

Hypoxic stress in diabetic pregnancy contributes to impaired embryo gene expression and defective development by inducing oxidative stress

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Li, Rulin, Martha Chase, Sung-Kwon Jung, Peter J. S. Smith, and Mary R. Loeken. Hypoxic stress in diabetic pregnancy contributes to impaired embryo gene expression and defective development by inducing oxidative stress. *Am J Physiol Endocrinol Metab* 289: E591–E599, 2005. First published May 31, 2005; doi:10.1152/ajpendo.00441.2004.—We have shown that neural tube defects (NTD) in a mouse model of diabetic embryopathy are associated with deficient expression of *Pax3*, a gene required for neural tube closure. Hyperglycemia-induced oxidative stress is responsible. Before organogenesis, the avascular embryo is physiologically hypoxic (2–5% O₂). Here we hypothesized that, because O₂ delivery is limited at this stage of development, excess glucose metabolism could accelerate the rate of O₂ consumption, thereby exacerbating the hypoxic state. Because hypoxia can increase mitochondrial superoxide production, excessive hypoxia may contribute to oxidative stress. To test this, we assayed O₂ flux, an indicator of O₂ availability, in embryos of glucose-injected hyperglycemic or saline-injected mice. O₂ flux was reduced by 30% in embryos of hyperglycemic mice. To test whether hypoxia replicates, and hyperoxia suppresses, the effects of maternal hyperglycemia, pregnant mice were housed in controlled O₂ chambers on embryonic day 7.5. Housing pregnant mice in 12% O₂, or induction of maternal hyperglycemia (>250 mg/dl), decreased *Pax3* expression fivefold, and increased NTD eightfold. Conversely, housing pregnant diabetic mice in 30% O₂ significantly suppressed the effect of maternal diabetes to increase NTD. These effects of hypoxia appear to be the result of increased production of mitochondrial superoxide, as indicated by assay of lipid peroxidation, reduced glutathione, and H₂O₂. Further support of this interpretation was the effect of antioxidants, which blocked the effects of maternal hypoxia, as well as hyperglycemia, on *Pax3* expression and NTD. These observations suggest that maternal hyperglycemia depletes O₂ in the embryo and that this contributes to oxidative stress and the adverse effects of maternal hyperglycemia on embryo development.

diabetic embryopathy; neural tube; hypoxia; *Pax3*

DIABETIC EMBRYOPATHY IS A COMPLICATION of diabetes in which the embryo of a diabetic mother develops congenital malformations. Diabetic pregnancy-induced malformations arise at the earliest stages of organogenesis, corresponding to approximately the first 8 wk of human gestation (36). Virtually any organ system is subject to malformation, but the neural tube and the heart are most commonly affected (4, 37). Offspring of women with either type 1 or type 2 diabetes are at risk for diabetic embryopathy (51), suggesting that hyperglycemia, and consequent increased glucose transport to the embryo, is responsible.

To understand the biochemical and molecular mechanisms by which maternal hyperglycemia leads to congenital malformations, we have employed a mouse model of diabetic pregnancy that leads to a significant increase in neural tube defects (NTD; see Ref. 41). We have shown that maternal diabetes induced by streptozotocin (STZ), or transient induction of hyperglycemia (≥ 13.9 mM) by glucose injection on embryonic day 7.5 leads to decreased expression of *Pax3* beginning on day 8.5 and increased incidence of NTD, which can be recognized by day 10.5 (20, 41). *Pax3* encodes a transcription factor that is expressed in neuroepithelium, neural crest, and somitic mesoderm (24). Homozygous *Splotch* embryos (*Sp/Sp*) carry loss of functional *Pax3* alleles and develop defects of the neural tube (exencephaly, spina bifida, or both), neural crest, and skeletal muscle with 100% penetrance (3, 7, 17, 23). They die by day 16.5 of gestation, resulting from defective migration of neural crest cells to the heart (13). Heterozygous *Sp/+* mice are viable, although they are recognizable by white patches of fur resulting from maldevelopment of neural crest-derived melanocytes. Because the exencephaly affecting embryos of diabetic mice and *Sp/Sp* embryos are morphologically quite similar, this suggests that deficient expression of *Pax3* phenocopies *Pax3* loss-of-function mutations. Undoubtedly, there are other genes in which expression could be inhibited by maternal diabetes and lead to defects of other organs or contribute to NTD. However, because *Pax3* is an absolutely essential gene for development, if its expression alone is reduced below a critical threshold, an NTD will occur. Therefore, investigating how *Pax3* is regulated by maternal diabetes is itself important, and may serve as a general model of how embryo gene expression is disturbed during diabetic pregnancy.

Many studies using rodent embryos have shown that reactive oxygen species are increased by maternal diabetes or high-glucose culture and that administration of antioxidants, or transgenic overexpression of Cu²⁺/Zn²⁺ superoxide dismutase, can prevent hyperglycemia-induced developmental defects (12, 18, 19, 25, 42, 46, 47, 54). We showed that oxidative stress, caused by maternal diabetes or administration of antimycin A (AA), a mitochondrial complex III inhibitor that increases superoxide production (52), inhibits *Pax3* expression and increases NTD (12). Increased oxidant status is likely to be complex, involving a combination of increased superoxide production as well as impaired free radical scavenging, and the pathways responsible for increased oxidant status have not been completely elucidated.

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The stage of early postimplantation development that is vulnerable for induction of NTD is just before the development of the circulatory system and coincides with a stage of development in which the embryo is relatively hypoxic (2–8% O₂, compared with 20% in maternal arterial circulation; see Refs. 21 and 44). Indeed, delivery of O₂ is diffusion limited, and the increasing mass of the embryo causes a state of physiological hypoxia that activates genes that induce formation of the circulatory system (1). Thus a slight increase in oxidative glucose metabolism resulting from increased glucose delivery to the embryo may accelerate O₂ utilization before establishment of the circulatory system can augment O₂ delivery. This could lead to a state of hypoxia in excess of the physiological hypoxia that is part of normal development. Hypoxia can stimulate mitochondrial production of O₂⁻ at complexes II and III (9–11, 38, 43). Thus it is possible that oxidative stress in the embryo might be the result of, at least in part, hypoxia-dependent superoxide production, as a consequence of O₂ depletion from increased glucose oxidation.

