Diacylglycerol Production and Protein Kinase C Activity Are Increased in a Mouse Model of Diabetic Embryopathy

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Activation of the diacylglycerol–protein kinase C (DAG-PKC) cascade by excess glucose has been implicated in vascular complications of diabetes. Its involvement in diabetic embryopathy has not been established. We examined DAG production and PKC activities in embryos and decidua of streptozotocin (STZ)-diabetic or transiently hyperglycemic mice during neural tube formation. STZ diabetes significantly increased DAG and total PKC activity in decidua (1.5- and 1.4-fold, respectively) and embryos (1.7- and 1.3-fold, respectively) on day 9.5. Membrane-associated PKC α, βII, δ, and ζ were increased in decidua by 1.25- to 2.8-fold. Maternal hyperglycemia induced by glucose injection on day 7.5, the day before the onset of neural tube formation, also increased DAG, PKC activity, and PKC isoforms (1.1-, 1.6-, and 1.5-fold, respectively) in the embryo on day 9.5. Notably, membrane-associated PKC activity was increased 24-fold in embryos of diabetic mice with structural defects. These data indicate that hyperglycemia just before organogenesis activates the DAG-PKC cascade and is correlated with congenital defects. Diabetes 51:2804–2810, 2002

Since the advent of insulin therapy, the survival of mothers with diabetes and their offspring has greatly improved. Most of the complications associated with diabetic pregnancy, such as spontaneous abortion, stillbirth, macrosomia, intrauterine growth restriction, and respiratory distress syndrome, can be significantly reduced by tight glycemic control before and throughout pregnancy (1,2). However, congenital malformations, which occur within the first 8 weeks of pregnancy, have been particularly difficult to avoid. The frequency of congenital malformations in the offspring of diabetic mothers is estimated to be 6–13%, a two- to fivefold increase over that in the nondiabetic population (3–7). The malformations associated with diabetic pregnancy affect many major organs, including the central nervous system, heart, skeleton, kidney, gastrointestinal system, and genitalia (5,6).

Studies using rodent models have attempted to provide biochemical and molecular explanations for diabetes-induced congenital defects. In vitro culture of rodent embryos has demonstrated that media containing elevated glucose or β-hydroxybutyrate can induce malformations like those caused by diabetic pregnancy (8,9). Several biochemical abnormalities have been observed in yolk sacs and embryos of diabetic rodents or after culture in high glucose, such as decreased uptake or membrane release of myo-inositol, arachidonic acid, and prostaglandin; increased lipid peroxidation; and decreased flux through the pentose shunt pathway (10–13). Moreover, the effects of antioxidants or overexpression of Cu²⁺/Zn²⁺ superoxide dismutase to prevent diabetes-induced defects suggest that oxidative stress is an important factor in hyperglycemia-induced embryopathy (14–17).

Previously, we demonstrated using a mouse model of diabetic pregnancy that expression of Pax-3, a gene required for neural tube development, is inhibited on day 8.5 and that increased neural tube defects (NTDs) are observed on day 10.5 (18). This suggests that disturbed expression of genes that regulate morphogenetic processes may be central to diabetes-induced congenital defects. Antioxidants can prevent the increase in oxidative stress as well as decreased Pax-3 expression and increased NTDs (Chang et al., submitted manuscript), supporting a role of oxidative stress in mediating the molecular and developmental abnormalities associated with diabetic pregnancy. Transient hyperglycemia caused by glucose injection only during day 7.5 is sufficient to inhibit Pax-3 expression and to increase NTDs (19), indicating that there is a critical period before organogenesis in which the molecular program that leads to neural tube formation can be disturbed. This observation is consistent with earlier findings, in which withdrawal of insulin treatment from pregnant diabetic rats just before organogenesis increased diabetes-induced malformations (20).

Excess glucose activates the diacylglycerol–protein kinase C (DAG-PKC) cascade and is associated with several diabetic complications that affect retinal, renal, neural, and cardiovascular systems in patients with diabetes and diabetic animal models (21–29). PKC signaling is pivotal.
for many cellular functions, including exocytosis, ion-exchange regulation, hormone and neurotransmitter release, and some processes that are particularly relevant to embryogenesis, such as cell proliferation, differentiation, and programmed cell death (21,30). Thus, if DAG-PKC signal transduction pathways are stimulated in embryonic tissues exposed to excess glucose, as they are in adult tissues affected by diabetic complications, then normal embryonic development may be disturbed. It is surprising that the ontogeny of DAG-PKC signaling during normal embryonic and fetal development has not been characterized extensively. In this report, we investigated whether DAG-PKC signaling was disturbed by diabetic pregnancy or transient hyperglycemia during organogenesis in the mouse embryo and whether this was associated with defective embryonic neural tube development.

