Evidence That Elevated Glucose Causes Altered Gene Expression, Apoptosis, and Neural Tube Defects in a Mouse Model of Diabetic Pregnancy

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Congenital malformations, including neural tube defects (NTDs), are significantly increased in the offspring of diabetic mothers. We previously reported that in the embryos of a mouse model of diabetic pregnancy, NTDs are associated with reduced expression of the gene *Pax-3*, which encodes a transcription factor that regulates neural tube development, and that reduced expression of *Pax-3* leads to neuroepithelial apoptosis. In this study, we used three approaches to test whether glucose alone could be responsible for these adverse effects of diabetes on embryonic development. First, primary culture of embryo tissue in medium containing 15 mmol/l glucose inhibited *Pax-3* expression compared with culture in medium containing 5 mmol/l glucose. Second, inducing hyperglycemia in pregnant mice by subcutaneous glucose administration significantly inhibited *Pax-3* expression ($P < 0.05$), as demonstrated by quantitative reverse transcription–polymerase chain reaction assay of Pax-3 mRNA, and also increased neural tube apoptosis ($P < 0.05$). NTDs were significantly increased in glucose-injected pregnancies when blood glucose levels were >250 mg/dl ($P < 0.002$) but not in moderately hyperglycemic pregnancies (150–250 mg/dl, $P = 0.37$). Third, phlorizin administration to pregnant diabetic mice reduced blood glucose levels and the rate of NTDs. As seen with glucose-injected pregnancies, the rate of NTDs in phlorizin-treated diabetic pregnancies was related to the severity of hyperglycemia, since NTDs were significantly increased in severely hyperglycemic (>250 mg/dl) diabetic pregnancies ($P < 0.001$) but not in moderately hyperglycemic pregnancies (150–250 mg/dl, $P = 0.35$). These two findings, that elevated glucose alone can cause the changes in *Pax-3* expression observed during diabetic pregnancy and that the NTD rate rises with significant increases in blood glucose levels, suggest that congenital malformations associated with diabetic pregnancy are caused by disruption of regulatory gene expression in the embryo in response to elevated glucose. *Diabetes* 48:2454–2462, 1999

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DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; NTD, neural tube defect; PBS, phosphate-buffered saline; PKC-β, protein kinase C-β; RT-PCR, reverse transcription–polymerase chain reaction; STZ, streptozotocin; TUNEL, Tdt-mediated dUTP nick end labeling.

The incidence of congenital malformations caused by maternal pregestational diabetes is two- to fivefold higher than during nondiabetic pregnancy (1–8). This syndrome, diabetic embryopathy, is the major source of neonatal mortality and morbidity associated with diabetic pregnancy (9). The malformations associated with diabetic embryopathy can affect any developing organ system (3,7). Understanding the underlying molecular basis for diabetic embryopathy is important to devise strategies aimed at reducing its occurrence.

Neural tube defects (NTDs), which are among the most common of the malformations associated with diabetic embryopathy in humans (3,7), are increased threefold in a mouse model of diabetic pregnancy (10). The increase in NTDs is associated with reduced expression of *Pax-3*, a gene that encodes a transcription factor that is required for neural tube development. As a result of reduced expression of *Pax-3*, neuroepithelial cells along the migrating ridge of the dorsal surface of the neural tube undergo apoptosis before midline fusion, thereby providing a cellular explanation for the NTDs. Expression of at least two Pax-3–regulated genes, *cdc46* and *Dep-1*, is disturbed in embryos of diabetic mice (11,12). In contrast, expression of two constitutively expressed genes, *fibronectin* and *36B4*, is not affected (10–12), indicating that the altered expression of *Pax-3*, *cdc46*, and *Dep-1* is not due to global disruption of all gene regulation in the embryo. Because loss of function mutations of *Pax-3* lead to NTDs with 100% penetrance (13–16), inhibiting the expression of *Pax-3* in the embryos of diabetic mothers below a critical threshold will most certainly result in malformation. Undoubtedly, there are additional critical developmental control genes whose expression is affected by maternal diabetes and that lead to other embryonic malformations. Therefore, by understanding the molecular mechanisms by which maternal diabetes inhibits expression of *Pax-3*, we may gain insight into the general mechanisms by which diabetic pregnancy leads to malformations.

Previous studies have not resolved the question of whether the adverse effects of diabetes on embryonic development could be attributed solely to exposure of the embryo to hyperglycemia, other metabolic abnormalities or altered serum components produced secondarily to hyperglycemia, or a combination of factors. One large multicenter study concluded that the increased malformation rate in diabetic pregnancies could not be attributed to hyperglycemia or elevated glycosylated hemoglobin (6). On the other hand, some other

studies, including the Diabetes Control and Complications Trial, have shown that the risk for diabetic embryopathy is correlated with glycosylated hemoglobin levels (17,18). In several studies using animal models, culture of postimplantation rat embryos in the presence of elevated glucose has replicated the growth and structural defects that are associated with diabetic pregnancy (19–22). However, β-hydroxybutyrate can also cause maldevelopment, and β-hydroxybutyrate and somatomedin inhibitors each synergize with glucose to cause maldevelopment (22). Other studies have shown that serum from insulin-treated diabetic rats was more teratogenic in postimplantation rat embryo culture than serum from nondiabetic rats (23,24). These results suggest that metabolic disturbances that are secondary to hyperglycemia have embryopathic potential. Perhaps the most compelling argument that glucose, or another metabolic abnormality resulting from hyperglycemia, is central to diabetic embryopathy is that type 2 diabetes poses the same risks for embryonic maldevelopment as type 1 diabetes (25), even though ketones are not characteristically elevated and somatomedin-binding proteins may even be lower than in nondiabetic subjects (26,27).

