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# COORDINATED INDUCTION OF ESTROGEN HYDROXYLASE AND CATECHOL-*O*-METHYL TRANSFERASE BY XENOBIOTICS IN FIRST TRIMESTER HUMAN PLACENTAL EXPLANTS

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**Summary**—The estrogen phenol A-ring metabolism was investigated in the first trimester placenta using radioenzymatic techniques. In untested explants cultured for 16 h, estrogen hydroxylase (EH) but not catechol-*O*-methyl transferase (COMT) activity was increased significantly 1.8-fold ($P < 0.05$). Cultures made in the presence of chemoprotectors, 25 $\mu$M of 1-phenylazo-2-naphtol (Sudan I) and coumarin but not 2(3)-tert-butyl-4-hydroxyanisole (BHA) caused a significant increase in EH activity, 1.8- and 2.2-fold, respectively ($P < 0.05$). This was coupled with a significant, $P < 0.05$, increase in the COMT activity by 25 $\mu$M of all three chemoprotectors, BHA, Sudan I, and coumarin, 2.7-, 2.3-, and 2-fold respectively. The carcinogens benzo(a)pyrene and 20-methylcholanterene at 50 $\mu$M concentration, however, had no effect upon both enzymes' activity. Finally, the two enzymes's activities were correlated under the experimental conditions tested. Except for zero time where no correlation was found ($r^2 = 0.3$), in all other experimental conditions, a significant ($r^2 = 0.75$) correlation was observed. In conclusion, EH and COMT enzyme activities appear to undergo a coordinated induction in cultured placental explants in the first trimester. The implications of catechol metabolism for embryonal development are discussed.

## INTRODUCTION

The placenta is a major steroid producing organ whose capacity increases markedly during advancing gestation [1]. Androgens are converted to estrogens via aromatase [2, 3], which is followed by the metabolism of primary estrogens (estradiol-17$\beta$ and estrone) at the D-phenol-ring leading to the formation of estriol [4, 5]. Estrogen can also be metabolized at the A-ring leading to the formation of catechol estrogens (CE) by estrogen hydroxylase (EH) [6]. The information available on this quantitatively important placental pathway is limited [7–9]. We have recently characterized placental EH enzyme activity at term [10]. The products formed by incubations with placental microsomes were principally 2-hydroxyestrogens, which are regarded as weak estrogen agonists or antagonists [11, 12]. The information on the formation and metabolism of CE in the first trimester, however, is scant [13].

At term, placental EH activity was shown to be induced by maternal cigarette smoking and benzo(a)pyrene (BP), a known carcinogen [14]. Several reports have also suggested that during the formation of CE, several highly unstable reactive intermediates are also produced [15]. These metabolites' involvement together with CE in the formation of cancer has been brought forward [16, 17].

Catechol estrogens are inactivated by conversion to methyoxyestrogens via catechol-*O*-methyl transferase (COMT). We have recently showed that this placental enzyme activity is lower in high risk conditions (toxemia and chronic hypertension) [18] and can be modulated by antihypertensive drugs *in vitro* at term [19]. Therefore it appears that at term local CE formation by EH and metabolism by COMT are affected by maternal pathologic conditions and xenobiotics at term. Whether catechol metabolism is responsive to environmental influences in the early placenta when embryogenesis occurs and is completed is currently unknown.

In the present study, we have examined the relationship between CE formation and metabolism in the first trimester placenta *in vitro*. This was carried out by determining EH and COMT activity under both basal conditions and following incubations with carcinogens and chemoprotectors.

## EXPERIMENTAL

### *Chemicals*

*S*-adenosyl methionine ([$^3$H]SAM, 14.5 Ci/mmol) was purchased from Amersham (U.K.). Unlabeled

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SAM, epinephrine, NADPH, estradiol-17$\beta$, and 20 methylcholanterene (MC), BP, 2-hydroxyestrone, coumarin, 2(3)-tert-butyl-4-hydroxyanisole (BHA), 1-phenylazo-2-naphtol (Sudan I) were purchased from Sigma (St Louis, Mo.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Beit Haemek (Israel). Other chemicals were of high analytical quality.

