1.2	0.9	1.1	1.7	1.7	0.9
Pregnenolone-16α-carbonitrile	9.4	29.3	1.6	13.4	19.8	1.9	3.4	6.8	3.7
Isosafrole	4.9	2.0	1.0	18.5	1.6	1.0	4.9	12.2	3.9
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	1.4	1.7	1.1	1.2	1.8	0.9	31.5	73.7	16.9

[a]Numbers in the table are ratios of mean specific activities (treated/control) calculated from a minimum of 3 (usually 4) pooled maternal hepatic tissues and 18 pooled fetal hepatic or placental tissues (3–4 treated pregnant animals/pool) for the inducing agents listed. Treatments were repeated at least once in order to verify the observed ratios. Mean specific activities for controls are reported in Table 2.

be under similar regulatory control, perhaps catalyzed by the same P-450 isozyme(s). However, close correlations may be partially accounted for by the possibility that hydroxylation of one position could possibly result in the eventual release of tritium at another (e.g. 2-hydroxylation could release tritium from [4-^{3}H]E_2 as the result of subsequent quinone formation and covalent binding at the 4 position). This possibility could also account for high 4-hydroxylase activities observed relative to the 2-hydroxylase activities in the tissues investigated. Nevertheless, the much greater response of the maternal hepatic 4-hydroxylation to the effects of isosafrole (and slightly greater response to PCN) would tend to argue for a more independent regulation and the possibility of differentially (but similarly) regulated P-450 isozymes specific for each reaction. Confirmation of this speculation will, of course, require additional reserarch. It was also interesting to note that the fetal hepatic 2-hydroxylation reaction was somewhat more responsive than 4-hydroxylation to PB and PCN (the latter was once regarded as a "PB-type" inducer) whereas in the placenta, 4-hydroxylation was somewhat more responsive than 2-hydroxylation to MC. These latter results are in general agreement with some earlier reported experiments with PB and MC [6–8].

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