In this study, we tested the hypothesis that maternal hyperglycemia increases the hypoxic state of the embryo, leading to inhibited *Pax3* expression and increased NTD, and whether this was associated with increased oxidative stress.

MATERIALS AND METHODS

Induction of diabetes and transient hyperglycemia. A flow chart of animal procedures is shown in Fig. 1. Diabetes was induced in 5- to 6-wk-old female ICR mice (Taconic, Germantown, NY), as described previously (41), with 100 mg/kg STZ (Sigma, St. Louis, MO) dissolved in 10 mM sodium citrate (pH 4.5) and treated with subcutaneously implanted insulin pellets (Linshin, Scarborough, Ontario). Blood glucose was monitored daily with a Glucometer Elite (Bayer, Mishawaka, IN). STZ-diabetic mice maintained euglycemia with insulin pellets before pregnancy but developed hyperglycemia beginning on *day 4.5* of pregnancy, thereby exposing the embryo to hyperglycemia during the entire postimplantation period. Transient hyperglycemia was induced by subcutaneous injection of 12.5% glucose dissolved in PBS at approximately hourly intervals to maintain blood glucose ≥ 16.7 mmol/l, as described previously (20).

Controls were injected in parallel with PBS. Oxidative stress was induced on *day 7.5* with AA (Sigma Chemicals) dissolved in 25% (vol/vol) propylene glycol and administered at 3 mg/kg body wt at noon on *day 7.5*, as described previously (12). Glutathione (GSH) ethyl ester (EE; Sigma Chemicals) dissolved in PBS was administered at 1 mmol/kg, in three doses on *day 7.5* as described (28). Vitamin E-treated animals received chow supplemented with 0.125% (wt/wt) (+)- α -tocopherol succinate, which increased dietary intake of vitamin E by 21-fold, beginning on *day 0.5* of pregnancy as described (12). Mice were killed to recover embryos for assay of markers of oxidative stress on *day 7.5*, for assay of *Pax3* expression on *day 8.5*, or to score NTD on *day 10.5*. All procedures performed using animals that are described herein were approved by the Institutional Animal Care and Use Committees of the Joslin Diabetes Center and the Marine Biological Laboratory.

Recording embryo O₂ flux. Preparation of O₂ microsensors and O₂ flux measurement were performed as described previously (31, 33, 48, 49) and as diagramed in Fig. 2A. Briefly, the microsensor, fabricated from platinum wire inserted in a pulled glass capillary, was placed adjacent to the target tissue and then oscillated with a frequency of 0.3 Hz over a distance of 10 μ m. One-third of the data, collected during the movement of the probe and during the period of settlement after the movement, was discarded automatically. Comparison of the current from the near pole and far pole allows a differential measurement directly proportional to flux while simultaneously removing the background signal.

Experiments were conducted in a Faraday box mounted on an air table, incorporating a Zeiss Axioskop upright microscope. Temperature was held at 37°C. The motion controllers, temperature controller and amplifiers, as well as coordinating software (IonView) were products of the BioCurrents Research Center (Marine Biological Laboratory, Woods Hole, MA; www.mbl.edu/BioCurrents).

For recording O₂ flux in embryos on *day 7.5* of pregnancy, nondiabetic mice were injected with 12.5% glucose to induce hyperglycemia as described above and in Ref. 20. After 3 h of hyperglycemia, mice were killed, and decidua were immediately removed from uteri and dissected in 37°C PBS to expose yolk sac-enclosed embryos. The yolk sac was pierced over the head fold, and O₂ flux was recorded adjacent to the cranial neuroepithelium (Fig. 2B). Oxygen tension in the medium was atmospheric, giving rise to a concentration of 210 μ M.

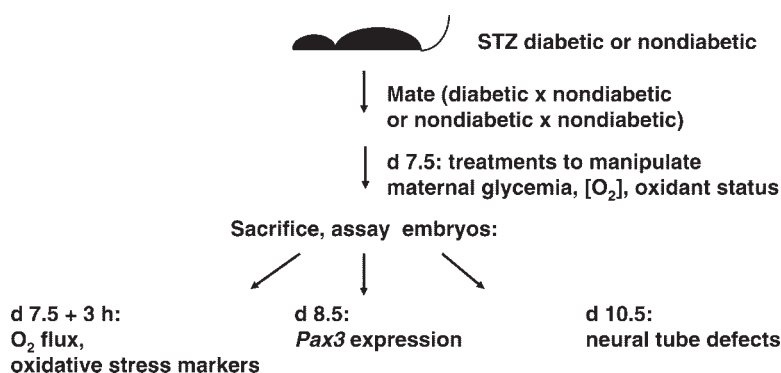


Fig. 1. Flow diagram of animal procedures as described in MATERIALS AND METHODS. For animals to be made diabetic, diabetes was induced with streptozotocin (STZ) about 3 wk before mating. Nondiabetic mice were either controls for the diabetic mice or were used for manipulation of glycemia, oxygenation, and/or oxidant status in the absence of diabetes. Mice were mated along with age-matched controls, and noon of the day on which a copulation plug was found was determined to be *day (d) 0.5*. Mice were maintained on control breeder chow, except for mice in which the effects of vitamin E were tested, which were given chow supplemented with 0.125% vitamin E succinate. On *day 7.5*, hyperglycemia was induced in some nondiabetic mice by subcutaneous glucose administration (controls were injected with saline); hypoxia was induced in nondiabetic mice by housing in a 12% O₂ chamber; hyperoxia was induced in diabetic and nondiabetic mice by housing in a 30% O₂ chamber; oxidative stress was induced in nondiabetic mice by administration of antimycin A; and the antioxidant glutathione (GSH) ethyl ester was administered to hyperglycemic, hypoxic, or saline-injected mice. Pregnant mice were killed for recovery of embryos at 1 of the following 3 time points: on *day 7.5* after 3 h of hyperglycemia, hypoxia, or oxidative stress to assay O₂ flux or markers of oxidative stress; on *day 8.5* to assay *Pax3* mRNA; or on *day 10.5* to score neural tube defects (NTD).