*Explant preparation*

Fifteen first trimester placentae (8–12 weeks of gestation), obtained by appropriate consent, were collected from elective pregnancy terminations done at Rambam Medical Center. The placental tissue was rinsed in 0.9% NaCl to remove the blood, and then washed in DMEM plus 2% antibiotics (10,000 U penicillin, 10 $\mu$g streptomycin and 10 $\mu$g fungizone). Subsequently, 20 mg wet wt explants, 1–3 mg protein, were prepared as previously reported [20], and incubated (6 explants per xenobiotic treatment group or vehicle only treated control) in DMEM plus 1% antibiotics with or without various xenobiotics at 37°C in an atmosphere of 95% air and 5% $CO_2$ for 6–24 h. At the end of incubation, the explants were removed and stored at −20°C until assayed within a few days. Explants were homogenized with 1.5 ml of cold 0.25 M sucrose using a glass homogenizer, and then centrifuged at 800*g* at 4°C for 5 min. The supernatant obtained was used for the assay.

The explants' viability in this model was assessed by the progressive glucose consumption in the media, and the nearly linear increase in hCG secretion during culture. This secretion was blocked by incubation with $10^{-5}$ M cyclohexamide, a protein synthesis inhibitor (data not shown).

*EH assay*

We have recently reported in detail the methodology for measuring EH activity in the placenta [10]. In that report, the products formed by the incubation were identified by HPLC. Briefly, the assay mixture consisted of Tris–HCl buffer 0.01 M, pH 7.4, 30–300 $\mu$g placental protein, 1 M $MgCl_2$, 200 $\mu$M ascorbic acid, 20 $\mu$l partially purified rat liver COMT, 25 $\mu$M estradiol-17$\beta$ in ethanol and tritiated (SA 12.8) SAM 9 $\mu$M in a total vol of 1 ml. Incubation mixtures were then vortexed, placed in glass vials, and immersed in a water bath at 37°C for 5 min. The enzymatic reaction was started by adding 1 mM NADPH to the mixture. After 30 min, incubation samples were placed on ice and the reaction was stopped by adding 0.5 ml of 0.05 M borate solution, (pH 10.5). Samples were extracted with 3 ml Lipoluma and counted in scintillation vials at 52% efficiency. Incubates without $E_2$ served as blank. They had an activity of $<10\%$ of actual samples. The reaction was linear for 0–30 min at protein concentrations of 0.1–2 mg/ml.

*COMT assay*

The assay for measuring placental COMT activity was previously reported by us [18]. We found that the only product formed during incubation is 2-methoxyestrone. Briefly, the incubation mixture consisted of phosphate buffer pH 7.7 (0.2 M), 18 mM $MgCl_2$, 10 $\mu$M 2-hydroxyestrone, 1.8 $\mu$M [3H]SAM, and placental protein in a total vol of 1 ml. Incubations were carried out in a water bath at 37°C for 30 min. After incubation, samples were placed on ice to cool, and the reaction was stopped by adding 500 $\mu$l of a 0.5 M (pH 10.5) sodium borate solution. The product formed during incubation was extracted with 3 ml of Lipuloma, decanted and counted at 52% efficiency. Recovery was $>90\%$. Samples without protein served as blanks. They had an activity of $<10\%$ of the actual samples.

The reaction was linear for 30 min at protein concentrations of 0.5 to 3 mg/ml. Explants' protein content was measured according to the method of Lowry *et al.*[21].

*Statistical analysis*

Statistical analysis was carried out by using one-way ANOVA, Student's *t*-test, and linear regression. $P < 0.05$ was considered statistically significant. Data represent mean ± SEM measurements of three or more different placentae. Both enzymes' activity is expressed as nmol/mg protein/30 min.

## RESULTS

Figure 1 shows the basal EH activity in placental explants. Compared to zero time, there was a significant (2-fold, $P < 0.05$) increase in activity after 16 h of culture. Therefore, in subsequent experiments the effect of xenobiotics was tested already after 6 h of culture.

