Developmental Damage, Increased Lipid Peroxidation, Diminished Cyclooxygenase-2 Gene Expression, and Lowered Prostaglandin E_2 Levels in Rat Embryos Exposed to a Diabetic Environment

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Previous experimental studies suggest that diabetic embryopathy is associated with an excess of radical oxygen species (ROS), as well as with a disturbance of prostaglandin (PG) metabolism. We aimed to investigate the relationship between these pathways and used hyperglycemia in vitro (embryo culture for 24–48 h) and maternal diabetes in vivo to affect embryonic development. Subsequently, we assessed lipid peroxidation and gene expression of cyclooxygenase (COX)-1 and -2 and measured the concentration of prostaglandin E_2 (PGE_2) in embryos and membranes. Both hyperglycemia in vitro and maternal diabetes in vivo caused embryonic dysmorphogenesis and increased embryonic levels of 8-epi-$PGF_{2\alpha}$, an indicator of lipid peroxidation. Addition of *N*-acetylcysteine (NAC) to the culture medium normalized the morphology and 8-epi-$PGF_{2\alpha}$ concentration of the embryos exposed to high glucose. Neither hyperglycemia nor diabetes altered COX-1 expression, but embryonic COX-2 expression was diminished on gestational day 10. The PGE_2 concentration of day 10 embryos and membranes was decreased after exposure to high glucose in vitro or diabetes in vivo. In vitro addition of NAC to high glucose cultures largely rectified morphology and restored PGE_2 concentration, but without normalizing the COX-2 expression in embryos and membranes. Hyperglycemia/diabetes–induced downregulation of embryonic COX-2 gene expression may be a primary event in diabetic embryopathy, leading to lowered PGE_2 levels and dysmorphogenesis. Antioxidant treatment does not prevent the decrease in COX-2 mRNA levels but restores PGE_2 concentrations, suggesting that diabetes-induced oxidative stress aggravates the loss of COX-2 activity. This may explain in part the antiteratogenic effect of antioxidant treatment. *Diabetes* 48:813–820, 1999

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Received for publication 29 July 1998 and accepted in revised form 20 December 1998.

ANOVA, analysis of variance; COX, cyclooxygenase; GSH, reduced glutathione; IL, interleukin; MD, manifest diabetes; NAC, *N*-acetylcysteine; PG, prostaglandin; PGE_2, prostaglandin E_2; ROS, radical oxygen species; RT-PCR, reverse transcriptase–polymerase chain reaction; SOD, superoxide dismutase; TNF, tumor necrosis factor.

Maternal diabetes leads to a higher frequency of congenital malformations in offspring than in offspring of nondiabetic mothers (1,2). The teratological processes in diabetic pregnancy are not completely understood (3). In recent years, however, a putative excess of radical oxygen species (ROS) has been observed in studies during which diabetes-induced embryopathy was blocked by antioxidants in vitro (4–8) and in vivo (8–13). The direct demonstration of ROS production has been controversial, in both short-term incubations of embryonic cells (14–17) and in vivo measurements (12). In the present study, we decided to assess possible embryonic oxidative stress by measuring the concentration of the F_2-isoprostane 8-epi-$PGF_{2\alpha}$, a prostaglandin-like compound generated by ROS-derived oxidation of arachidonic acid in cellular membranes (18). The isoprostanes are believed to be formed in situ in membrane phospholipids by ROS oxidation and subsequently cleaved off by phospholipase A_2 (19). Elevated levels of isoprostanes have been detected in different states of oxidative stress (20–22), including diabetes (23). To our knowledge, no studies of isoprostane levels in embryos of normal or diabetic mothers have been reported in the literature.

Earlier studies have implicated diabetes-induced alterations of several metabolites as having teratological capacity, among them arachidonic acid (7,24,25) and prostaglandins, in particular prostaglandin E_2 (PGE_2) (7,26,27). Addition of these compounds to embryos exposed to a diabetes-like environment in vitro or in vivo diminishes embryonic dysmorphogenesis (7,24–27). Embryonic PGE_2 may play a particularly important role in the developmental processes leading to neural tube closure. In mouse embryos, PGE_2 concentration is high during the gestational period when the neural groove is formed and immediately before neural tube closure is initiated (28). PGE_2 levels subsequently diminish during the neurulation process. If embryonic development is disturbed by maternal diabetes, the PGE_2 concentration decreases below the normal level, in particular in the early neurulation embryos, which subsequently show disturbed neural tube closure (28).