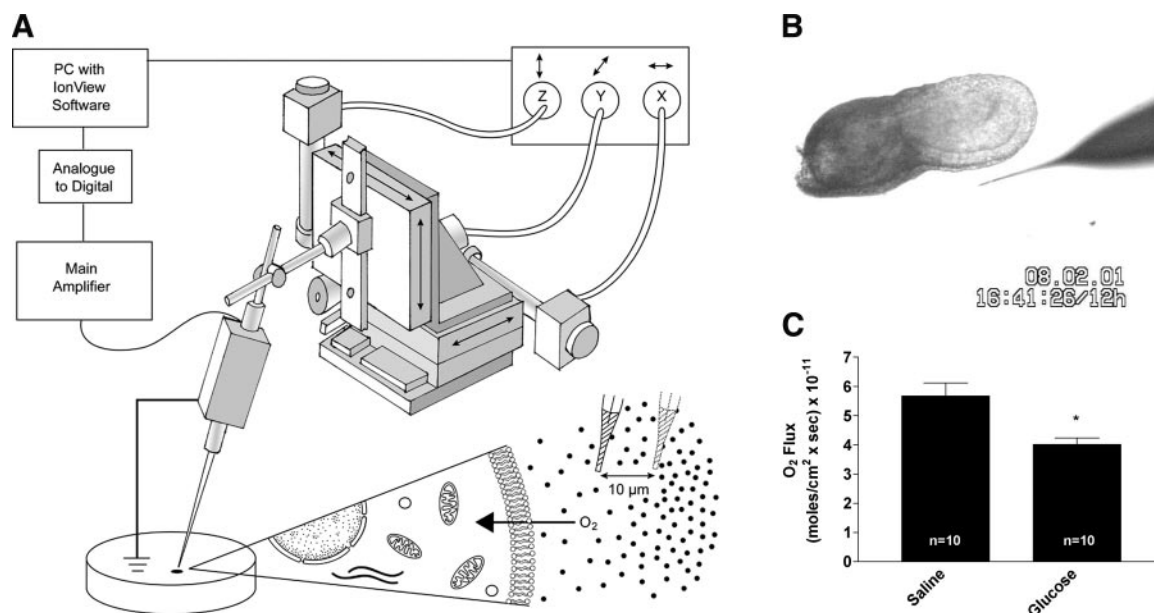


Fig. 2. A: diagram of O_2 flux recording. The computer [personal computer (PC) with IonView software] serves the following 2 functions: to control electrode movement and to record O_2 flux. Electrode movement is directed to cause the head stage to move the microelectrode back and forth within $10\ \mu\text{m}$ of the target tissue at a frequency of 0.3 Hz. The current generated by the O_2 gradient at the near pole (adjacent to the target tissue) and the far pole ($10\ \mu\text{m}$ from the target tissue) is transmitted through the main amplifier to the computer. B: image of *day 7.5* yolk sac-enclosed embryo next to microelectrode used to record O_2 flux. C: O_2 flux assayed in embryos of saline- or glucose-injected mice. * $P < 0.005$; n, no. of embryos assayed.

Hypoxic and hyperoxic exposure. For induction of hypoxia and hyperoxia, pregnant mice were housed on *day 7.5* in an OxyCycler chamber from Reming Bioinstruments (Redfield, NY), which is a semi-sealed, four-chamber Plexiglas incubator containing an O_2 sensor and a small, quiet fan inside to provide forced circulation and instant homogenization of gases. It is attached to a computer for programmable adjustment of chamber O_2 concentrations. To induce hypoxia, the chamber O_2 concentration is reduced to 12% in 2% increments over a 15-min period. To induce hyperoxia, the chamber O_2 concentration was increased to 30% within a 5-min period. Mice were exposed to hypoxia or hyperoxia for 3 h for experiments assaying markers of oxidative stress and 8 h for experiments assaying Pax3 mRNA or NTD.

Assay of malondialdehyde, GSH, and H_2O_2 . Embryos, including extraembryonic membranes, were recovered on *day 7.5*, and one-third of the embryos from each pregnancy was pooled and saved for assay of malondialdehyde (MDA), GSH, or H_2O_2 . MDA was assayed spectrophotometrically, as described previously (15), using purified MDA (Sigma Chemicals) to generate a standard curve. GSH and H_2O_2 were assayed using kits obtained from Cayman Chemicals (Ann Arbor, MI) according to the manufacturer's instructions. The GSH kit measures total GSH and oxidized GSH (GSSG); GSH was determined from the difference between total GSH and GSSG. MDA, GSH, and H_2O_2 were normalized to protein concentrations in cell extracts, which were assayed using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA).

Assay of Pax3 expression. Embryos from individual pregnancies were pooled, and RNA was prepared as described (40). Pax3 mRNA in each pregnancy was quantitated relative to one arbitrarily selected saline or nondiabetic/20% O_2 pregnancy, after normalization to rRNA, using real-time RT-PCR, as described previously (12).

Statistical analyses. Data were analyzed from a minimum of three pregnancies per treatment group by one-way ANOVA, followed by the Neuman-Keuls post hoc test, using GraphPad Prism software (San Diego, CA).

RESULTS

Maternal hyperglycemia suppresses aerobic respiration. Aerobic respiration depends on O_2 delivery. Thus, although glucose metabolism in the *day 7.5* mouse embryo is predominantly anaerobic (2), we hypothesized that an increase in glucose delivery to the embryo resulting from maternal hyperglycemia would transiently increase aerobic metabolism. Thus, if O_2 were consumed faster than it can be replenished, this would suppress aerobic metabolism by the embryo, leading to decreased O_2 flux. To test this, we assayed O_2 flux, which is an indicator of aerobic metabolism, in *day 7.5* embryos after 3 h of maternal hyperglycemia (≥ 16.7 mmol/l) using self-referencing O_2 microelectrodes (Fig. 2A). An image of a microelectrode next to a yolk sac-enclosed embryo is shown in Fig. 2B. As shown in Fig. 2C, O_2 flux was reduced significantly in embryos of hyperglycemic mice. This indicates that, consistent with the hypothesis, after 3 h of maternal hyperglycemia, aerobic respiration was suppressed, suggesting that a state of hypoxia in excess of that of normal *day 7.5* embryos was induced.