To characterize the biochemical pathways by which the diabetic environment interferes with embryo gene expression and causes congenital malformations, it is necessary to determine whether glucose is responsible for the adverse effects of diabetes on embryo gene expression that are associated with NTDs. In this study, we tested whether exposing the embryo to elevated glucose elicits the same changes in embryo gene expression and apoptosis that occur during diabetic pregnancy, and whether the NTD rate is correlated with blood glucose levels in pregnant mice.

RESEARCH DESIGN AND METHODS

Experimental animals. Insulin-dependent diabetes was induced in 9-week-old female ICR mice (Harlan Sprague Dawley) with streptozotocin (STZ) (Sigma, St. Louis, MO) as previously described (10). Briefly, STZ was administered ~4 weeks before pregnancy, and hyperglycemia was controlled using subcutaneously implanted sustained-release insulin pellets (Linshin; Scarborough, Ontario, Canada). Glycemic control was monitored for ~3 weeks by assay of tail blood with a Glucometer Elite (Miles, Elkhart, IN). Diabetic and age-matched control mice were mated with ICR males. Noon on the day on which a copulation plug was observed was considered day 0.5 of pregnancy. As previously described (10), STZ-insulin pellet-treated mice became hyperglycemic again (~270 mg/dl) beginning on day 4.5 of pregnancy.

Phlorizin (Sigma) was dissolved in 40% propylene glycol and administered intraperitoneally twice a day at 0.4 g/kg as described (28,29) to diabetic and nondiabetic mice on days 6.5–9.5 of pregnancy. Propylene glycol alone was injected in control diabetic and nondiabetic mice. Tail vein blood glucose was assayed every 60–90 min during the daytime on days 6.5–10.5, and these measurements were used to determine mean blood glucose levels of each mouse after phlorizin treatment.

Hyperglycemia was induced by subcutaneous injection of 1 ml of 25% glucose (Sigma) dissolved in phosphate-buffered saline (PBS). PBS alone was injected in control mice. Injections were performed approximately every 2 h (or when blood glucose levels fell below 200 mg/dl) during a 10-h period on day 7.5 and once in the morning on day 8.5. Tail vein blood glucose levels were measured every 60–90 min after glucose injection to determine mean blood glucose levels.

All pregnancies were terminated, and embryo material was recovered on day 8.5 (for assay of RNA) or 10.5 (for assay of apoptosis or NTDs) as described (10). Embryos from individual pregnancies were saved for RNA assay. NTDs were noted by the presence of open or underdeveloped NTDs as described (10) and were scored blindly.

Primary embryo culture. Embryos on day 9.5 of gestation were dissected from uteri and extraembryonic structures in PBS prewarmed to 37°C and placed in Dulbecco's minimal essential medium (DMEM) (Gibco BRL Life Technologies, Grand Island, NY) that had been prepared without glucose. Whole embryos were minced, first with jeweler's forceps then by passing through an 18-gauge needle, to generate tissue clumps and single cells. Tissues were placed into 35-mm culture dishes that had been coated with 1% gelatin (Sigma) containing 2 ml DMEM supplemented with 10% fetal calf serum (FCS) and 5 mmol/l glucose at the concentrations indicated in the figure legends. Tissues from approximately seven to nine embryos were cultured per dish. Cultures were incubated at 37°C with 5% CO_2. After 24 h of culture, media were removed from plates, and plates were rinsed with PBS and stored at –80°C until assay of RNA.

Assay of RNA by reverse transcription–polymerase chain reaction. RNA was prepared from whole embryos from individual pregnancies as described (10) using the commercial reagent Ultraspec (Cinna Biotecx, Houston, TX), and from embryo cultures, also using Ultraspec, according to the manufacturer's instructions. Embryos from individual pregnancies were pooled and RNA was prepared by homogenizing in 1 ml Ultraspec with a 1,000-μl micropipette. RNA from embryo culture was similarly prepared using 1 ml Ultraspec per 35-mm culture dish. Two hundred nanograms of whole or cultured embryo RNA was reverse-transcribed and amplified by polymerase chain reaction (PCR) using specific *Pax-3* or *36B4* primers as described (10,12). PCR products were visualized by autoradiography and quantitated by scanning densitometry.

Apoptosis assay. Whole-mount apoptosis assay of day 10.5 embryos was performed using a modified Tdt-mediated dUTP nick end labeling (TUNEL) procedure (30) as described (10). Embryos were scored blindly on a scale of 0–10 in comparison to positive and negative control embryos. Positive controls were pretreated with DNase I and scored as 10, and negative controls were incubated without terminal transferase enzyme and scored as 0.

Statistical analyses. Statistical analyses were performed using Student's unpaired *t* test to test for significance of single independent variables or multiple analysis of variance followed by Duncan's multiple range test to test for significance of more than one independent variable. Analyses were performed using Statistica software (StatSoft, Tulsa, OK) for Macintosh.

RESULTS

Glucose directly inhibits *Pax-3* expression on cultured embryo tissue. To test whether elevated glucose alone directly inhibits expression of *Pax-3* in the embryo, a method using in vitro culture of primary embryo tissues was employed. Embryos obtained on day 9.5 of development were dispersed for culture of single cells and cell clumps in medium containing normal (5 mmol/l) or elevated (15 mmol/l) glucose (Fig. 1*A*). As shown in Fig. 1*B*, culture in elevated glucose inhibited *Pax-3* expression. The inhibition of *Pax-3* expression by 15 mmol/l D-glucose was not due to an osmotic effect, because culture in medium containing 5 mmol/l D-glucose plus 10 mmol/l L-glucose did not affect Pax-3 mRNA levels. Expression of *36B4* was not affected by any of the culture conditions. These results demonstrate that glucose alone, at concentrations comparable to those in pregnant diabetic mouse serum, can inhibit expression of *Pax-3*. Furthermore, these results show that the adverse effects of glucose occur directly on embryo tissue.