RESEARCH DESIGN AND METHODS

Animals procedures. Female ICR mice (Taconic, Germantown, NY) were obtained at 6–8 weeks of age, and diabetes was induced and treated as described previously (18). Briefly, mice received three daily injections of doses of 75 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO). Tail-vein glucose concentrations were measured daily using a Glucometer Elite (Miles, Elhart, IN) starting at 1 week after STZ injection. Diabetes was controlled with subcutaneously implanted insulin pellets (Linshin, ON, Canada). Three weeks after implantation of insulin pellets, mice were mated along with age-matched nondiabetic mice. Noon on the day in which a copulation plug was found was determined to be day 0.5 of gestation. As reported previously (18), mice that had received STZ injections and been treated with insulin pellet, which had been euglycemic before pregnancy, became hyperglycemia on day 4.5 of pregnancy until the time they were sacrificed.

For testing the effects of transient hyperglycemia just before the beginning of organogenesis, nondiabetic mice were made hyperglycemic by subcutaneous injection of 1 ml of 25% glucose in PBS during an 8-h period on day 7.5 as of organogenesis, nondiabetic mice were euglycemic before pregnancy, became hyperglycemia on day 4.5 of pregnancy and neutralized with 1 N of HCl for protein quantitation by the Bradford method.

Immunoblot analysis. A total of 30–60 μg of protein extracts from whole embryos or membrane and cytosol fractions of decidua were separated by SDS/PAGE (8% PAGE) using a XCell II system (Novex, San Diego, CA) and transferred to nitrocellulose membranes. After transfer, membranes were blocked and then incubated with rabbit polyclonal antibodies raised against cPKC-α, PKC-β II, nPKC-δ, or nPKC-ζ (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were washed and then incubated with horseradish peroxidase–coupled donkey anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ). Bands were detected by ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and were quantified by densitometric scanning of the X-ray film and analysis by Image Quant software.

RESULTS

Maternal diabetes increases DAG production and PKC activity in embryos and decidua on day 9.5. In preliminary studies, a profile for DAG production and PKC activity during gestation was performed using decidua and placenta to determine whether DAG-PKC signaling was detectable during prenatal development. Decidua or placentas were obtained from normal, nondiabetic pregnant mice beginning on day 9.5 and every 3 days until day 18.5, the day before parturition. DAG was detectable (200 ± 86 pmol/mg wet wt) on day 9.5 and was increased by 1.7-fold on day 18.5 (data not shown). Similarly, membrane-associated PKC activity was detectable (25.6 ± 4.5 pmol · mg protein−1 · min−1) on day 9.5 and increased twofold by day 15.5 (data not shown). Immunoblot analysis demonstrated that PKC α, βII, δ, and ζ were expressed at these time points and increased on days 15.5 and 18.5.

Because DAG and PKC were detectable during early organogenesis (day 9.5) in decidua, the effects of maternal diabetes on DAG and PKC in the embryo and decidua were investigated. Similar to previous findings (18), diabetic mice were euglycemic on day 0.5 of pregnancy but were significantly hyperglycemic on day 9.5 (Table 1). Mice were sacrificed on day 9.5, and DAG and PKC were assayed in embryos and decidua.