Maternal hypoxia replicates, and hyperoxia suppresses, the effects of maternal diabetes on Pax3 expression and NTD. If hyperglycemia inhibits Pax3 expression and increases NTD by inducing hypoxic stress in the embryo, then reducing O_2 delivery to embryos should replicate the effects of maternal hyperglycemia, and increasing O_2 delivery should block the effects of maternal diabetes.

To test whether reducing O_2 delivery to embryos replicates the adverse effects of hyperglycemia, pregnant mice were housed in a programmable O_2 chamber, ramping down from room air (20% O_2) to 12% O_2 on *day 7.5*. Mice were made hyperglycemic by glucose injection or injected with saline as

Table 1. Mean blood glucose levels of pregnancies in Fig. 3, A and B

	Saline	Glucose	Hypoxia
Day 7.5 blood glucose, mM	7.0±0.08	19.3±0.46*	7.1±0.13

Values are means ± SE. **P* < 0.001 vs. saline and hypoxia.

controls. Table 1 shows that there was no effect of maternal hypoxia on blood glucose concentrations, whereas glucose injection significantly increased maternal blood glucose concentrations. Nevertheless, maternal hypoxia, like maternal hyperglycemia, significantly inhibited *Pax3* expression and increased NTD (Fig. 3, A and B).

To test whether increasing O₂ delivery blocks the adverse effects of maternal diabetes, diabetic or nondiabetic mice were housed at 30% O₂ on day 7.5 of pregnancy. Control diabetic and nondiabetic mice were kept in room air (20% O₂). As shown in Table 2, hyperoxia had no effect on maternal blood glucose concentrations, which were significantly elevated in diabetic mice. Nevertheless, although hyperoxia had no effect on *Pax3* expression and NTD in embryos of nondiabetic mice, it blocked the inhibition of *Pax3* expression and NTD in embryos of diabetic mice (Fig. 3, C and D).

Hypoxic stress induces oxidative stress in embryos. We have previously shown that oxidative stress, induced by maternal hyperglycemia or the mitochondrial complex III inhibitor, AA, inhibits *Pax3* expression and induces NTD (12). Noting that hypoxic stress can stimulate superoxide production (9–11, 38, 43), we hypothesized that the adverse effects of hyperglycemia-induced hypoxic stress on *Pax3* expression and NTD could be mediated, at least in part, by oxidative stress. To test this hypothesis, we assayed markers of oxidative stress (MDA and GSH, as well as H₂O₂, the production of which is increased upon increased superoxide production) after 3 h of maternal exposure to 12% O₂. As shown in Fig. 4A, MDA, a

Table 2. Mean blood glucose levels of pregnancies in Fig. 3, C and D

	ND/Normoxia	ND/Hyperoxia	D/Normoxia	D/Hyperoxia
Day 7.5 blood glucose, mM	7.2±0.084	7.1±0.131	30.1±0.833*	29.6±1.060*

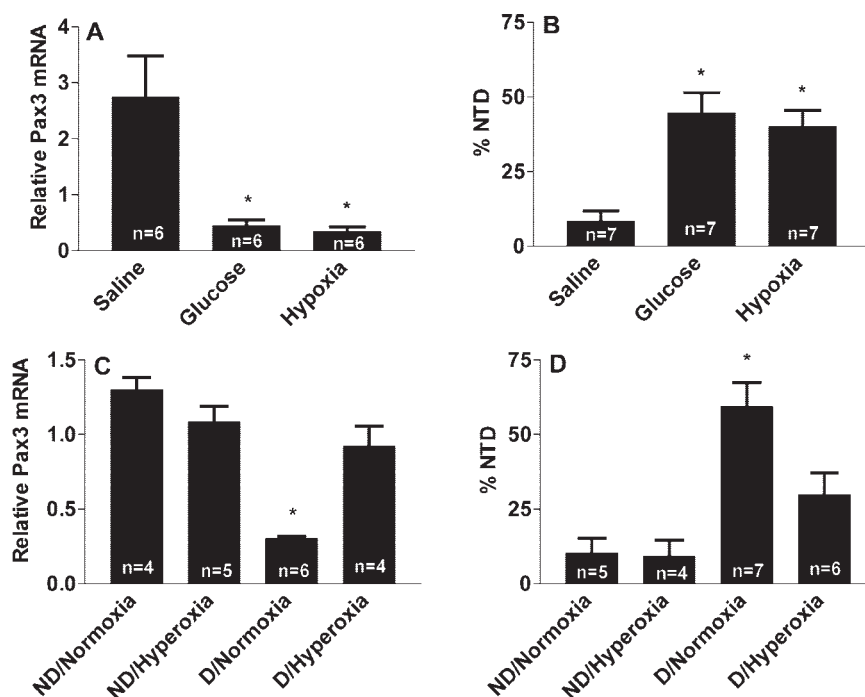
Values are means ± SE. ND, nondiabetic; D, diabetic. **P* < 0.001 vs. ND.

marker of lipid peroxidation, was significantly increased by maternal hypoxia to the same extent that it was increased by hyperglycemia or AA. Another indicator of oxidative stress, GSH, was significantly decreased by hypoxia to the same extent that it was decreased by hyperglycemia or AA (Fig. 4B). H₂O₂ was increased equally by hypoxia, hyperglycemia, and AA (Fig. 4C).