Elevated blood glucose inhibits expression of *Pax-3* in vivo. To test whether elevating blood glucose of pregnant animals causes the changes in *Pax-3* expression observed during embryo culture, the effect of hyperglycemia on *Pax-3* expression was examined. Hyperglycemia was induced in nondiabetic pregnant mice by subcutaneous glucose injection on day 7.5 and the morning of day 8.5, to coincide with the day preceding and the morning of the beginning of neural tube fusion (Fig. 2*A*). Glucose injection significantly elevated blood glucose levels in pregnant mice (Fig. 2*B*). Expression of *Pax-3* was assayed in embryos at day 8.5 of development, the day on which *Pax-3* expression and neural tube fusion begins (31), by a quantitative reverse transcription (RT)-PCR assay. As previously described (10), serial dilutions of reverse-transcribed RNA are amplified using primers specific to *Pax-3* or to a constitutively expressed gene, *36B4*, to demonstrate that the amount of PCR reaction product is limited by the amount of RNA used to generate the PCR template (11,32). Using this method, the amount of PCR product for

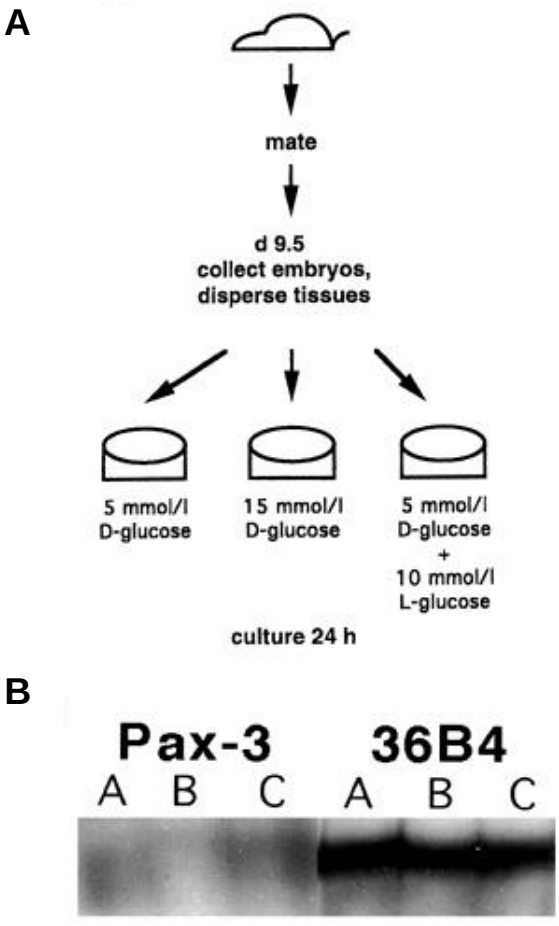

FIG. 1. Effect of glucose concentration on *Pax-3* expression during primary embryo culture. *A*: Experimental scheme for embryo culture. *B*: RT-PCR of 200 ng RNA amplified with *Pax-3* or *36B4* primers. RNA was obtained from cells cultured in medium containing 5 mmol/l (*lane A*) or 15 mmol/l (*lane B*) D-glucose or 5 mmol/l D-glucose plus 10 mmol/l L-glucose as an osmotic control (*lane C*).

Pax-3, as determined by scanning densitometry, at each dilution of RT reaction product was reduced using RNA obtained from glucose-injected compared with control pregnancies, whereas the amount of PCR product for *36B4* at each dilution was not reduced (Fig. 2*C*). The amount of *Pax-3* PCR product relative to *36B4* PCR product at the same dilution of RT reactions from five glucose-injected pregnancies (mean blood glucose 278 ± 38 mg/dl) was reduced 24% compared with four saline-injected pregnancies (mean blood glucose 122.5 ± 4.05 mg/dl) (Fig. 2*D*). These results demonstrate that induction of hyperglycemia for 24 h before the onset of *Pax-3* expression causes reduced expression of *Pax-3* without causing a global inhibition of gene expression.

Elevated blood glucose increases neural tube apoptosis. We previously demonstrated that the portion of the developing neural tube that is prone to malformation undergoes unscheduled apoptosis at increased frequency in embryos of diabetic mice, and that this apoptosis is related to reduced expression of *Pax-3* (10). This evidence provides a cellular mechanism by which reduced expression of *Pax-3* causes NTDs. To test whether induction of hyperglycemia in pregnant mice, like diabetic pregnancy, causes increased neural tube apoptosis, whole-mount in situ TUNEL assay was performed on a sample of embryos chosen at random from saline- and glucose-injected pregnancies. After TUNEL processing, embryos were assigned an apoptosis score on a scale of 0 to 10. As shown in Fig. 3*A* and *B*, apoptosis in embryos from glucose-injected pregnancies was particularly severe at sites of NTD. When scored blindly, embryos from glucose-treated pregnancies exhibited significantly more neural tube apoptosis (Fig. 3*C*). Thus, simply exposing the embryo to elevated blood glucose causes the same increase in neural tube apoptosis that is observed during diabetic pregnancy.

