O-Methylation of Catechol Estrogens by Human Placental Catechol-O-Methyltransferase: Inter-individual Differences in Sensitivity to Heat Inactivation and to Inhibition by Dietary Polyphenols

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d) **Abbreviations used:** COMT, catechol-O-methyltransferase; 2-OH-E2, 2-hydroxyestradiol; 4-OH-E2, 4-hydroxyestradiol; 2-MeO-E2, 2-methoxylestradiol; AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; EGCG, (-)-epigallocatechin-3-gallate.
ABSTRACT

The human catechol-O-methyltransferase (COMT) is a polymorphic enzyme that catalyzes the O-methylation of catechol estrogens. Recent animal studies showed that the placental COMT is involved in the development of the placentas and embryos, likely via the formation of 2-methoxyestradiol. In this study, we analyzed a total of 36 human term placentas to determine their cytosolic COMT activity for the O-methylation of catechol estrogens as well as their sensitivity to inhibition by heat and dietary compounds. Large variations (up to 4-fold) in the COMT activity for the formation of methoxyestrogens were noted with different human placental samples. The cytosolic COMT in different human placentas also displayed considerable differences in their sensitivity to heat inactivation. This differential sensitivity was not associated with the overall catalytic activity for the O-methylation of catechol estrogen substrates. It was observed that there was a positive correlation ($r = 0.760$) between the sensitivity of the human placental COMT to heat inactivation and its sensitivity to inhibition by (-)-epigallocatechin-3-gallate (a well-known tea polyphenol with COMT-inhibiting activity), but an inverse correlation ($r = 0.544$) between heat inactivation and inhibition by quercetin (another dietary COMT inhibitor). The differences in inhibition by these two dietary compounds are due to different mechanisms of COMT inhibition involved.
INTRODUCTION

Endogenous estrogens, such as $17\beta$-estradiol ($E_2$) and estrone ($E_1$), undergo rapid oxidative metabolism in humans, mostly catalyzed by cytochrome P450 enzymes, resulting in the formation of hydroxylated or keto metabolites (Zhu and Conney, 1998a; Zhu, 2002). Catechol estrogens, such as 2- and 4-hydroxyestradiols (2-OH-E$_2$ and 4-OH-E$_2$, respectively), are among the major oxidative estrogen metabolites formed in the liver. These estrogen metabolites are further O-methylated to form corresponding monomethyl ethers, catalyzed by catechol-O-methyltransferase (COMT) using S-adenosyl-L-methionine (AdoMet) as the methyl donor (depicted in Figure 1).

During human pregnancy, there is a dramatic increase in the daily production of endogenous estrogens. The placenta contributes importantly to the biosynthesis of endogenous estrogens. This organ also contains rather high levels of estrogen 2-hydroxylase (Osawa et al., 1993; Zhu, 2002) and COMT (Boadi et al., 1992; Zhu et al., 2000; Zhu et al., 2009), and the concerted actions of these two estrogen-metabolizing enzymes lead to increased formation of 2-methoxyestradiol (2-MeO-E$_2$). Earlier studies have shown that the concentration of 2-MeO-E$_2$ increased considerably during human pregnancy, and its circulating level at the third trimester was around 10 nM (Berg et al., 1983). However, the levels of 2-MeO-E$_2$ and COMT were found to be significantly lower in women with pre-eclampsia (Barnea et al., 1988; Rosing and Carlstrom, 1984). A recent study using the $\text{Comt}^{-/-}$ mice provided definitive evidence showing that COMT is important for the normal development of the placentas and embryos (Kanasaki et al., 2008). Furthermore, administration of 2-MeO-E$_2$ could effectively rescue the pregnant $\text{Comt}^{-/-}$ phenotype in these mice, thus suggesting that COMT plays an important role during pregnancy via the formation of 2-MeO-E$_2$. In that same study, it was shown that COMT and 2-MeO-E$_2$ also play a role in regulating the
It has been known for years that human COMT is a polymorphic enzyme. Genetic analysis showed that the Val108Met variant of S-COMT (or Val158Met variant of MB-COMT) is a common variation in the COMT gene resulting from a single nucleotide mutation (Lachman et al., 1996; Shield et al., 2004). This mutated COMT had significantly lower catalytic activity compared with the wild-type enzyme. An earlier study also showed that the maternal COMT polymorphism with a low enzyme activity was associated with an increased occurrence of fetal growth restriction, in addition to maternal pre-eclampsia (Sata et al., 2006).