The constitutive cyclooxygenase (COX)-1 has a physiologic function as a housekeeping enzyme with small alterations in

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activity despite varied exogenous conditions. Changes on the order of two- to fourfold stimulation of COX-1 have been recorded in response to hormones and growth factors (29). For instance, activation of COX-1 leads to production of prostacyclin as an antithrombogenic factor by the endothelium (30) and to cytoprotection of the gastric mucosa (31). An inducible cyclooxygenase, COX-2, was discovered some years ago as an isoform of COX-1 (32,33), showing 60% homology between the amino acid sequences. A large number of agents induce COX-2 activity, including cytokines, growth factors, and transcription factors (34–36). In addition, there appears to be a specific link between COX-2 induction (via increased gene expression) and increased PGE_2 concentration in a number of different tissues. For instance, interleukin (IL)-1β treatment of rheumatoid arthritis synoviocytes increases COX-2 gene expression and cytoplasmic phospholipase A_2 levels (mRNA and protein) and also enhances the intracellular PGE_2 concentration (35). Furthermore, IL-1β and tumor necrosis factor (TNF)-α stimulate PGE_2 production in lung fibroblasts by transcriptional stimulation of COX-2 (but not of COX-1) (37). Mitogenic stimulation of cell lines yields a large increase in PGE_2 release that parallels the induction of COX-2 (38). This release can be blocked by specific COX-2 inhibitors or by antisense oligonucleotides to COX-2 (but not to COX-1) (39). The causative relationship between induced COX-2 gene expression and increased PGE_2 concentration also seems to include an increased activity of the cytoplasmic phospholipase A_2, possibly acting as a mediator of the necessary arachidonic acid to COX-2, but not to COX-1 (35).

One mechanism for diminished PGE_2 production in the embryo, therefore, may be decreased COX-2 activity (35,39), constituting a possible link between the functional state of this enzyme and embryonic dysmorphogenesis.

The association between diabetes-induced developmental perturbations and the accompanying biochemical changes in the embryo has not been fully characterized. We have recently shown that high glucose levels and COX inhibitors cause severe embryonic damage, and that addition of arachidonic acid or PGE_2, as well as the antioxidative agents superoxide dismutase (SOD) and *N*-acetylcysteine (NAC), normalize embryonic development (7). These findings suggest that the ROS excess–related and arachidonic acid deficiency–related teratological pathways are linked, a relationship to be investigated in greater detail in the present study.

We subjected rat embryos either to a diabetes-like environment in vitro or to maternal diabetes in vivo. In whole embryo culture, we exposed day 9 embryos to low (10 mmol/l) and high (30 mmol/l) glucose concentrations with or without the scavenger NAC for 24 and 48 h before morphologic and biochemical evaluation. Embryos of corresponding age—day 10 and 11 of gestation—from normal and diabetic rats were also evaluated with respect to F_2-isoprostane concentration, COX-1 and COX-2 gene expression, and PGE_2 levels.

The aim of the study was to examine whether hyperglycemia in vitro or diabetes in vivo causes changes in lipid peroxidation, COX expression, and PGE_2 levels in exposed embryos, and whether treatment with an antioxidant, the reduced glutathione (GSH) precursor NAC, can alter any of these parameters.

RESEARCH DESIGN AND METHODS

Animals. Rats from a local Sprague-Dawley–derived outbred strain were used as embryo donors. This strain has been shown to exhibit increased incidence of skeletal malformations in diabetic pregnancy (9,12,13). All rats were fed a commercial pelleted diet (AB Analysen, Lidköping, Sweden) and had free access to food and tap water. They were maintained at an ambient temperature of 22°C with a 12-h light/dark cycle. Manifest diabetes (MD) was induced in some female rats by an intravenous injection of streptozotocin (40 mg/kg) in the tail vein, and the MD state was confirmed by a serum glucose level >20 mmol/l 1 week after the injection. Normal and MD female rats were caged together with nondiabetic male rats during the night, and the following morning was designated gestational day 0 if the vaginal smear was positive.