DAG concentrations and PKC activity were significantly increased (1.7- and 1.3-fold, respectively) in embryos of diabetic mice (Fig. 1A and B). Similarly, decidual DAG was increased 1.5-fold, and PKC activity in membrane and cytosolic fractions was significantly increased (1.2- and

<table>
<thead>
<tr>
<th>Gestational day</th>
<th>Nondiabetic</th>
<th>Diabetic</th>
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<tr>
<td>0.5</td>
<td>9.5 ± 0.79 (25)</td>
<td>5.36 ± 1.69 (26)</td>
</tr>
<tr>
<td>9.5</td>
<td>7.16 ± 0.39 (25)</td>
<td>23.61 ± 3.85* (15)</td>
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Diabetes was induced and treated as described previously (18). Briefer, mice received three daily injections of doses of 75 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO). Tail-vein glucose concentrations were measured daily using a Glucometer Elite (Miles, Elhart, IN) starting at 1 week after STZ injection. Diabetes was controlled with subcutaneously implanted insulin pellets (Linshin, ON, Canada). Three weeks after implantation of insulin pellets, mice were mated along with age-matched nondiabetic mice. Noon on the day in which a copulation plug was found was determined to be day 0.5 of gestation. As reported previously (18), mice that had received STZ injections and been treated with insulin pellet, which had been euglycemic before pregnancy, became hyperglycemia on day 4.5 of pregnancy until the time they were sacrificed.

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Statistical analysis. Statistical analyses were performed using Student’s unpaired t test. Analyses were performed using the Statview statistical package for Macintosh (Hulinks, Cary, NC). P < 0.05 was defined as statistically significant.
1.3-fold, respectively) in samples from diabetic mice (Fig. 1C and D). Immunoblot analysis demonstrated that steady-state concentrations of PKC isoforms \( \alpha, \beta II, \delta, \) and \( \zeta \) in whole embryo were not significantly affected by maternal diabetes (Fig. 2A), suggesting that increased PKC activity in cell extracts (Fig. 1B) resulted from activation of the enzymes, not from new mRNA or protein synthesis. In decidua, membrane association of all PKC isoforms examined (PKC \( \alpha, \beta II, \delta, \zeta \)) were increased in samples from diabetic pregnancies but not in the cytosolic fraction (Fig. 2B). This suggests that diabetes activated PKC located in the cytoplasmic compartment, causing it to be translocated to the membranous compartment.

**Transient hyperglycemia before organogenesis activates DAG and PKC during organogenesis.** We next investigated whether transient hyperglycemia occurring only on day 7.5 is sufficient to increase DAG production and PKC activity on day 9.5. As shown in Table 2, injection of glucose at approximately hourly intervals during an 8-h time period on day 7.5 significantly increased maternal blood glucose, and blood glucose returned to normal by day 9.5. Nevertheless, even though maternal glucose concentrations were normal on day 9.5, DAG concentrations and PKC activity were significantly increased in embryos of mice that received glucose injections (Fig. 3A and B). Similarly, PKC activity in membrane fractions of decidua and decidua of mice that received glucose injections was significantly increased, and decidual DAG was also increased, although the increase in DAG was not statistically significant (Fig. 3C and D). Western blot analysis demonstrated that the steady-state concentrations of PKC isoforms in whole embryos or in membrane or cytosolic fractions of decidua were variable but were not significantly increased by glucose injection (Fig. 4A and B). This suggests that, like maternal diabetes, effects of hyperglycemia on PKC activity were due to stimulation of enzymatic activity rather than to de novo mRNA or protein synthesis.

**Increased PKC in defective embryos of diabetic mice.** By day 10.5, formation of the neural tube is complete and embryos with defective neural tubes can be distinguished from normal embryos. Examples of a normal embryo and an embryo with an NTD caused by maternal diabetes are shown in Fig. 5.

To test whether PKC activity was affected in malformed embryos of diabetic mice, we obtained embryos on day 11.5 of gestation from diabetic and nondiabetic pregnancies. As on day 10.5, diabetic mice were significantly more hyperglycemic than nondiabetic mice (25.53 ± 2.33 and 7.58 ± 0.19 mmol/l, respectively; \( P < 0.0005 \)). Like day 10.5 embryos, normal and defective day 11.5 embryos can be
distinguished from each other, but they are significantly larger so that PKC in membrane and cytosol fractions can readily be assayed. All of the embryos of nondiabetic mice collected for this assay were normal. In contrast, 25% of the embryos of diabetic mice had defects affecting the neural tube (exencephaly, cerebral hemorrhage, midbrain/hindbrain underdevelopment), sometimes also including heart or gut defects.