In addition to catalyzing the O-methylation of catechol estrogens, COMT also catalyzes the O-methylation of endogenous catecholamines as well as many other catechol-containing xenobiotics, owing to its broad substrate specificity (Axelrod and Tomchick, 1958; Zhu, 2002). Therefore, the rate of catechol estrogen O-methylation in vivo is subject to modulation by other endogenous substrates, and particularly exogenous dietary catechol substrates that are usually present in much larger quantities. It is, however, not known whether the polymorphic COMT enzyme in human placenta has differential sensitivity to inhibition by various dietary catechols. In the present study, we compared the O-methylation of 2-OH-E₂ and 4-OH-E₂ in vitro catalyzed by the cytosolic COMT from a total of 36 human term placentas. In addition, we have also analyzed
their differential sensitivity to heat inactivation as well as to inhibition by representative polyphenolic components present in our diet.
MATERIALS AND METHODS

Chemicals

2-OH-E₂, 4-OH-E₂, AdoMet, dithiothreitol, (-)-epigallocatechin-3-gallate (EGCG) and quercetin were purchased from the Sigma Chemical Co. (St. Louis, MO). [Methyl-³H]AdoMet (specific activity 11.2–13.5 Ci/mmol) was purchased from Perkin Elmer (Maltham, MA). All solvents used in this study were of HPLC grade or better and were obtained from Fisher Scientific Co. (Springfield, NJ).

Preparation of human placental cytosolic fraction

Human term placental samples were obtained from thirty-six Caucasians (at 36-40 weeks of gestation) after normal vaginal delivery at the St. Peter's University Hospital (New Brunswick, NJ). The mean age of the thirty-six participants were 32.4 ± 9.5 years (mean ± S.D.). The procedure for procurement of human term placenta samples was approved by the Institutional Review Boards (IRBs) of the University of South Carolina (Columbia, SC), Rutgers University (Piscataway, NJ), University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School (New Brunswick, NJ), and the St. Peter’s University Hospital (New Brunswick, NJ). Within 30 min after the placenta was expelled, a portion (50-100 g) was collected and snap-frozen in liquid nitrogen for transport to the laboratory for storage in a -80°C freezer until it was used for the preparation of placental cytosol.

On the day of preparation of cytosolic fraction, the placental samples were first thawed at room temperature and then rinsed with ice-cold normal saline to remove blood clots. The
chorionic membrane tissues were removed with a pair of sharp eye-surgery scissors. The tissues were then minced in 3 volumes of ice-cold 0.1 M Tris-HCl and 0.1% KCl solution and were then homogenized with a Tri-R homogenizer (Model K41) for 2-3 min followed by a Teflon homogenizer for another 2-3 min. Tissue homogenates were centrifuged at 9,000 g (4°C) for 10 min, and supernatants were pooled and filtered through two layers of cheesecloth to remove lipid clots. The filtrates were then recentrifuged at 105,000 g (4°C) for 90 min. The supernatants (the cytosolic fractions from each placenta) were then combined, and aliquots of the cytosol preparations were stored at -80°C until use. The protein concentration was determined by using the BioRad protein assay kit with bovine serum albumin (BSA) as standard.

**Human placental COMT-mediated O-methylation of 2-OH-E_2 and 4-OH-E_2**

The COMT-catalyzed O-methylation of catechol estrogens was carried out as described previously (Zhu et al., 2000; Zhu et al., 2009). The reaction mixture consisted of 0.25 mg human placenta cytosolic protein, 1.2 mM MgCl_2, 250 μM AdoMet iodide (containing ~0.2 μCi [3H-methyl]AdoMet), 1 mM dithiothreitol, and varying concentrations of 2-OH-E_2 or 4-OH-E_2 in 0.5 mL Tris-HCl buffer (10 mM, pH 7.4). For the inhibitor experiments, the incubation mixture also contained the inhibitor at indicated concentrations. The reaction was initiated by addition of placental cytosolic protein, and carried out at 37°C for 10-45 min. Each reaction was run in duplicate. The reaction was arrested by immediately cooling to ice-cold temperatures, addition of 50 μL NaOH (0.5 M), and extraction with 3 mL ice-cold toluene. After centrifugation at 1000 g for 10 min, portions of the organic extracts (2 mL) were analyzed for radioactivity content by liquid scintillation counting (Beckman Instruments, model LS 5000TD).