Diabetic pregnancy. The pregnant MD females and some pregnant normal control females were killed on gestational day 10 or 11. The embryos were dissected out, morphologically evaluated, and processed for F_2-isoprostane measurement, COX-1 and COX-2 gene expression, or PGE_2 measurement, as described below.

Preparation of embryo culture serum. Male retired breeders, weighing 400–450 g, were used for serum preparation. They were anesthetized with ether and laparotomized, and blood was collected from the abdominal aorta and centrifuged immediately (40). The resulting serum was supplemented with sodium benzylpenicillinate and streptomycin to give final concentrations of 60 and 100 mg/l, respectively. The serum was stored frozen at –70°C until used. On the day of culture, the serum was thawed and heat-inactivated at 56°C for 45 min immediately before use.

Whole embryo culture. On gestational day 9, the pregnant rats were killed by cervical dislocation between 12:00 and 5:00 P.M. The conceptuses (embryo and yolk sac) were dissected out and subjected to whole embryo culture for 24 or 48 h, as previously described (4). Briefly, the conceptuses were explanted, and culture of whole embryos was performed according to New (40). The embryos, within their intact yolk sacs, were maintained in polypropylene tubes (Falcon 2070; Becton Dickinson, Lincoln Park, NJ) in a roller incubator at 38°C and 60 revolutions/min. Each tube contained four to five conceptuses in 5 ml culture medium consisting of 80% serum from normal rats (see above) and 20% saline. The glucose concentration of culture medium was adjusted to 10 or 30 mmol/l (10G or 30G) by the addition of a sterile solution of 1.67 mol/l D-glucose, and the medium was changed after 24 h. To some culture tubes 0.5 mmol/l NAC (Sigma, St. Louis, MO) and 200 µmol/l indomethacin (Sigma) was added.

At the start of the experiment and after 24 and 40 h of culture, the culture tubes were gassed with different $N_2/O_2/CO_2$ gas mixtures, as previously described (4). After 48 h of culture, the embryos were harvested, dissected out of their yolk sacs, and morphologically evaluated. Under a stereo microscope, the crown-rump length, somite number, and malformation score (no malformation = 0, minor malformation = 1, less severe malformation = 5, severe malformation = 10) were determined by one examiner who was not aware of the culture conditions to which the embryos had been exposed. Subsequently, the embryos were hydrolyzed in 1 ml Ultraspec RNA (Biotecx Laboratories, Houston, TX) for subsequent reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of COX-1 and COX-2 or snap-frozen in groups of four to five embryos in liquid nitrogen for subsequent PGE_2 analysis, which was usually performed within 1 week after sampling.

Measurement of 8-epi-$PGF_{2\alpha}$. The estimations of 8-epi-$PGF_{2\alpha}$ were largely performed in accordance with the instructions from the manufacturer, Cayman Chemical (Ann Arbor, MI). Each embryo and yolk sac was separately homogenized in 500 µl of 0.5 mol/l NaOH on ice, then 2 ml ethanol was added, and the sample was vortexed, stored on ice for 5 min, and centrifuged at 3,000*g* for 10 min. The precipitate was used for protein determination according to Lowry et al. (41). The supernatant was transferred to a new tube, and 2.5 ml of 15% KOH was added. The samples were incubated at 40°C for 1 h. Thereafter 5 ml Ultra Pure water (Milli-Q Plus; Millipore AB, Stockholm, Sweden) was added, and the pH was lowered below 4.0 with concentrated HCl. The sample was passed through a C-18 Reverse Phase Cartridge (Supelclean LC-18 SPE Tubes; Supelco, Bellefonta, PA) previously activated by rinsing with 5 ml methanol followed by 5 ml Ultra Pure water. The cartridge was rinsed with 5 ml Ultra Pure water followed by 5 ml hexane (high-performance liquid chromatography [HPLC]-graded, Sigma), and the absorbed 8-epi-$PGF_{2\alpha}$ was subsequently eluted from the column with 5 ml ethyl acetate containing 1% methanol (Sigma). The ethyl acetate was then evaporated from the 8-epi-$PGF_{2\alpha}$ samples in a vacuum centrifuge (SpeedVac SVC 100), and the samples were analyzed in a spectrophotometric plate reader (iEMS, Helsinki, Finland) at 412 nm wavelength.