As shown in Fig. 6, membrane-associated PKC activity in defective embryos of diabetic mice was increased 24-fold compared with embryos of nondiabetic mice and threefold compared with morphologically normal embryos from diabetic pregnancies. Although mean PKC activity was higher in normal embryos of diabetic mice compared with embryos from nondiabetic pregnancies, the increase was not statistically significant. There was no significant difference in PKC activity of cytosol fractions from any treatment group. In decidua, there was a slight increase in membrane and cytosol PKC activity in both normal and abnormal embryos of diabetic mice, although these increases were not significant.

DISCUSSION
The PKC family of serine/threonine kinases plays a critical role in the regulation of cellular differentiation and proliferation (34). Therefore, dysregulation of this pathway would be expected to have adverse consequences on embryonic development. Increases in DAG-PKC signaling have been noted in retina, aorta, heart, and glomeruli of patients with diabetes and diabetic animals and seem to be

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<tr>
<td>0.5</td>
<td>5.97 ± 0.47 (12)</td>
<td>12.47 ± 2.02* (31)</td>
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<tr>
<td>7.5</td>
<td>7.23 ± 0.44 (12)</td>
<td>7.48 ± 0.76 (22)</td>
</tr>
<tr>
<td>9.5</td>
<td>7.17 ± 0.46 (12)</td>
<td>7.48 ± 0.76 (22)</td>
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Data are means ± SD (n). *P < 0.0001 vs. control.

FIG. 3. Effects of glucose injections on day 7.5 on DAG and PKC in embryos and decidua on day 9.5 assayed as in Fig. 2. A: DAG concentrations in embryos; *P < 0.05 vs. control. B: PKC activity in embryos; *P < 0.001 vs. control. C: DAG concentrations in decidua. D: PKC activity in cytosolic and membrane fractions of decidua; *P < 0.001 vs. control. □, control (saline injection) results; ■, glucose injection results.

FIG. 4. A: Representative immunoblot analysis of PKC isoforms in two day 9.5 embryos of mice that received saline injections and in three embryos of mice that received glucose injections. Quantitation of bands in the upper panel after scanning densitometry is shown in the lower panel with band intensity from embryos of diabetic mice expressed as a percentage of bands from embryos of nondiabetic mice. B: Representative immunoblot analysis of two day 9.5 decidua of mice that received saline injections and in two decidua of mice that received glucose injections. Quantitation of bands from four replicate decidua of saline-injected pregnancies and four glucose-injected pregnancies is shown in the lower panel. Quantitation of membrane bands are shown in solid bars and of cytosol bands are shown in shaded bars.
directly related to elevated glucose concentrations (22,24–29). Activation of the DAG-PKC pathway associated with abnormal growth and differentiation during diabetic embryopathy has not previously been demonstrated.

Several PKC isotypes have been detected in the placenta and decidua of the human and the rat (35–39). In the human placenta, there are significant increases in PKC βII and ε on the microvillus membrane and in PKC γ and ε on the basal membrane between 16 and 40 weeks of gestation (40). PKC is involved in the regulation of human chorionic gonadotropin, estrogen, and progesterone production by trophoblasts. Downregulation of PKC activity by chronic exposure to the phorbol ester 12-myristate 13-acetate or 12,13-dibutyrate stimulates secretion of human chorionic gonadotropin β (37,39). Similarly, a general PKC inhibitor, isoquinolinesulfonamide (HT) attenuates DNA synthesis in proliferating and differentiating trophoblast cells and accelerates the acquisition of progesterone biosynthetic capabilities (38). In the experiments reported here, we investigated the activities and protein concentrations of PKC α, βII, δ, and ζ in embryos and extraembryonic tissues during early organogenesis. We selected these PKC isoforms for study because of their involvement in other diabetic complications (41–43). Notably, DAG production and PKC activity were increased in the extraembryonic structures (decidua, ectlaplacental cone, and visceral yolk sac) by diabetes or hyperglycemia. Because these membranes contribute to implantation, development of the placenta, hormone and growth factor production, and nutrient and gas exchange, increased DAG-PKC signaling could interfere with any of these processes. Thus, increased PKC signaling may be of consequence to the embryo during organogenesis as well as impair later placental function, which could disturb fetal development.