The rate for the enzymatic O-methylation of 2-OH-E_2 and 4-OH-E_2 in vitro was expressed
as pmol of the methyl ethers formed by 1 mg total cytosolic protein in 1 minute (abbreviated as “pmol/mg protein/min”). The enzyme kinetics ($K_M$ and $V_{MAX}$) for the $O$-methylation of these catechol estrogens were calculated using the curve regression program of the SigmaPlot software, version 9 (San Jose, CA).

**Temperature inhibition studies**

To determine the temperature stability of placental COMT, the cytosolic enzyme fraction was pre-incubated in a water bath set at a given temperature for 10 min, and then the enzyme was immediately assayed for its catalytic activity for the $O$-methylation of catechol estrogen substrates *in vitro*. The corresponding control enzyme was not pre-incubated and placed on ice. The remaining catalytic activity of the enzyme after pre-incubation was expressed as % of the control enzyme activity (without pre-incubation).
RESULTS

Human placental COMT-mediated O-methylation of 2-OH-E2 and 4-OH-E2

We first determined the effect of different incubation time, protein concentration, AdoMet concentration, and reaction pH on the rate of O-methylation of 2-OH-E2 and 4-OH-E2 for several representative placenta COMT preparations (data not shown). The optimized conditions for the \textit{in vitro} enzymatic O-methylation included an incubation time of 10 min, a final protein concentration at 0.5 mg/mL for the placental cytosolic protein preparation, an AdoMet concentration at 250 µM, and the reaction pH at 7.4.

Next we determined the \textit{K}_M and \textit{V}_\text{MAX} values for the cytosolic COMT from several representative human placentas (\textbf{Figure 2}). Based on the curve patterns, it was apparent that there was an auto-inhibition of the enzyme activity when high concentrations of the substrates were present. The apparent \textit{K}_M values for placentas 1, 4 and 7 were approximately 2 µM, and plateaus were reached when substrate concentrations reached approximately 10 µM. Notably, the apparent \textit{K}_M value for placenta 8 was substantially lower, at approximately 1 µM, and a plateau was reached at approximately 5 µM. The same assay for this placenta was repeated twice, and similar results were obtained.

Using 10 µM of 2-OH-E2 and 4-OH-E2 as substrates, the catalytic activity for the cytosolic COMT prepared from a total of 36 human term placentas was determined (data are summarized in \textbf{Figure 3, upper}). Considerable variations in the COMT activity were noted with different placenta samples, with activity for the O-methylation of 2-OH-E2 and 4-OH-E2 ranging from 48–197 and 35–125 pmol/mg protein/min, respectively.
Differential sensitivity to heat inactivation

Human cytosolic COMT is a polymorphic enzyme. Earlier studies with human liver and red blood cells have shown that the low activity form of the COMT is slightly more sensitive to heat inactivation than its high activity form (Scanlon et al., 1979; Weinshilboum and Dunnette, 1981). Using 8 representative human placenta samples, we determined in the present study their sensitivity to inhibition by gradually increasing the pre-incubation temperatures from 30 to 50°C (Figure 4). Our data showed that when the cytosolic fraction of the human placentas was preincubated for 10 min at 35 to 50°C, the COMT activity for the O-methylation of 2-OH-E2 and 4-OH-E2 was each inhibited in a temperature-dependent manner. However, the sensitivity of different placental cytosols to a given temperature showed substantial differences. While some of the samples were more sensitive to heat inactivation, with $IT_{50}$ values (the pre-incubation temperature that deactivates 50% of the COMT's catalytic activity) at approximately 40°C, other samples were less sensitive (with the $IT_{50}$ values of as high as 47°C). Notably, for the same placenta sample, the pattern of heat inhibition of the O-methylation of 2-OH-E2 and 4-OH-E2 remained almost the same, although the pattern for different placenta samples varied considerably.