RT-PCR analysis of COX-1 and COX-2. Embryos cultured (24–48 h) in vitro, as previously described, and in vivo embryos from normal and MD rats on gestational days 10 or 11 were used to determine the levels of COX-mRNA by RT-PCR. Total cellular RNA in embryos and yolk sacs was isolated with the aid of Ultraspec RNA Isolation System (Biotecx Laboratories). One microgram of total RNA was used for reverse transcription. First-strand cDNA synthesis used first-strand beads (Ready To Go; Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions. After incubation at 37°C for 60 min, the reaction was terminated by heating to 95°C for 5 min. The resulting cDNA was diluted threefold with diethylenepyrocarbonate (DEPC)-treated water. One microliter of the cDNA was amplified in a final volume of 50 µl buffer containing 2.5 mmol/l $MgCl_2$,

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0.2 mmol/l dNTP, 25 U/ml Amp-Taq Gold, and 5 µg/ml of the sense and antisense primers (Pharmacia Biotech). The sequences of the COX-1 primers were from Lee et al. (38): sense, 5′-TGCATGTGGCTGTGGATGTCATCAA-3′, and antisense, 5′-CACTAAGACAGACCCGTCATCTCCA-3′. The COX-2 primers were sense, 5′-ACTTGCTCACTTTGTTGAGTCATTC-3′, and antisense, 5′-TTTGATTAG TACTGTAGGGTTAATG-3′, the latter from Lee et al. (38). We used the following actin primers: sense, 5′-CTGACCGAGCGTGGCTAC-3′, and antisense, 5′-CCT GCTTGCTGATCCAGTCTGCTGG-3′. The mix was subsequently amplified; COX-1 used 33 cycles with annealing temperature 55°C, COX-2 used 38 cycles with annealing temperature 50°C, and actin used 22 cycles with annealing temperature 55°C in a OmniGene Thermal Cycler (Hybaid, Teddington, Middlesex, U.K.). The PCR products were run in a 1% agarose gel, stained with ethidium bromide (5 µg/ml), photographed under ultraviolet light (UVP, San Gabriel, CA), and densitometrically analyzed in a Kodak Digital Science Electrophoresis, Documentation, and Analysis System for Macintosh (Kodak AB, Stockholm, Sweden). For each set of primers, a number of PCR amplifications with different cycle numbers were tested, and the cycle number yielding a linear signal was subsequently chosen.

Measurement of PGE_2. The estimations of PGE_2 were largely performed in accordance with the manufacturer's instructions (Cayman Chemical). Briefly, each embryo and yolk sac was separately homogenized in 500 µl of 0.5 mol/l NaOH, then 2 ml methanol was added. The sample was vortexed, stored on ice for 5 min, and centrifuged at 3,000*g* for 10 min. The supernatant was transferred to a new tube, whereas the precipitate was used for protein determination according to Lowry et al. (41). Phosphate buffer (8 ml), pH 4.0, was added to the supernatant, the sample was vortexed and passed through a C-18 Reverse Phase Cartridge (Supelclean LC-18 SPE Tubes; Supelco) previously activated by rinsing with 5 ml methanol followed by 5 ml Ultra Pure water (Milli-Q Plus; Millipore AB). The cartridge was rinsed with 5 ml Ultra Pure water followed by 5 ml hexane (HPLC-graded, Sigma). The absorbed PGE_2 was subsequently eluted from the column with 5 ml ethyl acetate containing 1% methanol (Sigma). The ethyl acetate was evaporated from the PGE_2 samples in a vacuum centrifuge (SpeedVac SVC 100), and the samples were analyzed in a spectrophotometric plate reader (iEMS) at 412 nm wavelength.

Ethical and statistical evaluation. The research protocol including all experimental procedures involving animals was approved by the Animal Ethical Committee of the Medical Faculty of University of Uppsala. The comparisons between different experimental groups were based on individual embryos. Differences between means were evaluated by analysis of variance (ANOVA), where the applied test was Fisher's protected least significant difference (PLSD) at the 95% significance level (42), or with χ^2 statistics, whichever method was applicable (43). A value of $P < 0.05$ was considered to denote a statistically significant difference between means, whereas comparisons yielding $0.05 < P < 0.1$ are denoted "trend" in the text.