Conversely, we tested whether increasing O₂ delivery to embryos blocks oxidative stress induced by maternal diabetes. There was no effect of hyperoxia (30% O₂) on MDA in nondiabetic pregnancies, but the increase in MDA observed in diabetic pregnancies was significantly reduced by hyperoxia, although it was still significantly increased over MDA in nondiabetic pregnancies exposed to either normoxia or hyperoxia (Fig. 4D). Similarly, there was no effect of hyperoxia on GSH, but the decrease in GSH induced by maternal diabetes was significantly reduced by hyperoxia, although GSH was still significantly reduced in embryos of diabetic mice exposed to hyperoxia compared with embryos of nondiabetic mice exposed to either normoxia or hyperoxia (Fig. 4E). The increase in H₂O₂ observed in diabetic pregnancies was significantly suppressed by hyperoxia, although it remained significantly increased compared with nondiabetic pregnancies exposed to either normoxia or hyperoxia (Fig. 4F).

Antioxidant administration blocks the adverse effects of embryo hypoxia on neural tube development. To further test the hypothesis that hypoxic stress induces oxidative stress,

Fig. 3. Effects of hypoxia or hyperoxia on *Pax3* expression and NTD. A and B: pregnant mice were housed at 12% O₂ on day 7.5 of pregnancy or injected with glucose to induce hyperglycemia or saline as a control, as described in MATERIALS AND METHODS. C and D: pregnant diabetic (D) or nondiabetic (ND) mice were housed in room air (~20% O₂, normoxia) or at 30% O₂ on day 7.5, as described in MATERIALS AND METHODS. Embryos were recovered on day 8.5 to assay relative *Pax3* expression by RT-PCR (A and C), or on day 10.5 to score NTD (B and D). A: **P* < 0.01 vs. saline. B: **P* < 0.0005 vs. saline. C: **P* < 0.001 vs. all other treatment groups. D: **P* < 0.01 vs. all other treatment groups; n, no. of pregnancies assayed.



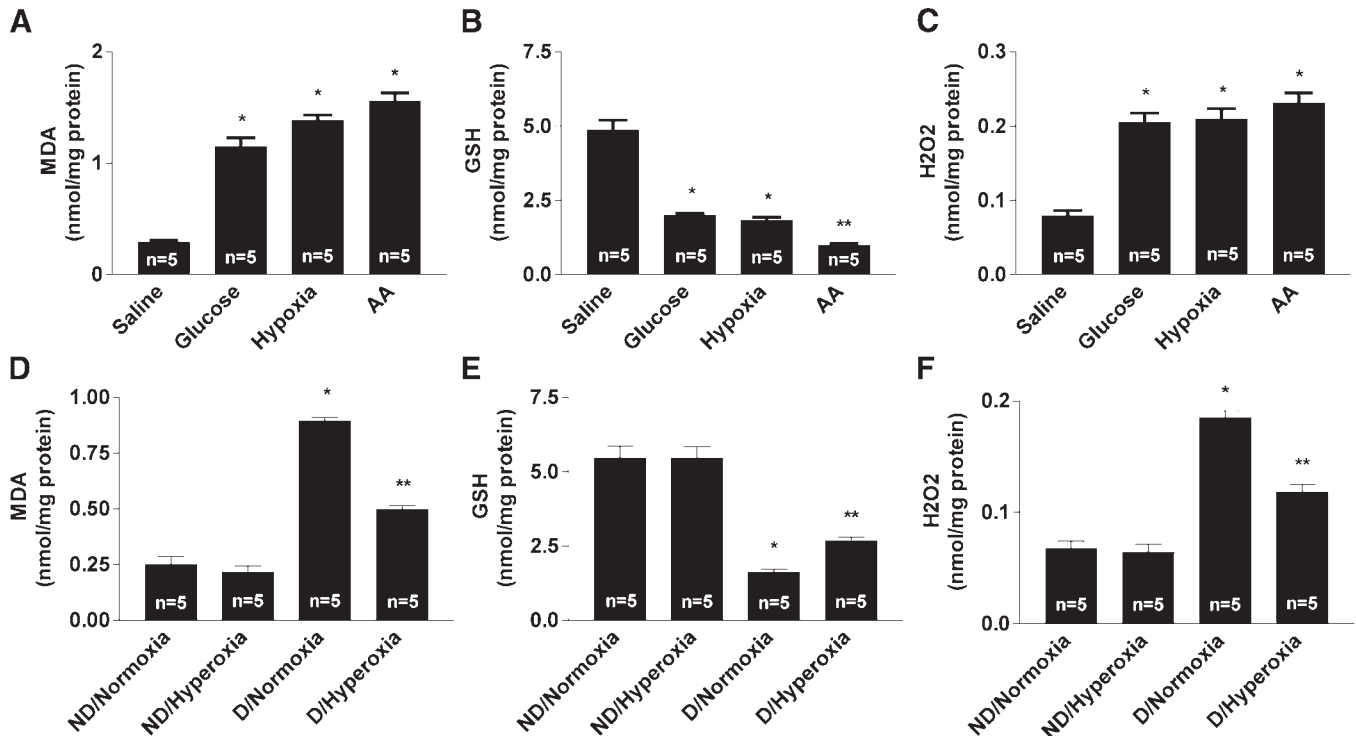


Fig. 4. Malondialdehyde (MDA), reduced GSH, and H₂O₂ in pregnancies treated as in Fig. 3. Antimycin A (AA) was administered as a control for stimulation of H₂O₂ production. A: **P* < 0.001 vs. saline. B: **P* < 0.001 vs. saline; ***P* < 0.001 vs. saline, glucose, and hypoxia. C: **P* < 0.001 vs. saline. D: **P* < 0.001 vs. ND (normoxia and hyperoxia); ***P* < 0.001 vs. all other treatment groups. E: **P* < 0.001 vs. ND (normoxia and hyperoxia); ***P* < 0.001 vs. all other treatment groups. F: **P* < 0.001 vs. ND (normoxia and hyperoxia); ***P* < 0.001 vs. all other treatment groups.

which thereby mediates the adverse effects of maternal hyperglycemia, the membrane-soluble GSH analog GSH-EE was administered on *day 7.5* to mice that were housed at 12% O₂, or to glucose- or saline-injected mice as controls. Noting that opening chamber door had the potential to restore normoxia during the three times that GSH-EE was administered to animals housed in the O₂ chamber, the effect of opening the door without GSH-EE administration was also tested as a control.