Elevated blood glucose is sufficient to induce NTDs. The NTD rate in saline- and glucose-injected pregnancies was compared to determine whether the induction of hyperglycemia that inhibited *Pax-3* expression and increased neural tube apoptosis was accompanied by increased NTDs. As shown in Fig. 4*A*, glucose injection of pregnant mice significantly increased in the rate of NTDs. When the rate of NTDs was examined as a function of blood glucose levels, however, it was significantly elevated only in severely hyperglycemic pregnancies (>250 mg/dl) but not in pregnancies that were only moderately hyperglycemic (150–250 mg/dl) (Fig. 4*B*). Examples of hyperglycemia-induced NTDs, exencephaly, and midbrain/hindbrain underdevelopment (defects also induced by diabetic pregnancy [10]) compared with a normal embryo are shown in Fig. 4*C–E*.

Elevated blood glucose is necessary for diabetes-induced NTDs. The previous results demonstrated that elevated glucose alone can replicate the effects of diabetic pregnancy on increasing NTDs. To test whether glucose is necessary to increase NTDs associated with diabetic pregnancy, the effect of selectively reducing only the elevated blood glucose levels in pregnant diabetic mice was tested. Phlorizin, which inhibits renal tubular reabsorption of glucose, thus reducing the hyperglycemia in diabetic animals, was administered to pregnant diabetic and nondiabetic mice on days 6.5–9.5 of pregnancy (Fig. 5*A*). These days were chosen so that blood glucose levels would be reduced before the onset of neural tube formation through completion of neural tube fusion. Phlorizin had no effect on mean blood glucose levels in nondiabetic mice but significantly reduced blood glucose levels in diabetic mice (Fig. 5*B*). Just as there had been no effect of moderate hyperglycemia induced by glucose injection on NTDs, there was no significant difference in the NTD rate between nondiabetic euglycemic pregnancies and phlorizin-treated diabetic pregnancies that were only moderately hyperglycemic (150–250 mg/dl). In contrast, diabetic mice that were not treated with phlorizin were severely hyperglycemic (>250 mg/dl), and the NTD rate was significantly greater than in either euglycemic or moderately hyperglycemic pregnancies (Fig. 5*C*). These results demonstrate that the rate of NTDs in diabetic pregnancies is related to the severity of hyperglycemia.

DISCUSSION

To investigate the biochemical mechanisms by which maternal diabetes disturbs expression of a critical embryonic gene, *Pax-3*, and leads to NTDs, it is important to determine whether elevated glucose is responsible for the effects of maternal diabetes in this system. The experiments presented here provide evidence supporting the hypothesis that hyperglycemia is necessary and sufficient to cause the molecular processes that culminate in NTDs. Primary cultures of mouse embryo tissue in medium containing glucose at con-