RESULTS

Morphologic outcome. The high glucose concentration in vitro and the diabetic condition in vivo caused severe changes in embryonic development (Table 1). After 24 h of culture in high glucose (30G) between days 9 and 10 in vitro, both somite number and crown-rump length were decreased in the embryos. These decreases remained during the second 24-h period of culture (Table 1). Addition of NAC to the culture medium normalized the somite number and crown-rump length in embryos after both 24 and 48 h of culture. The MD embryos showed decreased somite number and shorter crown-rump length compared with the normal embryos on both gestational days 10 and 11 (Table 1). Comparing the in vitro and in vivo development of the embryos showed that 10G-cultured embryos after 24 h had slightly fewer somites ($P < 0.05$) and smaller crown-rump length ($P < 0.05$) than normal embryos on gestational day 10. The difference in crown-rump length between 10G and normal embryos ($P < 0.05$) remained 24 h later (Table 1). In contrast, there was no difference between 30G embryos after 24 h culture and MD embryos on gestational day 10, whereas the 30G embryos were markedly smaller 24 h later compared with MD embryos on gestational day 11, with respect to both somite number and crown-rump length ($P < 0.001$).

F_2-isoprostane concentration. The 8-epi-$PGF_{2\alpha}$ concentration was increased in embryos and membranes exposed to 30 mmol/l glucose in vitro, and addition of NAC normalized the 8-epi-$PGF_{2\alpha}$ levels in both embryos and membranes (Fig. 1). In vivo, however, diabetes also increased 8-epi-$PGF_{2\alpha}$ levels in embryos, but only tended to enhance the 8-epi-$PGF_{2\alpha}$ concentration in the membranes ($0.05 < P < 0.10$), presumably due to large SEMs of the two membrane groups (Fig. 1).

TABLE 1
Morphological outcome in embryos

	n	Somites	Crown-rump length
In vitro			
Day 10 (24-h culture)			
10G	17	18.8 ± 0.4	2.02 ± 0.04
30G	17	12.6 ± 0.7*	1.40 ± 0.05*
30G + NAC	13	16.5 ± 0.7†	1.72 ± 0.06†
Day 11 (48-h culture)			
10G	11	29.3 ± 0.3	3.87 ± 0.07
30G	10	16.4 ± 1.3*	2.44 ± 0.19*
30G + NAC	7	26.4 ± 1.2†	3.41 ± 0.20†
In vivo			
Day 10			
Normal	17	21.1 ± 0.9	2.48 ± 0.15
MD	16	12.9 ± 1.4*	1.66 ± 0.15*
Day 11			
Normal	11	31.5 ± 0.3	4.32 ± 0.04
MD	9	25.7 ± 1.7*	3.53 ± 0.29*

Data are means ± SE. *$P < 0.05$ vs. 10G or normal on same gestational day; †$P < 0.05$ vs. 30G on same gestational day (ANOVA).

COX-1 and COX-2 gene expression. COX-1 expression did not differ between the cultured embryos and membranes, regardless of treatment and duration of culture (Table 2). In vivo, the COX-1 expression of the normal and MD offspring did not differ between groups on the same gestational day, but there was a decrease in COX-1 expression from day 10 to day 11 in all groups ($P < 0.05$, Table 2).

COX-2 gene expression tended to decrease in embryos and membranes cultured in 10 mmol/l glucose from day 10 to day 11 (Table 3). This decrease was more pronounced in normal embryos and membranes between days 10 and 11. The percentage of embryos with no RT-PCR–detectable COX-2 expression (denoted "n_0") increased from 30 to 90% in embryos exposed to 30 mmol/l glucose for 24 h, and a similar trend was noted for the corresponding membranes (10G vs. 30G, 38 vs. 86%, $P = 0.0572$, χ^2 statistics). Addition of NAC to the high glucose culture medium did not alter the high rate of zero expression of COX-2, 78 and 100% in the embryos and membranes, respectively (Table 3). The expression of the COX-2 gene, given as densitometer units, numerically followed the zero expression pattern, but the large SEMs of the groups precluded any statistical differences. After a further 24 h of culture, there was no difference between the groups with respect to COX-2 expression in embryos or membranes (Table 3).

The embryos from normal and MD mothers showed a pattern of expression for COX-2 similar to those of the embryos cultured in vitro. Thus, in the MD embryos and membranes, the zero expression (nondetectable) percentage increased compared with the normal tissues on gestational day 10 (from 29 to 78% in embryos and from 38 to 100% in membranes, Table 3), and the densitometric values reflected those changes. In the day 11 embryos and membranes, we did not detect any COX-2 expression at all (Table 3).

