EFFECTS OF GESTATIONAL AND OVERT DIABETES ON HUMAN PLACENTAL CYTOCHROMES P450 AND GLUTATHIONE S-TRANSFERASE

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(Received August 26, 1997; accepted December 11, 1997)

This paper is available online at http://www.dmd.org

ABSTRACT:

The placenta possesses the ability to metabolize a number of xenobiotics and endogenous compounds by processes similar to those seen in the liver. Animal and in vivo studies have observed that the presence of diabetes alters the expression of hepatic metabolizing enzymes (cytochrome P450 and glutathione S-transferase); however, it is unknown whether similar alterations occur in the human placenta. To evaluate whether diabetes has any effect on placental xenobiotic metabolizing activity, the catalytic activities of 7-ethoxresorufin O-deethylation (EROD, CYP1A1), chloroxazone 6-hydroxylation (CYP2E1), dextromethorphan N-demethylation (CYP3A4), dextromethorphan O-demethylation (CYP2D6), and 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with glutathione (glutathione S-transferase, GST) from placentas of diet (class A1) and insulin-dependent (class A2) gestational diabetics and overt diabetics were compared with matched controls. EROD activity (CYP1A1) ranged from 0.29 to 2.67 pmol/min/mg protein. However, no differences were observed among overt or gestational diabetics and their respective matched controls. CDNB conjugation (GST) ranged from 0.275 to 1.65 units/min/mg protein. In contrast to that observed with CYP1A1, a small but statistically significant reduction in GST activity was noted in overt diabetics as compared with their matched controls and gestational diabetics. CYP2E1, 2D6, and 3A4 enzymatic activities were not detected in human placental tissue. GST protein was detectable in all tissues studied, but no CYP protein could be detected in any of the tissues. Thus, it seems that pregnant women with overt diabetes have reduced GST activity in the placenta, which could potentially result in the exposure of the fetus to harmful electrophiles. However, the full clinical significance of this finding remains to be elucidated.

Diabetes complicates 1–3% of all pregnancies (Brinkman, 1987; Hollingsworth, 1984). Infants born to diabetic mothers tend to be large for gestational age and have a 2-fold increase in the incidence of being born with a major anomaly (Zonana, 1976). Likewise, neonatal morbidity is increased as a direct result of fetal macrosomia and congenital malformations associated with maternal diabetes (Greene and Brown, 1995; Kuhl and Moller-Jensen, 1989).

The primary function of the human placenta is to ensure an optimal environment for fetal growth and development. Xenobiotics, nutrients, and endogenous substances enter the placenta via maternal circulation. Placental transfer to the fetus is dependent upon the compound’s lipid solubility, molecular weight, degree of protein binding, and placental metabolism (Pacifici and Nottoli, 1995; Reynolds, 1987). The placenta possesses the capabilities to metabolize these compounds through cytochrome P450 enzymes and/or glutathione conjugation pathways (Juchau, 1980; Meigs and Ryan, 1968; Pasanen and Pelkonen, 1989–1990). Although such metabolites produced are usually inactive, reactive or toxic metabolites may also be generated. Therefore, alterations in placental metabolizing capabilities can potentially cause fetal injury.

With respect to placental metabolism, the mRNAs of CYP1A1, -2E1, -2F1, -3A3/4, -3A5, and -4B1 have been detected in full-term placentas by RT-PCR1 (Hakkola et al., 1996a) and seem to be expressed at a very low level compared with the liver. In this regard, most research in this area has focused on women who smoke during pregnancy, showing that smoking induces placental CYP1A1 (Pasanen et al., 1990; Sesardic et al., 1990) but has no or little effect on glutathione S-transferase or aromatase (CYP19A1) (Pasanen and Pelkonen, 1990). More recently, we have demonstrated that in normal patients, placental xenobiotic metabolizing activity (CYP1A1, CYP19A1, and glutathione S-transferase) does not vary throughout various regions of the placenta (McRobie et al., 1996).

Animal and in vivo human studies suggest diabetes alters the liver’s xenobiotic metabolizing enzymes (Schenkman, 1991). Recently, we reported that diabetes does not alter overall placental aromatase activity (CYP19A1) responsible for the conversion of androgens to estrogens (McRobie et al., 1997), but the effect of diabetes on placental xenobiotic metabolizing enzymes is unknown. Numerous ultrastructural abnormalities have been observed in placentas from diabetic women (Fox, 1989). These structural changes could alter the placenta’s enzymatic function and result in alterations in maternal-fetal exchange. Therefore, we hypothesized that diabetes might affect placental P450 isozymes and glutathione S-transferase activities. A series of experiments was conducted with placental tissues obtained...
from gestational and overt diabetic patients and their matched controls to determine if the presence of diabetes alters placental xenobiotic metabolizing activity.