*EH activity: effect of chemoprotectors*

Figure 2 shows that incubations made with 25 $\mu$M Sudan I and coumarin increased EH enzyme activity

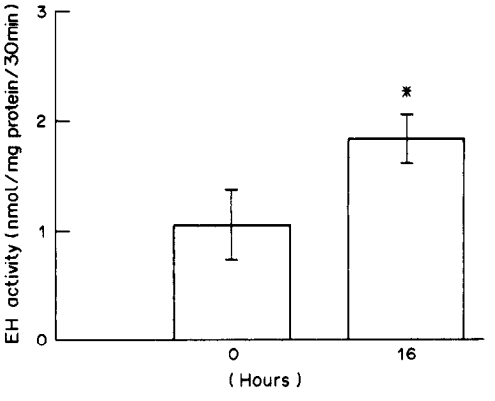


Fig. 1. Expression of EH activity in placental explant cultures. Compared to zero time, there was a significant increase in the mean ± SEM enzyme activity following 16 h of culture. *$P < 0.05$.

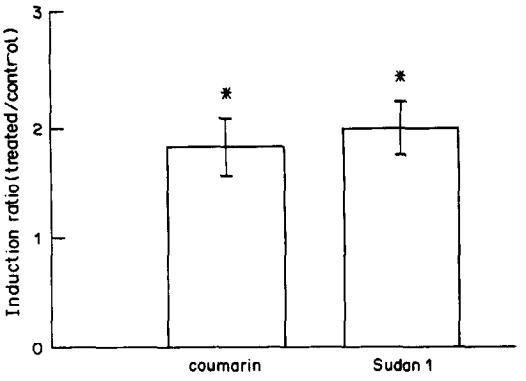


Fig. 2. Effect of 25 $\mu$M of chemoprotectors upon placental EH activity in culture. Compared to the vehicle only treated controls, there was a significant increase in coumarin and Sudan I induced mean $\pm$ SEM enzyme activity. Data is expressed as induction ratio treatment/vehicle only treated control, $^*P < 0.05$.

2.2- and 1.8-fold respectively, $P < 0.05$. Only in one placenta incubations with 25 $\mu$M BHA caused a significant increase in the EH activity (Table 1).

*EH activity: effect of carcinogens*

The effect of 50 $\mu$M BP upon placental explants' EH activity of the 5 different placentae was tested (Fig. 3A). Only one placenta had a significant increase in the enzyme activity following exposure to BP at 6 h, $P < 0.05$. This activity, however, returned to control levels after 24 h (data not shown). Incubations with 50 $\mu$M MC had no significant effect on the EH activity (Fig. 3B).

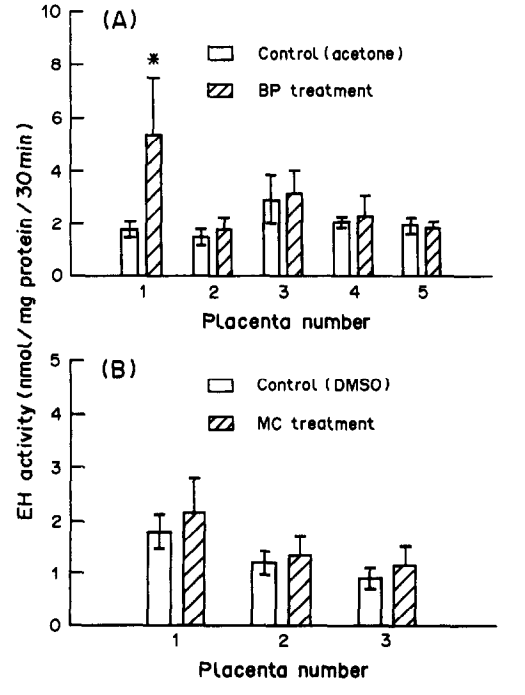


Fig. 3. Effect of 50 $\mu$M BP (panel A) and MC (panel B) upon placental explants EH activity following 6 h incubation. $^*P < 0.05$. Data is expressed as mean $\pm$ SEM.

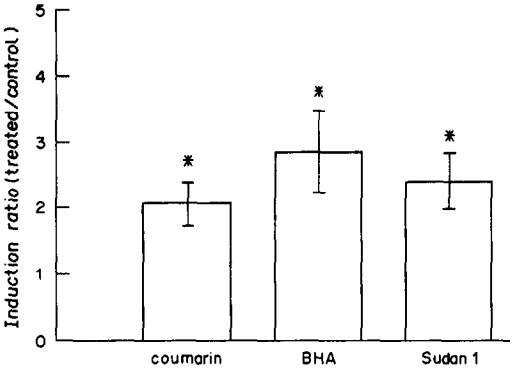


Fig. 4. Effect of 25 $\mu$M of chemoprotectors upon placental COMT activity in culture. Compared to vehicle only treated controls, there was a significant increase in the mean $\pm$ SEM enzyme activity following exposure to coumarin, BHA, and Sudan I. Data is expressed as COMT induction ratio treatment/vehicle only treated control, $^*P < 0.05$.