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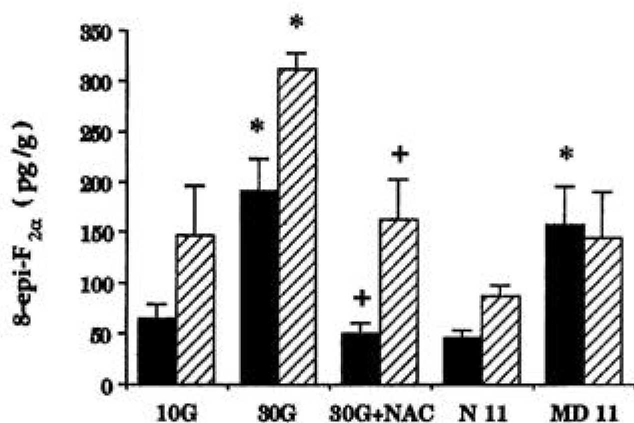

FIG. 1. Concentration (pg/g) of 8-epi-$PGF_{2\alpha}$ in day 9 rat embryos (■) and membranes (▨) exposed to 10 or 30 mmol/l glucose for 48 h in vitro (10G or 30G), with the possible addition of 0.5 mmol/l NAC (30G + NAC), or in embryos of normal (N) or manifestly diabetes (MD) rats in vivo on gestational day 11; 3 = n = 8. Tissue 8-epi-$PGF_{2\alpha}$ concentration is displayed as mean + SE. *P < 0.05 vs. 10G or N on same gestational day; +P < 0.05 vs. 30G on same gestational day (ANOVA).

TABLE 2
COX-1 expression in embryos and membranes

	Embryo		Membranes	
	n	COX-1	n	COX-1
In vitro				
Day 10 (24-h culture)				
10G	10	0.76 ± 0.09	8	0.56 ± 0.16
30G	10	0.62 ± 0.11	7	0.73 ± 0.12
30G + NAC	9	0.52 ± 0.15	5	0.58 ± 0.23
Day 11 (48-h culture)				
10G	4	0.73 ± 0.16	4	1.42 ± 0.42
30G	4	0.96 ± 0.27	4	0.72 ± 0.09
30G + NAC	2	0.57 ± 0.28	2	0.48 ± 0.08
In vivo				
Day 10				
Normal	14	0.83 ± 0.15	8	1.18 ± 0.36
MD	9	1.29 ± 0.42	7	1.40 ± 0.45
Day 11				
Normal	14	0.33 ± 0.04	11	0.21 ± 0.05
MD	15	0.58 ± 0.11	12	0.48 ± 0.18

Data are means ± SE.

PGE_2 concentration. The concentration of PGE_2 in embryos and membranes showed a distribution with clear resemblance to the COX-2 expression pattern (Table 4). Thus, the PGE_2 concentration decreased from day 10 to day 11 in embryos and membranes exposed to 10 mmol/l glucose in vitro, as well as in normal embryos. Furthermore, increasing the glucose concentration from 10 to 30 mmol/l decreased PGE_2 concentration in embryos and tended to do the same in membranes (0.05 < P < 0.1). Addition of NAC had a pronounced effect: the PGE_2 concentration of the embryos and membranes in the 30G + NAC group reverted back to the 10G levels with an excess in the membranes (from 0.42 to 1.07 pg/µg protein) and a similar trend in the embryos (from 0.42 to 1.02 pg/µg protein; 0.05 < P < 0.1) (Table 4). The PGE_2 levels were generally lower after 48-h culture compared with 24-h culture, but there were no differences between the groups.

In vivo, we found a similar pattern to the in vitro distribution of COX-2 and PGE_2 values. Thus, maternal diabetes decreased PGE_2 concentration in the embryos and membranes on gestational day 10. Likewise, on day 11, the PGE_2 levels were all low, and similar, in all the normal and MD groups (Table 4).