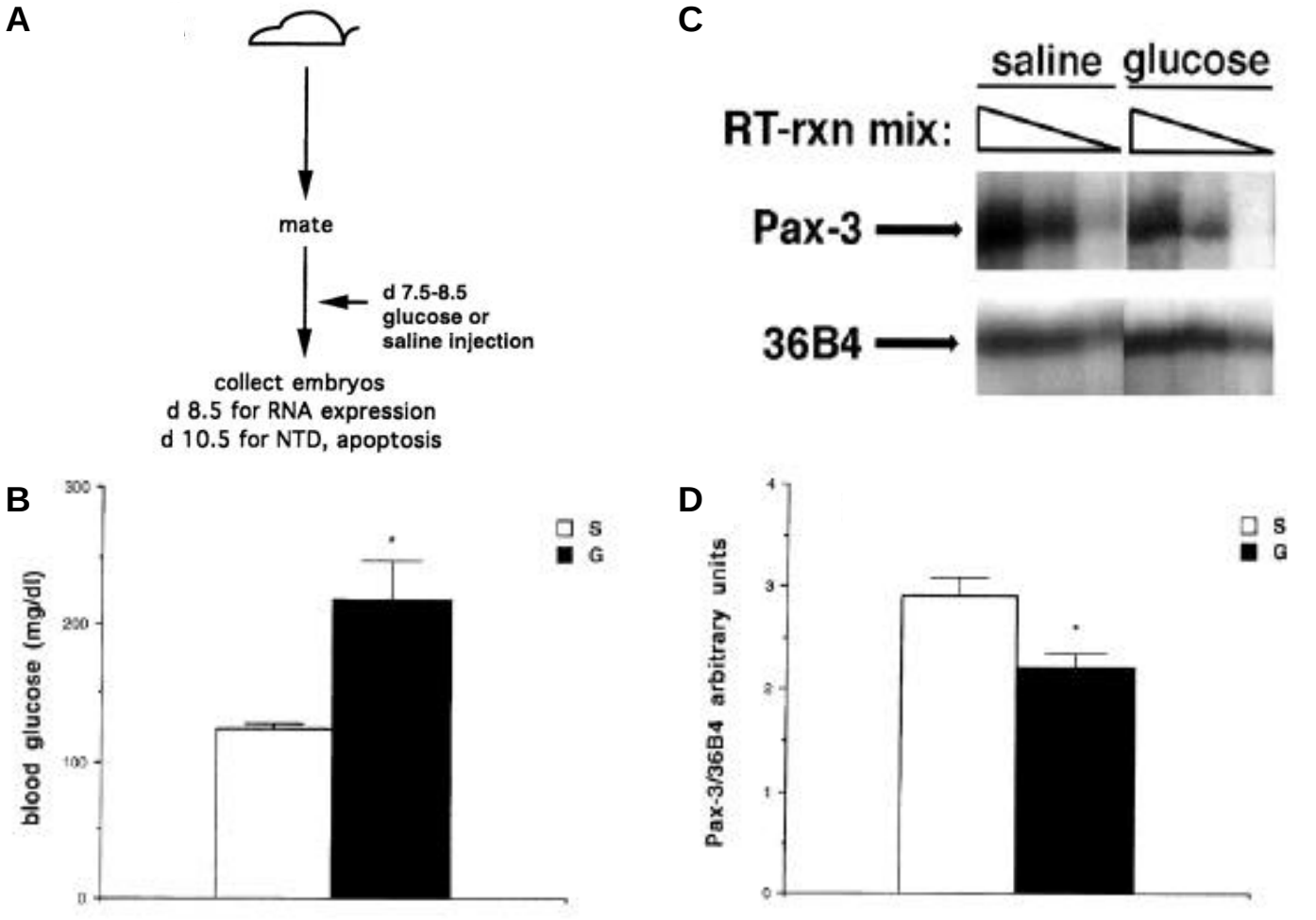

FIG. 2. Effect of glucose injection on blood glucose levels and *Pax-3* expression in day 8.5 embryos. *A*: Experimental scheme to induce hyperglycemia in pregnant mice. *B*: Mean blood glucose levels in mice injected with saline (S, n = 11) and glucose (G, n = 10). Values represent the means ± SE of blood glucose measurements taken every 60–90 min during day 7.5 and the morning of day 8.5 of pregnancy. *P < 0.005 vs. saline control by Student's unpaired t test. *C*: Representative RT-PCR assay of Pax-3 RNA from embryos of a glucose-injected mouse compared with a saline-injected pregnancy. RT reaction products were serially diluted and amplified using specific *Pax-3* or *36B4* primers. *D*: Pax-3:36B4 PCR product of saline-injected (n = 4) compared with glucose-injected (n = 5) pregnancies. *P < 0.02 compared with saline controls by Student's unpaired t test.

centrations similar to those that occur during diabetic pregnancy in the mouse caused expression of *Pax-3* to be inhibited, similar to the effect during diabetic pregnancy. Furthermore, simply inducing hyperglycemia in nondiabetic mice caused the same inhibition of *Pax-3* expression and increase in apoptosis that occurs during diabetic pregnancy. Finally, manipulations that either lowered blood glucose levels in diabetic mice or elevated blood glucose levels in nondiabetic mice demonstrated that increased NTDs are correlated with severity of hyperglycemia.

Primary embryo tissue culture was employed to test whether high glucose is responsible for inhibition of *Pax-3* expression and whether this effect occurs directly on the embryo. In addition, embryo culture provides information that cannot be obtained from in vivo approaches. For example, the adverse effects of glucose injection of pregnant mice could have been caused either by glucose uptake and metabolism or by osmotic effects of the high-glucose solution. We were unable to distinguish these possibilities by testing the effect of L-glucose injection, because mice that were injected with solutions of L-glucose, instead of D-glucose, died within 24 h of L-glucose injection, presumably as a result of extreme diuresis and subsequent dehydration (data not shown). However, lack of effect of L-glucose on survival of embryo tissue or on *Pax-3* expression in vitro indicated that hyperglycemia, not hyperosmolarity, induced by D-glucose injection is responsible for inhibition of *Pax-3* expression. In addition, the inhibition of *Pax-3* expression by glucose that can be observed in embryo culture demonstrates that the effects of high glucose can occur directly on the embryo tissue and does not need to involve other metabolic defects, for example, increased levels of β-hydroxybutyrate, somatomedin-binding proteins (22,24,33), branched-chain amino acids (19,34), or effects on yolk sac structure or physiology (35–37). Although these effects of diabetes could exacerbate the effects of high glucose, our results indicate that study of the molecular regulation of diabetic embryopathy should focus on the primary involvement of glucose in the regulation of gene expression in the embryo.

L-Glucose is not transported into cells by specific carriers, suggesting that inhibition of *Pax-3* expression by high glucose requires uptake by specific glucose transporters and, per-