As shown in Table 3, only glucose-injected mice were hyperglycemic, and GSH-EE had no effect on glycemia in any treatment group. In contrast, the inhibition of *Pax3* expression induced by hypoxia or hyperglycemia was prevented by coincident administration of GSH-EE (Fig. 5A). This effect could not be attributed to transient normoxia during the time the OxyCycler chamber doors were opened to administer GSH-EE, since *Pax3* expression was still reduced in embryos of mice exposed to hypoxia during *day 7.5* when the chamber doors were opened for times comparable for injection of GSH-EE. Correspondingly, the increase in NTD induced by hypoxia or hyperglycemia was prevented by GSH-EE, and this cannot be explained by transient normoxia during administration of GSH-EE (Fig. 5B).

To further test whether the adverse effects of hyperglycemia-induced hypoxia are the result of oxidative stress, the effects of another antioxidant were tested. Vitamin E succinate, which we previously showed prevented the adverse effects of maternal diabetes on *Pax3* expression and NTD (12), was administered by dietary supplementation beginning on *day 0.5* of pregnancy. As shown in Fig. 5, C and D, supplemental vitamin E prevented the inhibition in *Pax3* expression and the increase in NTD induced by hypoxia and hyperglycemia but had no effect on glycemia (Table 4).

DISCUSSION

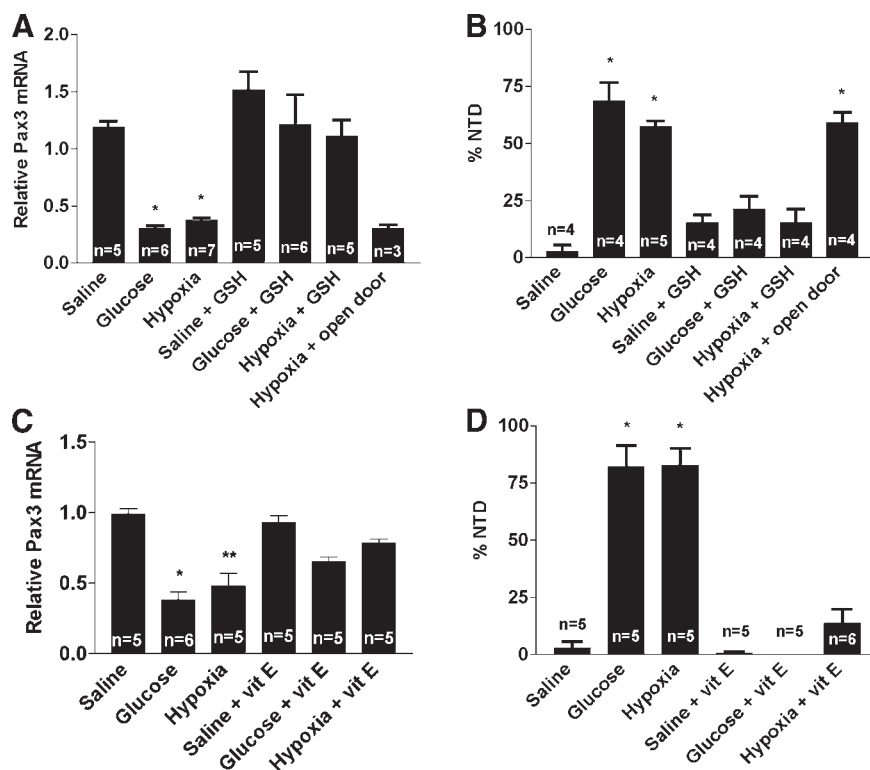
There is much evidence implicating oxidative stress in the pathogenesis of diabetic embryopathy (12, 18, 19, 25, 42, 46, 47, 54). More recently, we have shown that oxidative stress causes diabetic pregnancy-induced NTD because it disrupts expression of at least one gene (*Pax3*), which is crucial for neural tube development (12). However, the possibility that maternal hyperglycemia could cause excessive hypoxia in the embryo, and that this could be a source of oxidative stress, has not been reported previously.

Table 3. Mean blood glucose levels of pregnancies in Fig. 5, A and B

	Saline	Glucose	Hypoxia	Saline + GSH	Glucose + GSH	Hypoxia + GSH	Hypoxia + Open Door
Day 7.5 blood glucose, mM	7.4±0.09	18.6±0.35*	7.6±0.17	7.6±0.12	18.4±0.28*	7.1±0.11	7.5±0.17

Values are means ± SE. GSH, glutathione. **P* < 0.001 vs. all saline and hypoxia groups.

Fig. 5. Effects of GSH ethyl ester (EE) or vitamin (vit) E succinate on *Pax3* expression or NTD induced by maternal hypoxia. Pregnant mice were housed at 12% O₂ or made hyperglycemic by glucose injection on day 7.5 as in Fig. 3, A and B. Controls were injected with saline. GSH-EE was administered 3 times during day 7.5 (A and B), or pregnant dams were fed control chow or chow supplemented with vitamin E succinate from day 0.5 (C and D). As a control for transient normoxia while administering GSH to animals housed at 12% O₂, the OxyCycler chamber door was left open for some hypoxia-exposed dams for the same duration as required to administer GSH (Hypoxia + open door). A: **P* < 0.001 vs. saline, saline + GSH, glucose + GSH, or hypoxia + GSH. B: **P* < 0.001 vs. saline, saline + GSH, glucose + GSH, or hypoxia + GSH. C: **P* < 0.001 vs. saline and saline + vit E, *P* < 0.01 vs. glucose + vit E; ***P* < 0.001 vs. saline and saline + vit E, *P* < 0.01 vs. hypoxia + vit E. D: **P* < 0.001 vs. saline, saline + vit E, glucose + vit E, and hypoxia + vit E.