Effect of indomethacin and NAC addition. The 10G cultures with 200 µmol/l indomethacin added showed pronounced dysmorphogenesis, after both 24 and 48 h of culture, a dysmorphogenic effect markedly diminished with the addition of 0.5 mmol/l NAC (data not shown), which is in line with our previous studies of the teratogenicity of COX inhibitors (7). The PGE_2 levels in the embryos and membranes showed a marked decrease after 24-h culture, a decrease rectified by NAC treatment (Fig. 2). A similar pattern could be discerned in the PGE_2 values after 48-h culture, but the large SEs of the data precluded detection of any firm statistical differences.

DISCUSSION

Two pivotal findings were made in the present study. High glucose concentration in vitro and diabetes in vivo causes a selective decrease in COX-2 gene expression and yields an increased concentration of an F_2-isoprostane compound, 8-epi-$PGF_{2\alpha}$. Both of these observations may prove to be of importance for understanding the etiology of diabetic embryopathy.

The gene expression of the constitutive cyclooxygenase enzyme, COX-1, was not altered in embryonic tissue by exposure to hyperglycemia in vitro or diabetic environment in vivo, as was expected due to the constitutive nature of this enzyme (29). In contrast, the expression of the inducible enzyme, COX-2, was decreased by both conditions during the time period between gestational days 9 and 10. This decrease leads to decreased embryonic PGE_2 levels during this critical time of neural tube closure, a fact earlier demonstrated in a mouse model in vivo (28) and in human yolk sacs from diabetic pregnancies (44), but not previously seen as a result of hyperglycemia in vitro.

The fundamental question, how a diabetes-like environment specifically prevents the expression of the COX-2 gene, is not easily answered. In previous studies, ROS excess has mostly been associated with increased COX-2 expression, and, as outlined below, the F_2-isoprostane data indicate the opposite in hyperglycemia/diabetes-exposed embryos—that is, ROS excess in conjunction with lowered PGE_2 levels. Of the known endogenous stimulators of COX-2 gene expression—IL-1β (35,36), TNF-α, and the transcription factors NF-κB, NF-IL-6 (34), and cyclic AMP responsive element binding protein (CREBP)—none has been found to exhibit lowered concentration in embryos exposed to hyperglycemia/diabetes. Likewise, the known inhibitors of COX-2 gene expression—glucocorticoids (36) and IL-4 (45)—have not been shown to display increased levels in diabetic pregnancy. Speculatively, however, since the promoter area of the COX-2 gene has binding sites for NF-κB, a lowered NF-κB activity in embryos exposed to hyperglycemia/diabetes (N.W., H. Forsberg, U.J.E., unpublished observations) may explain the diminished COX-2 expression in the present investigation, but the nature of such a decrease has to await further studies.

Increased F_2-isoprostane (8-epi-$PGF_{2\alpha}$) concentration in embryonic tissue exposed to hyperglycemia in vitro and dia-

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TABLE 3
COX-2 expression in embryos and membranes

	Embryo			Membranes		
	n	n_0 (%)	COX-2	*n*	n_0 (%)	COX-2
In vitro						
Day 10 (24-h culture)						
10G	10	3 (30)	0.23 ± 0.07	8	3 (38)	0.33 ± 0.13
30G	10	9 (90)*	0.02 ± 0.02	7	6 (86)	0.04 ± 0.04
30G + NAC	9	7 (78)*	0.13 ± 0.09	5	5 (100)*	0.00 ± 0.00
Day 11 (48-h culture)						
10G	4	1 (25)	0.13 ± 0.04	4	2 (50)	0.15 ± 0.09
30G	4	2 (50)	0.28 ± 0.19	4	3 (75)	0.18 ± 0.18
30G + NAC	2	1 (50)	0.05 ± 0.05	2	0 (0)	0.26 ± 0.09
In vivo						
Day 10						
Normal	14	4 (29)	0.33 ± 0.12	8	3 (38)	0.65 ± 0.37
MD	9	7 (78)*	0.06 ± 0.05	7	7 (100)*	0.00 ± 0.00
Day 11						
Normal	14	14 (100)	0.00 ± 0.00	11	11 (100)	0.00 ± 0.00
MD	15	15 (100)	0.00 ± 0.00	12	12 (100)	0.00 ± 0.00

Data are means ± SE. "n_0 (%)" denotes the number and percentage of observations with zero COX-2 expression. *$P < 0.05$ vs. 10G or normal on same gestational day (χ^2 statistics).