Based on the above data, next we chose to determine the sensitivity of all 36 human placenta cytosolic COMT preparations to heat inactivation at two selected pre-incubation temperature points (42 and 46°C) (data shown in Figure 3, middle and lower). Our data showed that the COMT present in different human placenta samples had markedly different sensitivity to heat inactivation. There was no apparent correlation between the apparent COMT activity present in the cytosolic fraction of different human placentas and the sensitivity to heat inactivation either at 42°C or 46°C (Figure 5, upper and middle panels). However, there was a high degree of correlation between the sensitivity to heat inactivation at 42°C and 46°C (Figure 5, lower panel).
We also determined the effect of heat treatment on the kinetic parameters for the O-methylation of 2-OH-E2 and 4-OH-E2 by two representative placenta samples (placentas 13 and 15). Heat treatment had nearly the same effect on the O-methylation of 2-OH-E2 and 4-OH-E2. For instance, heat treatment at 42°C not only decreased the apparent $V_{\text{MAX}}$ values of the enzyme for the O-methylation of 2-OH-E2 and 4-OH-E2, but it also increased its apparent $K_M$ values (data not shown).

In summary, the cytosolic COMT activity in different human placentas had substantial differences in sensitivity to heat inactivation. This differential sensitivity appears to be dissociated with the overall catalytic activity for the O-methylation of catechol estrogen substrates.

**Differential sensitivity to inhibition by dietary polyphenols**

We showed earlier that many catechol-containing dietary polyphenols are excellent substrates for placental COMT that O-methylates catechol estrogens (Zhu et al., 2000). In the present study, we compared the sensitivity of all 36 placental cytosolic COMT preparations to inhibition by two representative dietary polyphenols, EGCG and quercetin, using 4-OH-E2 as a substrate (data are summarized in Figures 6, 7). We found that there was a positive correlation ($r = 0.760$) between the sensitivity of the human placental COMT to heat inactivation and the sensitivity to inhibition by EGCG (a common tea polyphenol) (Figure 6). Interestingly, there was an inverse relationship ($r = 0.544$) between these two when quercetin (a dietary bioflavonoid) was tested as a COMT inhibitor, i.e., the higher sensitivity of the placental COMT to heat inactivation was associated with a decreased sensitivity to inhibition by quercetin.

Our earlier studies showed that EGCG and quercetin inhibit human COMT-mediated O-
methylation of catechol estrogens via different mechanisms (Zhu et al., 1994; Zhu and Liehr, 1994 and 1996). Whereas EGCG inhibits COMT mainly through direct binding to the enzyme, quercetin (a catechol-containing bioflavonoid) inhibits COMT through a combination of two mechanisms: one was through the formation of S-adenosyl-L-homocysteine (AdoHcy) as a result of its own rapid O-methylation catalyzed by COMT (a major mechanism), and the other one was its direct competitive inhibition of the enzyme by serving as a substrate. Therefore, we also determined the sensitivity of these human placental COMT preparations to inhibition by AdoHcy (data are shown in Figure 8). We found that the sensitivity to AdoHcy inhibition was also inversely correlated with their sensitivity to heat inactivation, which was similar to the pattern seen with the differential sensitivity to inhibition by quercetin.
DISCUSSION

A recent study showed that COMT, likely via the formation of 2-MeO-E₂, plays an important role in the development of placenta and embryos as well as in the regulation of utero-placental vascular homeostasis, blood pressure, kidney glomerular structure, and hypoxia response during pregnancy (Kanasaki et al., 2008). In the present study, we analyzed the cytosolic COMT activity contained in 36 human term placenta samples to evaluate their differential sensitivity to inhibition by heat and dietary polyphenolic compounds. We found that the cytosolic COMT activity in different human placentas displayed a large inter-individual variation, with up to 4-fold differences noted. In addition, substantial differences (up to 3-fold) were also noted in their sensitivity to heat inactivation. These observations are mostly in agreement with an earlier study showing that considerable inter-individual variability existed in human placental COMT activity (Barnea et al., 1988).

Earlier studies of the human COMT activity in red blood cells (Scanlon et al., 1979) and also other tissues (Sladek-Chelgren and Weinshiboum, 1981) had led to the discovery of a common polymorphism in the two co-dominant alleles of the COMT gene. Grossman et al. (1992) and Goodman et al. (2002) later found that the Val-108 and Met-108 variations of the human COMT correlated with the phenotypes of high and low levels of COMT catalytic activity, respectively. However, a more detailed analysis of the catalytic activity of these two forms of COMT revealed a similar catalytic property, although the Met-108 variant was more thermolabile (37°C), which could be stabilized by AdoMet binding (Weinshilboum and Dunnette, 1981). Studies have also shown that there was a significant correlation between the COMT heated/control ratios and the levels of enzyme activity in the lysates prepared from hepatic tissue and red blood
cells. However, the results of our present study with human term placental samples did not show a significant correlation between the COMT heated/control ratios and the enzyme activity (Figure 5). This likely suggests that other factors besides genetic polymorphism, such as differences in the levels of COMT gene expression in the placentas of different individuals and/or in the post-translational modifications of the enzyme proteins, may contribute to the difference in the overall cytosolic COMT activity and/or thermoliability of the enzyme in human placentas.