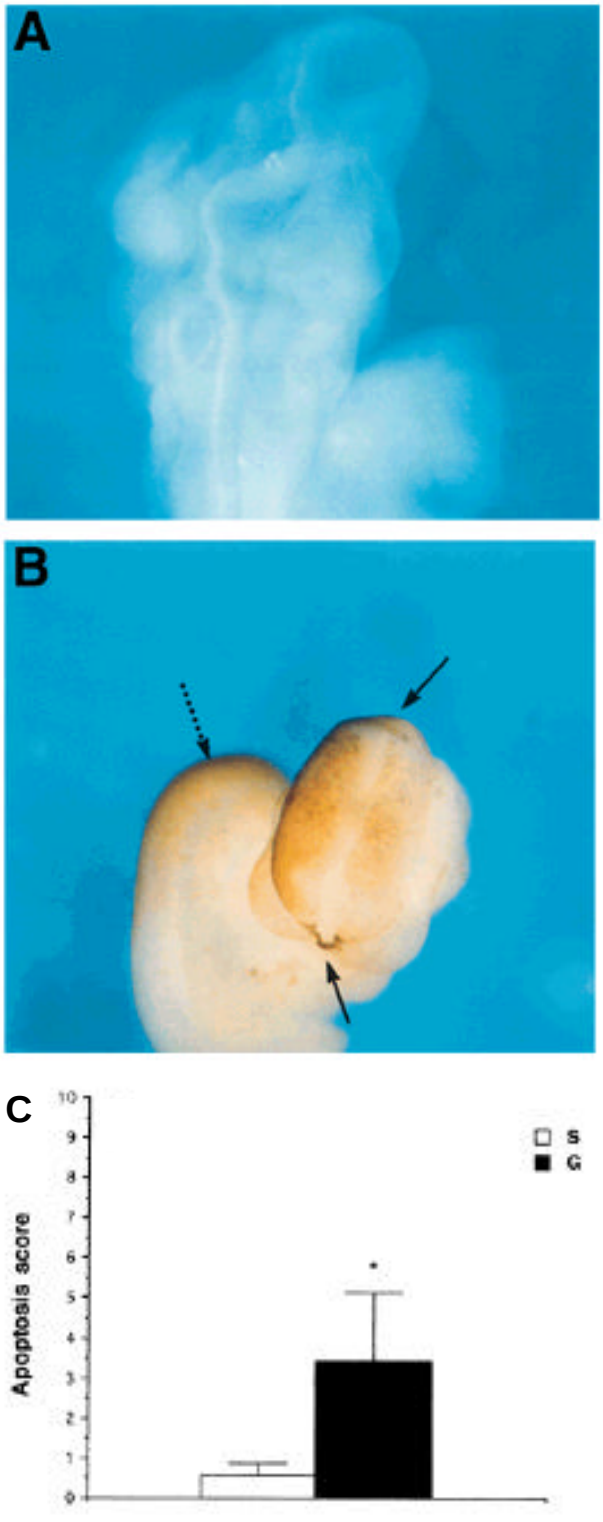

FIG. 3. *A*: **Example of embryo from saline-injected pregnancy (viewed from the dorsal surface of the embryo) after TUNEL assay (score = 0) for apoptosis.** *B*: **Example of embryo from glucose-injected pregnancy (viewed looking down on the exposed mesencephalon) after TUNEL assay (score = 9). Region of high apoptosis in the tail region of the neural tube is indicated with a dotted arrow, and the region of midbrain exencephaly is between the solid arrows.** *C*: **Relative apoptosis score (on a scale of 0 to 10) of embryos of saline-injected (n = 9) compared with glucose-injected (n = 4) pregnancies. A representative sampling of embryos was chosen at random for TUNEL assay from saline or glucose-injected pregnancies. *P < 0.05 compared with saline controls by Student's unpaired t test.**

haps, metabolism. How, exactly, elevated intracellular glucose could disturb gene expression is not known at present, but several intriguing possibilities could be considered. There is not likely to be a direct effect of glucose on gene expression, as in processes involved in regulation of glucose-responsive genes in the β-cell or the liver. Instead, indirect mechanisms are likely to be operational. For example, excess glucose transport could disturb transport or membrane incorporation of arachidonic acid or myo-inositol or utilization of the pentose phosphate shunt pathway, as observed by others (20,33, 38–40). Supplementation of the diets of pregnant diabetic rats with arachidonic acid and myo-inositol reduces malformations (41), suggesting that either altered lipid uptake or metabolism, secondary to increased glucose uptake, could trigger molecular processes that lead to altered expression of developmental control genes or else that increasing the pool size of lipid substrates reduces glucose flux, thereby preventing glucose-dependent inhibition of gene expression. Some of the pathways that have been implicated in other diabetic complications could also be operational in the embryo. For example, stimulation of protein kinase C-β (PKC-β) activity by high glucose has been shown to be important for processes, including altered gene expression, in the pathogenesis of diabetic complications affecting the retina, kidney, and vasculature (42–47). Conceivably, activation of PKC-β could interfere with signaling pathways that regulate morphogenesis. In addition, advanced glycation end products (AGEs), formed by the nonenzymatic reaction of aldoses with free amino groups of proteins, lipoproteins, and nucleic acids, are thought to contribute to the pathogenesis of diabetic complications affecting adult tissues (48–52). However, the rate of accumulation of nonenzymatically glycosylated proteins or nucleic acids is not likely to be sufficient to account for the dramatic and global inhibition of *Pax-3* expression that occurs within 24 h of hyperglycemia in the embryo. Similarly, nonenzymatic glycation of DNA has been shown to be mutagenic in vitro (52), and DNA mutations have been found to be associated with dysmorphogenesis in rodent models exposed to diabetic pregnancy or high-glucose culture (53,54). Nevertheless, it has not been demonstrated that the mutagenesis preceded the appearance of malformations or occurred in genes that direct the development of defective structures. Therefore, it is not clear whether the abnormalities in gene expression result from mutagenesis or impairment of transcriptional regulatory mechanisms.

Several studies have suggested that oxygen free radicals, which are increased in embryos of diabetic rats and in embryos cultured in high glucose, contribute to diabetic embryopathy and that antioxidants and free radical scavenging enzymes reduce the effects of diabetic embryopathy (55–59) (T.I.C., G.F., A.L. Hill, M.R.L., unpublished observations). Free radicals may be elevated as a result of increased or precocious oxidative metabolism or reduction of the endogenous pool of cellular antioxidants, including reduced glutathione, secondary to increased glucose utilization (21,59,61). There are several mechanisms by which elevated oxygen free radicals could activate transcriptional responses. For example, genes that are regulated by the NFκB transcription factor can be activated by oxidative stress (62–64). Free radicals, by altering the redox state of transcription factors, could alter their abilities to regulate transcription, as has been shown for factors such as p53, zinc finger proteins, and