*COMT activity in culture*

Compared to zero time basal COMT activity did not change after culture for 16 h (data not shown).

*COMT activity: effect of chemoprotectors*

Figure 4 shows that COMT activity in culture is induced by all three chemoprotectors tested, 25 $\mu$M BHA, Sudan I, and coumarin, 2.7-, 2.3-, and 2-fold respectively, $P < 0.05$.

*COMT activity: effect of carcinogens*

Only in one placenta out of the five tested, COMT activity was induced by 50 $\mu$M BP (Fig. 5A) after 6 h

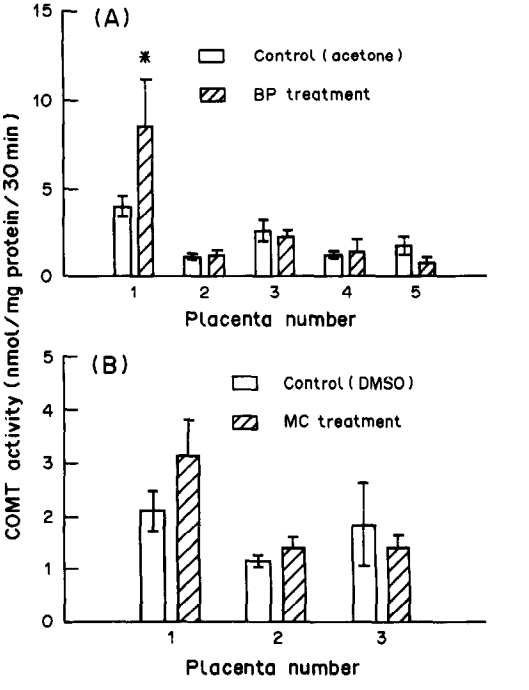


Fig. 5. Effect of 50 $\mu$M BP (panel A) and MC (panel B) upon COMT activity in placental explants after 6 h incubation. Data is expressed as mean $\pm$ SEM. $^*P < 0.05$.

Table 1. Effect of 25 $\mu$M BHA incubated for 6 h upon placental explants EH activity

| Placenta number | Control | BHA treatment | |
|---|---|---|---|
| 1 | 0.44 ± 0.04 | 0.32 ± 0.06 | $P > 0.1$ |
| 2 | 1.3 ± 0.19 | 6.17 ± 2.16 | $P < 0.05$ |
| 3 | 1.06 ± 0.2 | 1.52 ± 0.4 | $P > 0.1$ |

Data is expressed as mean ± SEM nmol/mg protein/30 min.

incubation. In the same dose, incubations with MC had no effect upon the enzyme activity (Fig. 5B).

*Correlation between EH and COMT activities*

Finally, the correlation between basal and induced EH and COMT activity was examined. At zero time, no correlation between the activity of the two enzymes was found ($r^2 = 0.3$ data not shown). In contrast, a significant positive correlation was found between the two enzymes' activity in culture at both basal conditions and following exposure to the five classes of xenobiotics used in the study, $r^2 = 0.75$, $P < 0.05$ (Fig. 6).

## DISCUSSION

Previous studies have suggested that the early placenta has a very limited capacity to either metabolize xenobiotics or to respond to environmental toxins [22]. Such a view led to the belief that no link exists between environmental toxins and genotoxicity at that time. In this report we present evidence that the mixed function oxidase enzyme EH, which leads to CE formation, is active in the early trimester placenta and that catechol metabolism through COMT is also affected by exposure to xenobiotics *in vitro*. Similarly, direct correlation between the two enzymes activity was found under both control and experimental conditions. This, however, was not observed at zero time before incubation. The reasons behind these last findings are not clear. In our view, they may be related to the stimulatory effect that the products formed by EH in culture have upon their further metabolism by COMT [16] which led to the observed coordinated induction of the two enzymes by xenobiotics *in vitro*. The direct effect of the xenobiotics themselves upon the two enzymes' activity is also not excluded. In contrast, zero time enzyme activity reflects a situation which is similar to that found *in vivo* where environmental influences could differently modulate the two enzymes. In our view, catechol amines could be a major factor that affect COMT activity [18, 24].