**Materials and Methods**

**Chemicals and Reagents.** Glutathione, resorufin, 7-ethoxresorufin, chlorozoxazone, and dextromethorphan were purchased from Sigma. 3-Methoxyphosphinian, dextrophan, and 6-hydroxychlorzoxazone were obtained from Research Biochemical International (Natick, MA). 1-Chloro-2,4-dinitrobenzene was purchased from Pfaltz and Bauer, Inc. Anti-rat CYP1A1, -2E1, and -3A antibodies were purchased from Gentest (Woburn, MA). Recombinant human glutathione transferase P1-1 was obtained from the PanVera Corporation (Madison, WI). All other chemicals were obtained from commercial sources and were of the highest purity available.

Subjects were recruited through the Obstetrics and Gynecology Department at West Virginia University Hospital. Placentas were collected from women (ages 18 – 40 years, mean 27 ± 5.8) between 34 and 41 weeks gestation. Placental samples were obtained by a protocol approved by the West Virginia University Institutional Review Board for the Protection of Human Research Subjects. Participants provided informed written consent prior to obstetric delivery. Healthy subjects who remained febrile and normotensive throughout their entire hospital course were included in this study. In addition, participants had no history of ingesting foods and medications known to alter cytochrome P540 activity for 3 months prior to enrollment. Alcohol consumption and smoking status were determined during patient interview. On admission, urine cotinine levels were determined to confirm nonsmoking status. A 50-g oral glucose test was performed between 26 and 28 weeks gestation to screen for diabetes. Subjects demonstrating a blood glucose > 135 mg/dl were considered to be glucose intolerant and were eliminated from participation as a control. The criteria of Carpenter and Coustan (1982) were used for diagnosis of gestational diabetes. For the purpose of this study, diabetic subjects were classified as overt diabetics (those having diabetes prior to pregnancy and controlled by insulin), class A1 gestational diabetics (diet-controlled), or class A2 gestational diabetics (insulin-controlled). Twenty subjects were recruited per group and matched with controls as to age, body mass index, gravida, and parity. Multiple gestations, placental anomalies, clinical evidence of chorioamnionitis, and a history of frequent charcoal broiling of foods were exclusion characteristics.

**Human Placental Tissue.** Samples were collected within 30 min after placental delivery. Specimens were labeled and stored at −70°C. Placental microsome and cytosol fractions were prepared according to established methods (Vaz et al., 1992). Protein content was determined according to Lowry et al. (1951) with bovine serum albumin as a standard.

**Enzymatic Activity Assays.** Glutathione S-transferase activity, measured as the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, was determined by the method of Habig et al. (1974). CYP1A1 activity, measured as ethoxyresorufin O-deethylation (EROD), was determined according to the fluorometric method described by Burke et al. (1985). The activity of CYP2E1 was estimated by measuring chlorozoxazone 6-hydroxylation (Peter et al., 1990) by the method of Chittur and Tracy (1997). Finally, dextromethorphan N-demethylation was used as a putative marker for CYP3A4 (Gorski et al., 1994) and dextromethorphan O-demethylation as a marker for CYP2D6 (Dayer et al., 1989). Incubations consisted of 250 μg of placental microsomal protein, potassium phosphate buffer (100 mM, pH 7.4) containing MgCl₂ (5 mM), dextromethorphan (800 μM), NADP (1 mM), and glucose-6-phosphate dehydrogenase (0.4 units/400 μl) in a final volume of 250 μl. Formation of dextromorphan and 3-methoxyphosphinian was measured by the high performance liquid chromatography method of Ducharme et al. (1996).

**Western Blot Analysis.** For the cytochrome P450 isoforms, microsomal fractions were resolved by a 10% SDS-PAGE by the methods of Laemmli (1970). Following transfer to a polyvinylidene fluoride membrane, the blots were treated with the appropriate P450 antibody solution and then a secondary antibody (alkaline phosphatase-conjugated anti-goat IgG/HRabbit serum), followed by soaking in a nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphatase (100:1) solution and subsequently allowed to dry in the dark. Identification of GST protein in the cytosolic fraction followed a similar procedure, except that the protein was resolved using a 5–17% SDS-PAGE gradient gel, and Coomassie Brilliant Blue was used to stain and fix the proteins. A primary antibody to GST P1-1 (Crystal Chem, Chicago, IL) was used during preliminary studies but was found to cross-react with several proteins in the cytosolic fraction and result in decreased detection sensitivity.