Many catechol-containing dietary polyphenols (including those tested in the present study) can serve as both substrates and inhibitors of the human and rodent COMTs with different mechanisms (Zhu et al., 1994; Zhu and Liehr, 1994 and 1996). An earlier study showed that EGCG inhibited the COMT activity mainly through tight binding interaction with the catalytic site of the enzyme while the inhibitor itself was essentially not methylated by the enzyme (Zhu et al., 2008). In comparison, quercetin is a good substrate of COMT (Zhu and Liehr, 1994), and our earlier study showed that it inhibits the COMT activity largely via the formation of AdoHcy (a non-competitive feedback inhibitor of the COMT). In this study, we also tested, for comparison, the ability of these dietary compounds to inhibit the O-methylation of 4-OH-E2 catalyzed by representative human placental cytosolic COMTs that exhibited differential sensitivity to heat inactivation. We found that there was a marked difference in the sensitivity of different human placental samples to inhibition by these two dietary compounds. A positive correlation was noted between the sensitivity of the cytosolic COMT in different human placentas to heat inactivation and its sensitivity to EGCG inhibition. However, an inverse correlation was seen between the two when quercetin or AdoHcy was used as the inhibitor. The similarity between the inhibition patterns of quercetin and AdoHcy supports the mechanistic explanation that COMT inhibition by quercetin is mostly mediated by the formation of AdoHcy. Accordingly, it was suggested that
whereas EGCG might have a relatively higher binding affinity for the thermo-unstable COMT, AdoHcy might have a relatively higher binding affinity for the thermo-stable COMT. This explanation was supported by our recent study (Bai et al., 2008) showing that a novel mutant form of the human COMT protein that also exhibits an increased sensitivity to heat inactivation has a decreased sensitivity to AdoHcy inhibition when compared to the wild-type COMT.

It should be noted that in ancient Chinese folklore medicine, pregnant women, particularly at late stages of gestation, were strongly advised against drinking concentrated tea, for it was believed that drinking tea would cause various abnormalities, ranging from pregnancy complications, miscarriages, to stillbirth. Interestingly, recent studies have made a similar observation that pregnant women in Western countries who consumed 4–7 cups of coffee a day have a markedly increased risk for stillbirth and fetal abnormalities (Bech et al., 2005). Although the mechanisms for the adverse effects of drinking tea and coffee on human pregnancy are still not understood, it has been suggested that these adverse effects might be partially associated with the intake of caffeine from these beverages. Since the polyphenolic components abundantly present in tea and coffee can modulate DNA methylation and epigenetic control of gene expression (Fang et al., 2003; Lee et al., 2005; Lee and Zhu, 2006; Yang et al., 2008), it has also been suggested that alterations of these vital processes during pregnancy and fetal development may contribute to the occurrence of the adverse effects. The ability of the dietary phenolic compounds to inhibit the placental COMT activity as well as the formation of methoxyestrogens (such as 2-MeO-E2) as observed in the present study likely is another factor that may contribute to the development of adverse effects associated with tea and coffee beverage during pregnancy. In this content, it is of note that since placentas from different women have a rather wide difference in their sensitivity to inhibition by dietary polyphenolic compounds, this difference may partially contribute to the
The differential sensitivity of pregnant women to the adverse actions of drinking tea or coffee beverages.