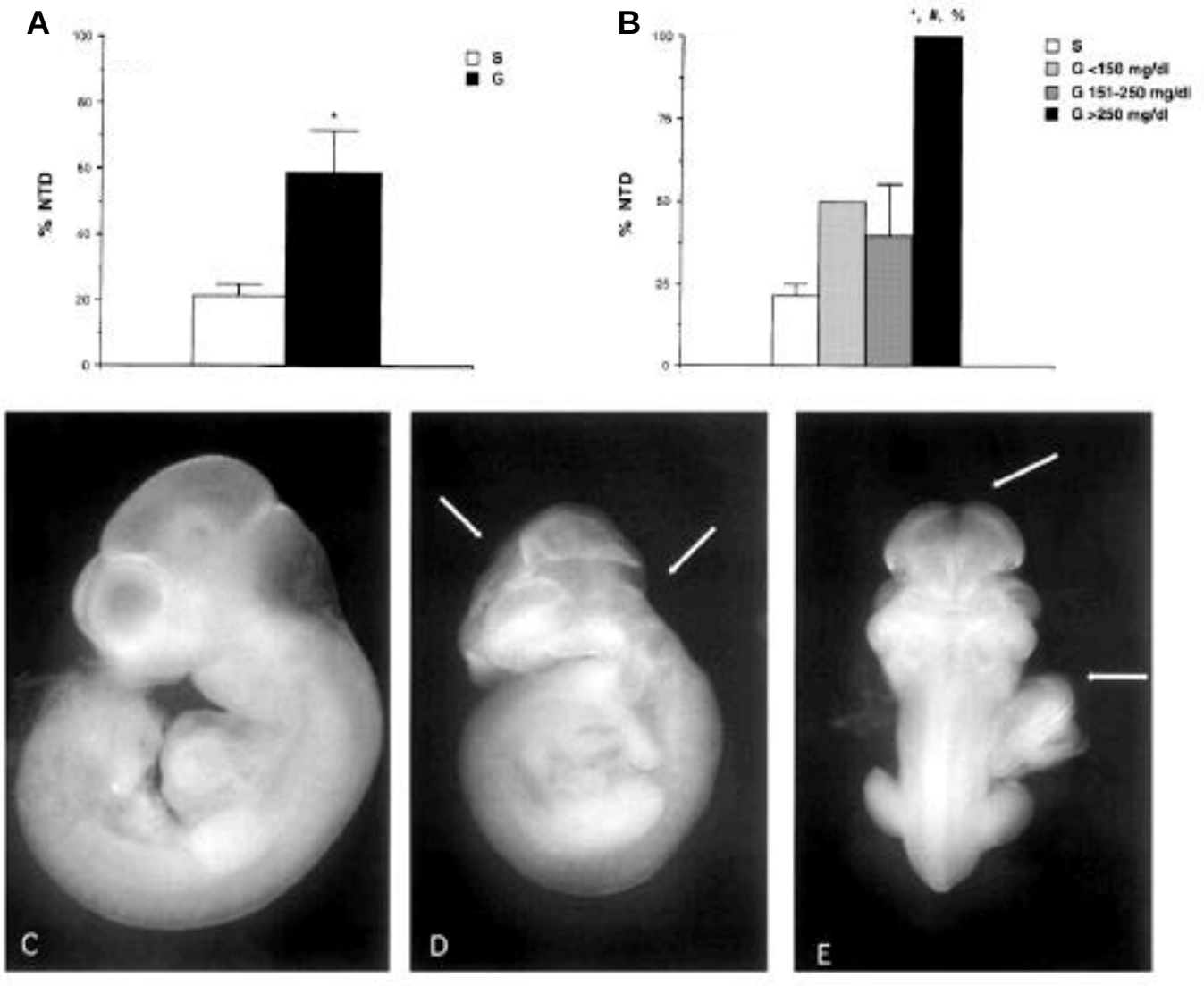

FIG. 4. ***A*****: Rate of NTDs in glucose-injected pregnancies. ****P* **< 0.01 vs. saline control by Student's unpaired** *t* **test.** ***B*****: Rate of NTDs in saline control pregnancies (***n* **= 11) compared with euglycemic glucose-injected (***n* **= 1), moderately hyperglycemic (***n* **= 6), and severely hyperglycemic (***n* **= 3) pregnancies. ****P* **< 0.002 vs. saline control; #***P* **< 0.05 vs. euglycemic glucose-injected pregnancy; %***P* **< 0.01 vs. moderately hyperglycemic glucose-injected pregnancy by analysis of variance and Duncan's multiple range test.** ***C*****: Normal day 10.5 embryo from saline-injected pregnancy.** ***D*****: Lateral view of day 10.5 embryo from glucose-injected pregnancy with open NTD (exencephaly, between arrows).** ***E*****: Dorsal view of embryo shown in** ***D*****.**

another Pax family member, Pax-8 (65–68). Alternatively, free radicals may activate hypoxia-inducible factors by causing a pseudohypoxic state (69–72). Although we have evidence that supports the results of others (55–59) that the antioxidant vitamin E reduces the rate of NTDs induced by diabetic pregnancy (T.I.C., G.F., A.L. Hill, M.R.L., unpublished observations), whether free radicals are embryopathic because they inhibit *Pax-3* expression has not been determined.

It is not yet clear how elevated glucose can affect expression of some genes, *Pax-3* for example, and genes that are regulated as part of a *Pax-3*-dependent process, such as *cdc46* and *Dep-1*, while other genes, such as *fibronectin* and *36B4*, are completely unaffected (10–12). An intriguing explanation is that glucose inhibits the induction of specific genes that must be switched on at specific times in development but does not attenuate the expression of genes that have already been activated. Thus, distinguishing between induction failure, rather than suppression of previously induced gene expression, will be important to characterize the mechanism by which glucose inhibits expression of *Pax-3*. Indeed, we have found that simply inducing hyperglycemia for a 10-h period on day 7.5, the day before the onset of *Pax-3* expression, is sufficient to significantly increase NTDs (data not shown). Because blood glucose levels in glucose-injected mice have returned to normal by day 8.5, these observations are consistent with a model in which glucose inhibits inductive processes that take place on day 7.5 of gestation and, furthermore, indicates that once the inhibition of *Pax-3* expression takes place, it cannot be reversed. Most certainly, identification of *Pax-3*–associated regulatory elements that mediate glucose transcriptional responses will allow determination of the molecular mechanism by which glucose inhibits *Pax-3* expression and, more importantly, suggest ways to prevent it.