Physiological hypoxia, caused by the increasing mass of the avascular early postimplantation embryo, plays a critical role during normal embryogenesis. As the embryo, which is already located in a relatively hypoxic environment (21, 44), increases in mass, O₂ delivery becomes increasingly diffusion limited. This physiological hypoxia activates the Hif-1 α /ARNT heterodimeric transcription factor to induce expression of genes that trigger formation of blood islands in the yolk sac (1, 22, 30). The primitive circulatory system of the embryo proper is also dependent on hypoxia-responsive transcription, since embryos lacking *EPAS-1*, which is hypoxia inducible, fail to develop a normal heart and circulatory system (50). Thus, in response to normal growth of the embryo, diffusion-limited O₂ delivery induces physiological hypoxia, and this activates expression of genes whose products induce hematopoiesis and structures forming the circulatory system, thereby increasing O₂ delivery to embryonic tissues and reducing the hypoxic state.

Increased O₂ delivery may be critical for the development of organ systems in which primordia appear just after this hypoxia-responsive period, such as the neural tube, because embryos that lack *Hif-1 α* , *ARNT*, *VEGF*, or sufficient gene copy numbers of *p300* and/or *CBP*, which encode coactivators of Hif-1 α /ARNT-dependent transcription, develop NTD (6, 32, 34, 50, 57). Thus failure to increase O₂ delivery at this stage of development may impair activation of genes needed

for formation of the neural tube. Conversely, increased O₂ delivery may be a developmental signal of metabolic fitness to support further development, which induces expression of certain genes that control critical developmental programs.

In the rat embryo, only 1.2% of glucose is metabolized oxidatively on day 10 (corresponding to approximately day 8 in the mouse), but it increases to 8.3% on day 11 (2). The increase in aerobic metabolism is made possible by increased O₂ delivery from establishment of the primitive yolk sac circulation. This period of development coincides with the stage of development that is sensitive to maternal hyperglycemia (20). As shown here, 3 h of maternal hyperglycemia on day 7.5 decreases O₂ flux. This suggests that increased glucose metabolism, which acutely increases O₂ consumption, leads to relative O₂ depletion, thereby inhibiting oxidative metabolism and suppressing O₂ flux. It is also possible that decreased O₂ flux could be the result of mitochondrial damage. Indeed, it has been suggested that exposure of rat embryo or fetal neural tissues to 50 mM glucose or maternal diabetes can suppress oxidative metabolism, perhaps because of the Crabtree effect, in which increased glucose metabolism leads to diminished supply of ADP, which, by inducing state 4 respiration, increases the production of superoxide (8, 14, 56). Another possibility is that hyperglycemia-induced increased ratio of NADH to NAD⁺ could cause a "pseudohypoxic" state, despite normal O₂ tension, which has been postulated to be associated

Table 4. Mean blood glucose levels of pregnancies in Fig. 5, C and D

	Saline	Glucose	Hypoxia	Saline + Vit E	Glucose + Vit E	Hypoxia + Vit E
Day 7.5 blood glucose, mM	7.2 \pm 0.07	18.9 \pm 0.4*	7.1 \pm 0.13	7.3 \pm 0.05	19.8 \pm 0.26*	7.4 \pm 0.19

Values are means \pm SE. Vit, vitamin. **P* < 0.001 vs. all saline and hypoxia groups.