Lastly, many earlier studies have shown that 2-MeO-E2 has strong apoptotic, antiangiogenic, and antitumorigenic activities (Zhu and Conney, 1998; Pribluda et al., 2000; Fukui and Zhu, 2009). Since the circulating 2-MeO-E2 levels are dramatically increased during human pregnancy, it has been speculated that the large amounts of 2-MeO-E2 formed during pregnancy may be an important endogenous protective factor against the development of estrogen-inducible tumors in various target organs (discussed by Zhu and Conney, 1998; Zhu, 2002). In partial support of this notion, an earlier study in an animal model showed that chronic administration of dietary quercetin enhanced 17β-estradiol-induced, but not diethylstilbestrol (DES)-induced, kidney tumor formation in male Syrian hamsters (Zhu and Liehr, 1994; B.T. Zhu, unpublished data). It is of note that quercetin did not increase, but instead inhibited 7,12-dimethylbenz[a]anthracene-induced mammary tumors in rats (Verma et al., 1988) and azoxymethanol-induced colonic neoplasms in mice (Deschner et al., 1991). These results suggest that the selective enhancing effect of quercetin on 17β-estradiol-induced carcinogenesis may be due to its inhibitory effect on the COMT-mediated O-methylation metabolism of catechol estrogens. Similarly, human epidemiological studies have also shown that the occurrence of the low-activity COMT polymorphism, under certain conditions, is associated with an increased risk for developing human hormone-associated cancers (Lavigne et al., 1997; Thompson et al., 1998).

In summary, we found that there are large person-to-person differences in the cytosolic COMT activity and sensitivity to heat inactivation with different human placenta samples. This differential sensitivity was not associated with the overall catalytic activity for the O-methylation of the catechol estrogen substrates. Overall, there was a positive correlation between the
sensitivity of the human placental COMT to heat inactivation and its sensitivity to inhibition by EGCG, but an inverse relationship between heat inactivation and inactivation by quercetin. The difference was due to the different mechanisms of COMT inhibition exerted by EGCG and quercetin. Our data suggest that the placental cytosolic COMT activities from different individuals have different sensitivities to heat inactivation as well as to inhibition by dietary compounds. The observed inter-individual differences may contribute to the person-to-person differences in the sensitivity of pregnant women to the possible adverse effects associated with tea or coffee drinking during pregnancy.
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FOOTNOTES:

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FIGURE LEGENDS

Figure 1  Metabolic formation of catechol estrogens and COMT-mediated further O-methylation of the catechol metabolites. Note that only the structure for 2-OH-E2 and its methylated products are shown as examples. The formation of 4-OH-E2 and its O-methylation follow the same metabolic pathways. The metabolic O-methylation of endogenous catechol estrogens is subject to modulation by various endogenous factors (such as catecholamines, S-adenosyl-L-homocysteine, and homocysteine) as well as exogenous factors (such as various catechol-containing dietary polyphenolic compounds). Abbreviations: CYP, cytochrome P450 enzymes; COMT, catechol-O-methyltransferase; AdoHcy, S-adenosyl-L-homocysteine; 2-OH-E2, 2-hydroxyestradiol; 2-MeO-E2, 2-methoxyestradiol; 2-OH-3-MeO-E2, 2-hydroxyestradiol 3-methyl ether.

Figure 2  Substrate concentration dependence for the O-methylation of 2-OH-E2 and 4-OH-E2 catalyzed by four human placental cytosolic COMT preparations. The incubation mixture consisted of 0-36 μM 2-OH-E2 or 4-OH-E2, 250 μM AdoMet (containing 0.2 μCi [3H-Methyl]AdoMet), 0.5 mg/mL of placental cytosolic protein, 1 mM dithiothreitol, and 1.2 mM MgCl2 in a final volume of 0.5 mL Tris-HCl buffer (10 mM, pH 7.4). The incubations were carried out at 37°C for 10 min. Each point is the mean of duplicate determinations (average variation <5%).

Figure 3  Human placental cytosolic COMT-mediated O-methylation of 2-OH-E2 and 4-OH-E2 (upper panel) and their sensitivity to heat inactivation at 42°C (middle panel) and 46°C (bottom panel). The incubation mixture consisted of 10 μM 2-OH-E2 or 4-OH-E2, 250 μM [3H-methyl]AdoMet (containing 0.2 μCi), 0.5 mg/mL of human placental cytosolic protein, 1 mM...
dithiothreitol, and 1.2 mM MgCl$_2$ in a final volume of 0.25 mL Tris-HCl buffer (10 mM, pH 7.4). Incubations were carried out at 37°C for 10 min. For heat inactivation, the cytosolic proteins were first incubated at 42°C or 46°C for 10 min before analysis of their catalytic activity. Each point is the mean of duplicate determinations (with variations <5%).