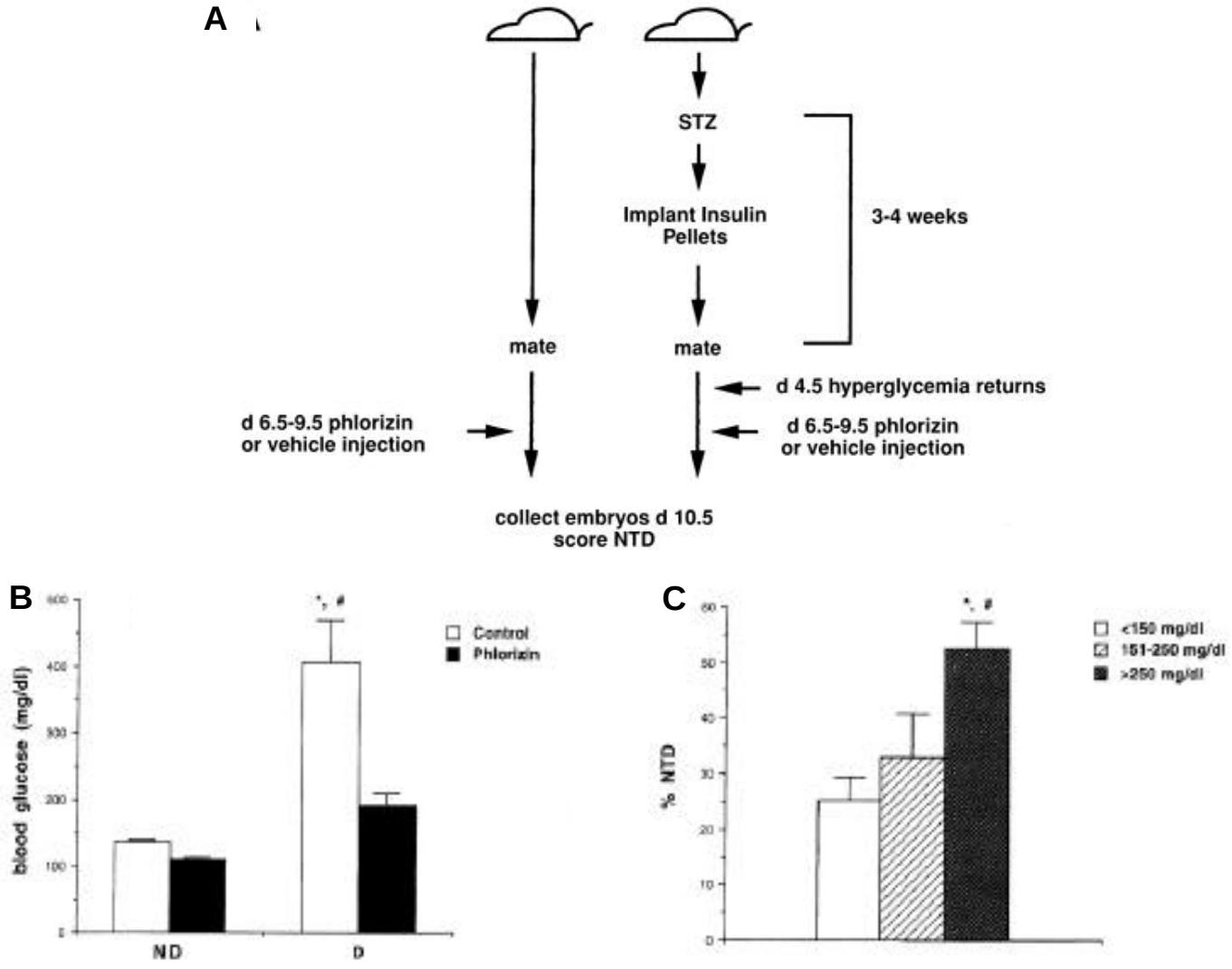

FIG. 5. Effect of phlorizin injection on blood glucose levels and NTDs. *A*: Experimental scheme for phlorizin treatment of pregnant diabetic and nondiabetic mice. *B*: Mean blood glucose levels of vehicle-injected nondiabetic (ND, $n = 3$), phlorizin-injected nondiabetic ($n = 4$), vehicle-injected diabetic (D, $n = 5$), or phlorizin-injected diabetic ($n = 4$) mice. Values represent the means ± SE of blood glucose measurements taken every 60–90 min during days 6.5–9.5 and before termination on day 10.5 of pregnancy. *$P < 0.001$ vs. nondiabetic pregnancies (control and phlorizin-injected). #$P < 0.005$ vs. phlorizin-injected diabetic pregnancies. *C*: Rate of NTDs in euglycemic nondiabetic pregnancies ($n = 6$) compared with moderately hyperglycemic phlorizin-treated diabetic (151–250 mg/dl, $n = 4$) and severely hyperglycemic untreated diabetic (>250 mg/dl, $n = 4$) pregnancies. *$P < 0.01$ vs. euglycemic pregnancies. #$P < 0.05$ vs. moderately hyperglycemic pregnancies. Statistical analyses were performed by analysis of variance and Duncan's multiple range test.

In conclusion, our data support a model in which elevated glucose levels alone are responsible for the molecular, cellular, and developmental defects in embryogenesis that have previously been observed in a mouse model of diabetic pregnancy. Investigation of the biochemical pathways by which glucose triggers transcriptional responses in embryo cells will provide increased understanding into the molecular mechanisms by which diabetic pregnancy causes congenital malformations.

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