In the embryo, inhibition of PKC activity in rat embryos causes NTDs, indicating that PKC activity is required for normal neural tube development (44,45). However, our results suggest that excess PKC signaling is also associated with defective development. It should be noted that increased DAG-PKC activity occurred on day 9.5, while the embryo is undergoing dramatic morphogenesis, particularly the establishment of the neural tube and the heart. There are three obvious interpretations of the correlation between increased DAG-PKC in embryos of diabetic dams and defective organogenesis: 1) that altered PKC signaling is responsible for abnormal morphogenesis, 2) that DAG-PKC signaling is increased in embryos of diabetic dams as a consequence of an already disturbed developmental program, or 3) that sustained increased DAG-PKC signaling disturbs the formation of structures that form at successively later times in gestation. Because the increase in DAG and PKC on day 9.5 occurs after the onset of gene expression that induces neural tube formation (day 8.5), this suggests that the increase in DAG and PKC on day 9.5 could not be a cause but could be a consequence of defective development of the neural tube. The increase in PKC activity in embryos of diabetic mice, which was even greater in defective embryos than in embryos that were morphologically normal, supports this interpretation. However, there are many structures that continue to form throughout the embryonic period and that depend on inductive influences from previously formed structures. Therefore, increased PKC activity in defective day 11.5 embryos could be due to abnormal signaling pathways in structures undergoing morphogenesis at that stage of development and that are adversely affected by previously existing structural defects. Because we did not cellularly localize the sites of increased PKC activity, it is not possible to distinguish these possibilities at this time. Future research will be necessary to determine the relationship between abnormal DAG-PKC signaling and defective development.

Another interpretation is that increased DAG-PKC signaling originates from vascular stem cells rather than from neuroepithelial or other primordial cell types. Because neovascularization is important for establishment of early organ structures, diabetic embryopathy secondary to defective neovascularization may be a novel type of diabetic vascular complication. In this regard, it should be noted that vitamin E prevents diabetes-induced vascular complications by inhibiting glucose activation of DAG-PKC signaling (46–48). Several studies have demonstrated that antioxidants can prevent diabetic embryopathy in animal models (14–17), and we have shown that hyperglycemia-induced oxidative stress inhibits expression of Pax-3 (Chang et al., submitted manuscript). Thus, it will be important in the future to determine whether antioxidants normalize DAG and PKC in the embryo and decidua, as it does in retinal and renal tissues (48,49).

It is interesting that induction of hyperglycemia during day 7.5 alone was sufficient to cause a sustained activation of this pathway that could still be measured on day 9.5.

FIG. 6. PKC activity in membrane and cytosolic fractions of day 11.5 embryos and decidua from nondiabetic (control) mice or in normal or abnormal embryos of diabetic mice. □, results from control (nondiabetic) embryos; ■, results from diabetic normal (without malformations) embryos; ▲, results from diabetic abnormal (with structural defects) embryos; n = 3 replicate samples of pooled embryos; *P < 0.01 vs. control and diabetic, normal.
This indicates that, just as there is a sensitive period of development before organogenesis in which withholding insulin treatment from diabetic rats will cause NTDs or inducing hyperglycemia will disturb gene expression and cause NTDs (19,20), this same period of development is susceptible to sustained hyperglycemia-induced DAG production and PKC signaling. Because there was no consistent increase in the steady-state amount of any PKC isozyme by immunoblot analysis, this suggests that there is a stable activation of PKC activity, most likely due to increased production of DAG rather than to increased expression of PKC mRNA or protein. For example, maternal hyperglycemia may induce expression of genes that encode the regulatory enzymes in this pathway, such as phospholipase C. A sustained effect of hyperglycemia on DAG and PKC is consistent with our previous observations that DAG and PKC remain elevated even years after the onset of diabetes in patients, but the increase in activity may take several days of exposure to hyperglycemia to appear (24). Indeed, increased activity of PKC, rather than increased synthesis, may explain the failure of others to see an increase in mRNA for PKC α, β, or γ in rat embryos of diabetic mothers or cultured in high glucose (45).

This is the first report that PKC activity is increased by experimental procedures (maternal diabetes or transient hyperglycemia) that cause diabetic embryopathy and, furthermore, that PKC activity is even more increased in defective embryos than in normal embryos of diabetic mice after completion of neural tube fusion. Certainly, further investigation will be necessary to localize cellularly the sites of increased PKC activity, both in embryonic and in extraembryonic tissues, at critical stages during organogenesis and to determine whether increased PKC activity contributes to abnormal development.

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