**Figure 4**  The sensitivity of eight representative human placental cytosolic preparations (placenta 3, 5, 7, 13, 18, 20, 24, and 33) to a 10-minute heat inactivation at 37 to 50°C. The incubation mixture consisted of 10 μM 2-OH-E$_2$ or 4-OH-E$_2$, 250 μM AdoMet (containing 0.2 μCi $[^3]$H-methyl] AdoMet), 0.5 mg/mL of human placental cytosolic protein, 1 mM dithiothreitol, and 1.2 mM MgCl$_2$ in a final volume of 0.25 mL Tris-HCl buffer (10 mM, pH 7.4). Incubations were carried out at 37°C for 10 min. Each point is the mean of duplicate determinations (with variations <5%).

**Figure 5**  Lack of correlation between total COMT catalytic activity and the heat sensitivity (upper and middle panels) from 36 human placental cytosolic preparations. The bottom panel shows the correlation between the sensitivity to heat inactivation at 46°C or 42°C. The original data were obtained from **Figure 3**.

**Figure 6**  Comparison of the sensitivity of 36 placental cytosolic COMT preparations to inhibition by EGCG. The incubation mixture consisted of 10 μM 4-OH-E$_2$, 250 μM AdoMet (containing 0.2 μCi $[^3]$H-methyl] AdoMet), 0.1 μg/mL EGCG, 0.5 mg/mL of placental cytosolic protein, 1 mM dithiothreitol, and 1.2 mM MgCl$_2$ in a final volume of 0.5 mL Tris-HCl buffer (10 mM, pH 7.4). The incubations were carried out at 37°C for 10 min. Each point is the mean of duplicate determinations (average variation <5%).
Figure 7  Comparison of the sensitivity of 36 placental cytosolic COMT preparations to inhibition by quercetin. The incubation mixture consisted of 10 μM 4-OH-E2, 250 μM AdoMet (containing 0.2 μCi [3H-methyl] AdoMet), 2 μM quercetin, 0.5 mg/mL of placental cytosolic protein, 1 mM dithiothreitol, and 1.2 mM MgCl$_2$ in a final volume of 0.5 mL Tris-HCl buffer (10 mM, pH 7.4). The incubations were carried out at 37°C for 10 min. Each point is the mean of duplicate determinations (average variation <5%).

Figure 8  Comparison of the sensitivity of 36 placental cytosolic COMT preparations to inhibition by $S$-adenosyl-$L$-methionine (AdoHcy). The incubation mixture consisted of 10 μM 4-OH-E2, 250 μM AdoMet (containing 0.2 μCi [3H-Methyl] AdoMet), 5 μM SAH, 0.5 mg/mL of placental cytosolic protein, 1 mM dithiothreitol, and 1.2 mM MgCl$_2$ in a final volume of 0.5 mL Tris-HCl buffer (10 mM, pH 7.4). The incubations were carried out at 37°C for 10 min. Each point is the mean of duplicate determinations (average variation <5%).
Figure 1

17β-Estradiol (E₂) → 2-OH-E₂ → AdoMet → 2-OH-3-MeO-E₂ → 2-MeO-E₂

CYP

COMT

AdoHcy
Figure 2

![Graphs showing the O-methylation of 2-OH-E₂ and 4-OH-E₂ in different placenta samples.](attachment:Figure2-graphs.png)
Figure 3

- **A. Control (without heat inactivation)**
- **B. 42°C heat inactivation for 15 min**
- **C. 46°C heat inactivation for 15 min**

The graphs show the levels of 2-OH-E₂ and 4-OH-E₂ in placenta samples over time, with heat inactivation at different temperatures and durations.
Figure 4

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Figure 5

2-OH-E₂

42°C

\[ r = 0.072 \]

4-OH-E₂

42°C

\[ r = 0.032 \]

46°C

\[ r = 0.207 \]

46°C

\[ r = 0.105 \]

\[ r = 0.925 \]

\[ r = 0.895 \]
Figure 6

Inhibition of 4-OH-E₂ methylation by 0.1 μg/mL EGCG

Placenta identification number

$r = 0.760$

$r = 0.779$
Figure 7

Inhibition of 4-OH-E₂ methylation by 2 μM quercetin

Inhibition of methylation (% of total activity left)

Placenta identification number

$H/C$ (at 42°C)

$H/C$ (at 46°C)

$r = 0.544$

$r = 0.550$
Figure 8

Inhibition of 4-OH-E₂ methylation by 5 μM AdoHey

Placenta identification number

Inhibition of methylation (% of total activity left)

H / C (at 42°C)

H / C (at 46°C)

r = 0.555

r = 0.539