**Data Analysis.** Results have been expressed as means ± SD. Data on human placental GST and EROD activities were analyzed using one-way ANOVA followed by Student-Newman-Keuls multiple comparisons. The null hypothesis was rejected at p ≤ 0.05.

**Results**

EROD (CYP1A1) activity was readily detected in all human placentas tested and ranged from 0.29 to 2.67 pmol/min/mg protein (fig. 1). However, no differences were noted among overt or gestational diabetics and their respective matched controls.

With respect to CDNB conjugation (a putative measure of GST), activity ranged from 0.275 to 1.65 units/min/mg protein (fig. 2). In contrast to the results observed for CYP1A1, a statistical difference in GST activity was observed in overt diabetics as compared with their matched controls and both class A1 and A2 gestational diabetics.

Placental microsomes from diabetics and their matched controls were also assessed for their activity toward other CYP substrates. In this regard, no activity toward chlorozoxazone 6-hydroxylation (CYP2E1), dextromethorphan N-demethylation (CYP3A4), or dextromethorphan O-demethylation (CYP2D6) were noted in placentas from overt or gestational diabetics or their matched controls.

Immunoblotting techniques using polyclonal antibodies were conducted to determine cytochromes P450 -1A, -2E1, and -3A content in the microsomal fractions of human placenta from these patients. No CYP1A1, -2E1, or -3A protein was detected in any of the patient groups, but GST protein was detected in all tissues tested (fig. 3). These results correlate with the detection of CDNB conjugation and lack of chlorozoxazone 6-hydroxylation, dextromethorphan N-demethylation, and dextromethorphan O-demethylation activities observed in the placenta. However, the inability to detect CYP1A1 protein contrasts with the observations of enzymatic activity.

**Discussion**

The human placenta exhibits the potential to metabolize numerous endogenous compounds and xenobiotics. Diabetes has been shown to alter a number of hepatic cytochrome P450 enzymes (Schenkman,
1991), though the effect of diabetes on placental xenobiotic metabolizing enzymes is unclear. It would be of considerable interest to elucidate the significance of the alteration in placental enzymatic capabilities by diabetes, particularly with regard to how this might affect fetal toxicity.

Most research on placental drug metabolism has focused on women who have smoked during pregnancy. Cytochrome P450 1A1 is induced by cigarette smoke and is responsible for the metabolism of polycyclic aromatic hydrocarbons to carcinogenic intermediates (Pasanen and Pelkonen, 1990). EROD activity has been proposed as a putative marker for CYP1A activity, with both CYP1A1 and -1A2 involved (Guengerich et al., 1982). However, it has been proposed that only CYP1A1 exists in the term human placenta (Hakkola et al., 1996a; Sesardic et al., 1990), and thus our measurement of EROD activity should be reflective of only CYP1A1. In the present study, EROD activity was measured in all placental tissues, but no differences in enzymatic activity were detected among the diabetic classes or their matched controls. These results are in contrast to those observed in livers of streptozotocin-induced diabetic rats, which exhibited a 59% increase in activity as compared with controls (Raza et al., 1996). Thus, it seems that either CYP1A1 in human placenta responds differently to diabetes than rat liver CYP1A (possibly 1A1 and 1A2), or it is CYP1A2 that is induced by diabetes in rat liver, and thus CYP1A1 does not respond to this induction in human placenta. Finally, it is possible that the coadministration of insulin, as occurred in our patients, attenuates the effect of diabetes on CYP1A1, whereas the rats were not given insulin in the previously cited study (Raza et al., 1996). Interestingly, CYP1A1 protein was not measurable in any of the patient groups despite measurement of EROD activity. It could be argued that this EROD activity is due to another enzyme in the placenta, though this seems unlikely given previous studies (Hakkola et al., 1996a) but is more likely explained by the relative insensitivity of Western blotting as compared with EROD measurements and given the low levels of activity present. In support of this explanation, Hakkola et al. (1996a) observed CYP1A1 protein (by immunoblotting) in only one placenta of those tested, and this subject was a smoker who had the highest EROD activity observed among any of the subjects studied.

Induction of hepatic cytochrome P450 2E1 by diabetes has been reported and is closely correlated to plasma ketone levels (Thomas et al., 1987). In our study, neither placent al chlorozoxazone 6-hydroxylation activity (a putative marker of CYP2E1) nor placent al CYP2E1 protein was detected, and these results are in direct agreement with those reported previously in full-term placentas despite the presence of CYP2E1 mRNA (Hakkola et al., 1996a). It has been suggested that chlorozoxazone is metabolized by CYP1A1 as well as 2E1 (Yamazaki et al., 1995), but this seems not to be the case for the human placenta because no chlorozoxazone 6-hydroxylation was observed.