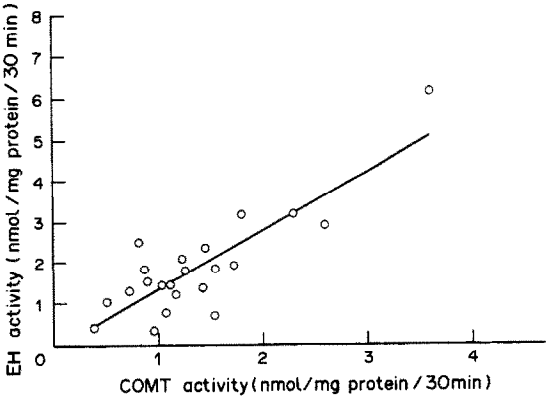


Fig. 6. Correlation between EH and COMT activity in cultured placental explants. Under both basal and experimental conditions, there was a significant correlation ($r^2 = 0.75$), $P > 0.01$, between the two enzyme activities.

EH is a microsomal enzyme, while COMT has a cytosolic location. Recently however COMT was shown to also have a membranous location [24], which would facilitate coordination between the two enzyme systems. The direct significant correlation found between EH and COMT activity also indicates that the placenta is geared towards inactivation of CE through methylation. This may be important for a number of reasons: first, CE are potent compounds that we have shown to affect local steroidogenesis via catecholaminergic receptors [18]; second, CE antagonizes primary estrogens action by binding to the estrogen receptor, as we and others have reported previously [11, 12]. At which point they may counteract the vasodilatory function [25] of primary estrogens which is critical for pregnancy maintenance [1]; third, during CE formation highly unstable reactive intermediates are formed. These epoxydiols could also be inactivated by COMT, which is similar to what was observed for BP in other systems [26].

Cigarette smoking and BP induce placental EH activity in microsomes at term [14]. However, except for one placenta where both EH and COMT activity were induced by exposure to BP but not MC, the activity in other placentas was not changed following these exposures. Since all placentas tested were at the same gestational age, it is likely that the hyperresponsiveness and high basal levels found for both enzymes are caused by either an unknown environmental exposure or an increased gene expression. It is of interest to note that in this specific case, both basal enzyme activities were elevated, which suggests that, although COMT activity does not increase in culture, it adapts to the high EH activity present.

As seen in Fig. 2, EH activity was induced by two groups of chemoprotectors; Sudan I, a food additive that commonly reaches the placenta by maternal ingestion and by coumarin, an anticoagulant. This last drug is a teratogen that causes a typical warfarin embryopathy [27]. Our data indicate that in therapeutic concentrations, such a drug can interact with the placenta and induce EH activity which could also be one of the avenues by which the toxicity of this drug is enhanced. Such an effect could be in addition to the direct effect that the drug may have on the placenta and the embryo.

It was of interest to note that COMT activity was also induced by chemoprotectors which generally induced type II enzyme activity (those responsible

for inactivating xenobiotics) [28] which raises the possibility that COMT may also serve as a type II enzyme in the organism.

Since xenobiotics activate placental EH activity and, as recently shown by us, aryl hydrocarbon hydroxylase activity in the first trimester (Sanyal M. K. and Barnea E. R., unpublished observations), COMT is likely to assume an important role in this period by protecting the placenta and the vulnerable developing embryo from activated hydroxylated compounds formed by these two enzymes. This induction of COMT is compatible with our previously reported effect of antihypertensive drugs on the placenta at term [19]. Whether COMT has such a role outside the placenta remains to be demonstrated.

Overall, it appears that the catechol metabolism as expressed by EH and COMT activity is sensitive to exposure to xenobiotics with a demonstrable direct correlation between CE formation and inactivation. Chromatographic data previously reported by us support such a possibility [10, 18].

In conclusion, placental CE formation and inactivation is modulated by xenobiotics in the first trimester which could be an important pathway by which the early placenta and embryo are affected by the environment.

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