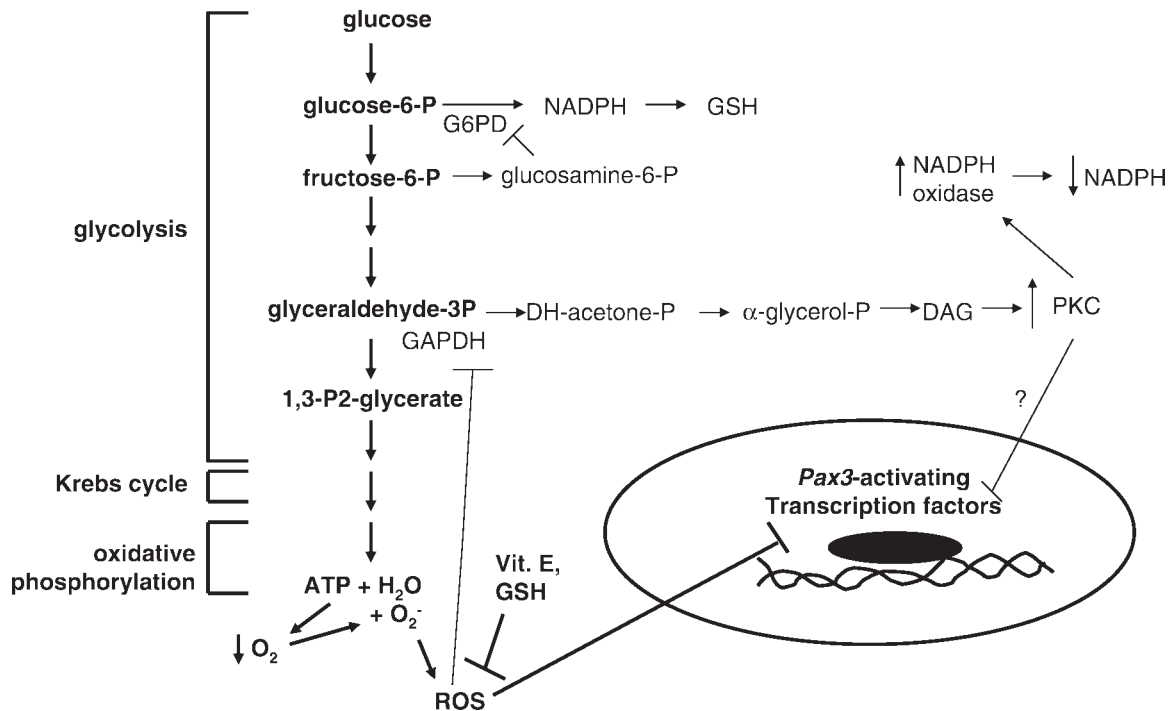


Fig. 6. Flow diagram of pathways affected by excess glucose in the embryo leading to impaired *Pax3* expression. During diabetic pregnancy, increased glucose transport from maternal circulation increases glucose metabolism by the embryo. Increased oxidative metabolism increases O₂ consumption; however, because O₂ cannot be replaced as quickly as it is consumed, this will lead to decreased O₂ availability. Excessive hypoxia increases superoxide and H₂O₂ production, thereby increasing reactive oxygen species (ROS), which inhibit *Pax3* expression by a yet unidentified mechanism. The effects of oxidative stress can be blocked by GSH precursors and vitamin E. Other studies have shown that oxidative stress inhibits GAPDH activity and that accumulation of glycolytic intermediates stimulate the diacylglycerol (DAG)/protein kinase C (PKC) and hexosamine flux pathways. Activation of PKC activity can stimulate NADPH oxidase activity, leading to decreased production of reduced GSH (although whether PKC regulates *Pax3* expression is not known). Activation of the hexosamine flux pathway inhibits the pentose shunt pathway, thereby decreasing NADPH and reduced GSH production. P, phosphate; G6PD, glucose-6-phosphate dehydrogenase; DH, dihydroxy; P₂, biphospho.

with diabetic vascular complications (55). Although our results are not inconsistent with either a Crabtree or pseudo-hypoxic effect, the fact that the adverse effects of maternal diabetes could be prevented by transient hyperoxia would suggest that they are not the result of irreversible mitochondrial damage and that limiting availability of O₂ mediates some of the adverse effects of maternal hyperglycemia. Thus our findings suggest that excess glucose stimulates mitochondrial O₂ utilization, leading to a state of hypoxia in excess of that of the normal *day* 7.5 embryo.

The data reported here support the hypothesis that hyperglycemia-induced O₂ depletion contributes to the adverse effects of maternal diabetes on the embryo. Specifically, when O₂ depletion was experimentally induced by restricting O₂ delivery to pregnant mice on *day* 7.5, all of the responses induced by maternal hyperglycemia (decreased *Pax3* expression, increased NTD and markers of oxidative stress) were observed. Conversely, when increased O₂ consumption induced by maternal diabetes was experimentally compensated by increasing O₂ delivery to pregnant diabetic mice on *day* 7.5, all of the responses of maternal diabetes were diminished. This suggests that excessive embryo hypoxia caused by increased glucose metabolism suppresses the increase in O₂ availability that would normally occur at this stage of development. Consequently, the processes leading to induction of *Pax3*, which may depend on increased O₂ delivery, are impaired. A further implication of these findings is that if, during normal develop-

ment, induction of the blood or circulatory systems in response to physiological hypoxia fails, for example, because of deficient activation of Hif-1 α - or Epas-1-dependent transcription, oxidative stress, leading to insufficient expression of *Pax3*, would result. This could explain the NTD that arise in such animal models.

In considering mechanisms by which glucose-induced excess hypoxic stress might inhibit *Pax3* expression and increase NTD, we considered several mechanisms, including activation of Hif-1 α -dependent transcription (39, 45) or stimulation of AMP kinase activity (27, 35). These pathways notwithstanding, noting that hyperglycemia-induced oxidative stress inhibits *Pax3* expression (12) and that hypoxia has the potential to increase superoxide production (9–11, 38, 43), we tested whether oxidant status was increased by hypoxia. Remarkably, all measures of oxidative stress (MDA, GSH, and H₂O₂) were equally affected by hypoxia and by hyperglycemia, and these markers of oxidative stress were equivalently attenuated by hyperoxia in embryos of diabetic mice.

Additional evidence that the adverse effects of glucose-induced hypoxic stress are mediated by oxidative stress was that the adverse effects of hypoxia were prevented by GSH-EE. Because GSH could have effects independent of its antioxidant properties, we tested another antioxidant, vitamin E, which we previously showed to inhibit the adverse effects of maternal diabetes on *Pax3* expression and NTD (12). Like GSH-EE, vitamin E also prevented the decrease in *Pax3* expression and

the increase in NTD induced by hypoxia or by hyperglycemia, suggesting that the adverse effects of hyperglycemia, leading to hypoxic stress, are mediated by superoxide-generated oxidative stress.

It is interesting to note that hyperoxia did not increase markers of oxidant status or inhibit *Pax3* expression in nondiabetic pregnancies. This suggests that, at this stage of development, maternal exposure to 30% O₂ does not increase superoxide production if glucose delivery to the embryo does not also increase. In diabetic pregnancies, markers of oxidative stress were significantly improved by hyperoxia. Even though oxidant status was not completely normalized in diabetic pregnancies by hyperoxia, it was apparently corrected sufficiently to prevent inhibition of *Pax3* expression.

The multiple biochemical pathways affected by high glucose are complex (Fig. 6). For example, we have shown that the diacylglycerol (DAG)/protein kinase C (PKC) pathway is activated in embryos of diabetic or hyperglycemic mice (26), which could contribute to oxidative stress by activation of NADPH oxidase activity (29), and that stimulation of the hexosamine flux pathway by hyperglycemia causes oxidative stress and inhibits *Pax3* expression through depletion of GSH (28). These effects may actually be secondary to hypoxia-induced superoxide production. Du et al. (16) have shown that increased mitochondrial superoxide production inhibits glyceraldehyde-3-PO₄ dehydrogenase (GAPDH) activity, which causes glycolytic intermediates to accumulate. The glycolytic intermediates, glyceraldehyde-3-PO₄ and fructose-6-PO₄, stimulate synthesis of DAG and glucosamine-6-PO₄, respectively (5, 16). In support of this model in diabetic pregnancy, inhibition of GAPDH has been observed in rat embryos exposed to a hyperglycemic environment (53). Thus the data presented here, in which maternal hyperglycemia of ~19 mmol/l, or 12% O₂, caused the same quantitative changes in markers of oxidant status, *Pax3* expression, and NTD, suggest that many of the effects of hyperglycemia result from O₂ depletion-induced oxidative stress. Further research will be necessary to determine whether the activation of PKC and hexosamine pathways is solely the result of increased mitochondrial superoxide production and inhibition of GAPDH activity and exactly how oxidative stress leads to inhibition of *Pax3* expression.

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