Because mRNA for CYP2E1 is present and diabetes can induce this isof orm, the possibility certainly existed for detection of catalytic activity. Using Western blotting techniques, Rasheed and coworkers (1997) were able to detect CYP2E1 protein in placentas from six heavy drinkers, although it is unclear whether this protein possessed catalytic activity. In our study, if the protein was functional, activity may still be below the limits of detection by the methods employed. Furthermore, the administration of insulin has been shown to attenuate the effects of diabetes on CYP2E1 (Barnett et al., 1992) and may also have served to counteract any inductive effects. Finally, as additional evidence that placent al CYP2E1 activity is absent or at extremely low levels in humans, placental tissue from patients with a history of alcohol abuse also did not exhibit CYP2E1 activity (Jones et al., 1992).

Utilizing PCR and immunoblotting techniques, CYP3A7 mRNA has been detected in first- and second-trimester placent al samples (Hakkola et al., 1996b; Schuetz et al., 1993). Interestingly, CYP3A7 is the predominant form found in fetal liver (Kitada et al., 1985). Furthermore, CYP3A3 and CYP3A5 but not CYP3A7 mRNA were detected in full-term placentas using RT-PCR techniques, but immunoreactive protein was not detected (Hakkola et al., 1996a). These same authors were unable to measure any CYP3A catalytic activity using testosterone 6β-hydroxylation as a probe (Hakkola et al., 1996a). Using dextromethorphan N-demethylation as a probe of CYP3A activity, we also were unable to demonstrate any activity in placentas from either diabetics or normal controls. Similarly, no immunoreactive CYP3A protein was observed in any of the placent al tissues tested. Thus, our results in diabetic patients are in agreement with those seen previously in full-term placentas from both non-smokers and smokers (Hakkola et al., 1996a) and suggest that human placenta does not possess measurable CYP3A activity.
In addition, we examined whether placentas from diabetic and normal patients possess CYP2D6 metabolic activity. CYP2D6 mRNA has been detected in first-trimester placentas (Hakkola et al., 1996b) but not in full-term placentas (Hakkola et al., 1996a). Using dextromethorphan O-demethylation as a probe for this reaction, we were unable to detect any CYP2D6 catalytic activity in any of the patient classes.

The final xenobiotic metabolizing enzyme studied was GST. The GST enzyme system conjugates biologically active electrophiles with the endogenous peptide glutathione (Hayes and Pulford, 1995). Placental GST seems to be active early in pregnancy (Datta et al., 1994; Polidoro et al., 1980); however, its activity does not increase with gestational age (Pacifici et al., 1988). The presence of GST in early pregnancy suggests that it plays a crucial role in protecting the fetus from electrophiles and other cell-damaging compounds. Diabetes seems to have no effect on human platelet and polymorphonuclear cell GST activity (Di Simplicio et al., 1995; Ratliff et al., 1996), but both increases and decreases in GST activity have been reported in the livers of diabetic rats (Raza et al., 1996; Suchocka et al., 1995). In the present study, a small but statistically significant decrease in human placental GST activity was noted in overt diabetics compared with gestational diabetics or control subjects, although the clinical significance of this finding is unclear. These results suggest that infants born to overt diabetic patients might be exposed to a higher amount of electrophilic cell-damaging compounds, assuming placental GST activity plays a substantial role in determining fetal exposure. The significance of this exposure to fetal toxicity has yet to be verified, but these results may contribute to understanding the anomalies observed in infants born to diabetic mothers.

Finally, it must be recognized that results observed following labor may not completely reflect placental activity during the 9 months prior to delivery. During labor, cytokine production increases tremendously (Stallmacher et al., 1995), especially tumor necrosis factor-alpha, interleukin-1, and interleukin-6. These cytokines have been shown to have a profound effect on cytochrome P450 (Shedlovsky et al., 1994) and may affect GST as well. Virtually all of our patients went through labor (or induction therapy), and thus the issue of cytokine release may have confounded our results. Patients undergoing elective Cesarean section would not experience this phenomenon but comprise such a small segment of our patient population that sufficient numbers of diabetic patients would have been difficult to recruit.

In conclusion, neither gestational nor overt diabetes seems to have any effect on placental cytochrome P450 enzymes. However, a statistically significant reduction in glutathione S-transferase activity was noted in placentas from overt diabetics as compared with gestational diabetics and controls. The mechanism responsible for this alteration as well as the clinical significance of this finding are unknown at this time, but future studies will be directed at answering these questions.

References