betes in vivo was also seen. This finding indicates an embryonic increase in lipid peroxidation rate (19), an increase that can be blocked by antioxidative treatment with NAC. These results are in line with recent reports of increased serum F_2-isoprostane levels (46) and increased electron spin clearance rate (47) in diabetic rats, indicative of oxidative stress, and normalized by vitamin E treatment. The present result contributes to the ongoing discussion regarding the oxidative status of dispersed embryonic cells. After exposure to high glucose in vitro and diabetes in vivo, dispersed embryonic cells showed no (15,17) and some (14) evidence of ROS excess, whereas other studies have yielded indirect evidence of embryonic ROS production and ROS excess in a diabetes-like environment, at least in neuroepithelial cells (8,16,48). We found previously that day-11 embryos of diabetic rats showed no clear increase of the thiobarbituric acid–reactive species (TBARS) levels, although their α-tocopherol tissue concentration was decreased, indicative of oxidative stress (12). Against this background of ambiguous results, we elected to make use of the sensitive and specific assay of the F_2-isoprostanes as an indicator of oxygen-radical–generated lipid peroxidation (18). Our present finding demonstrates an increased accumulation of embryonic lipid peroxidation products in intact embryos after 48 h of high glucose exposure or 11 days of diabetic intrauterine development. This increase suggests that long-term exposure to high glucose cre-

TABLE 4
PGE_2 concentration in embryos and membranes

	Embryo		Membranes	
	n	PGE_2 (pg/μg protein)	*n*	PGE_2 (pg/μg protein)
In vitro				
Day 10 (24-h culture)				
10G	6	0.85 ± 0.08	6	0.63 ± 0.16
30G	6	0.42 ± 0.06*	6	0.42 ± 0.06
30G+NAC	8	1.02 ± 0.11†	8	1.17 ± 0.10*†
Day 11 (48-h culture)				
10G	4	0.13 ± 0.05	3	0.33 ± 0.20
30G	5	0.11 ± 0.02	5	0.29 ± 0.08
30G+NAC	5	0.10 ± 0.02	5	0.23 ± 0.04
In vivo				
Day 10				
Normal	5	0.97 ± 0.13	6	0.88 ± 0.13
MD	8	0.44 ± 0.07*	8	0.37 ± 0.03*
Day 11				
Normal	7	0.07 ± 0.01	7	0.35 ± 0.09
MD	7	0.08 ± 0.01	7	0.29 ± 0.06

Data are means ± SE. *$P < 0.05$ vs. 10G or normal on same gestational day; †$P < 0.05$ vs. 30G on same gestational day (ANOVA).

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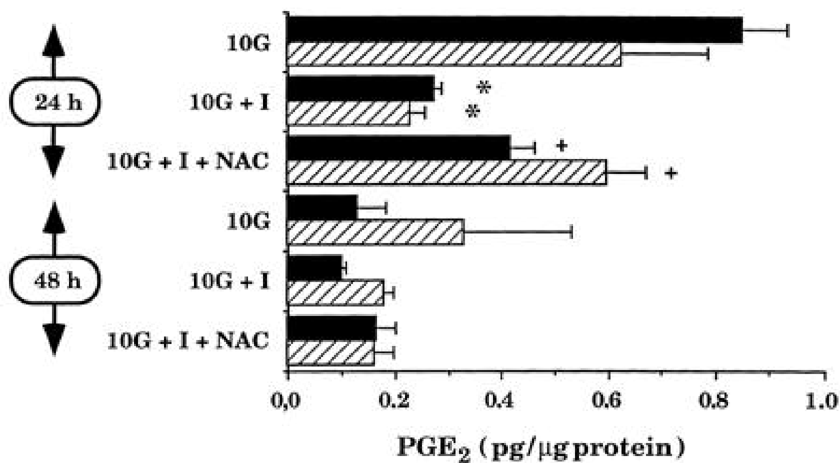

FIG. 2. PGE_2 concentration (pg/µg protein) in day 9 rat embryos (■) and membranes (▨) exposed to 10 mmol/l glucose (10G) for 24 h or 48 h in vitro, with the possible addition of 10 µmol/l indomethacin (10G + I) and 0.5 mmol/l NAC (10G + I + NAC); 4 = n = 6. PGE_2 concentration is displayed as mean + SE. *P < 0.05 vs. 10G on same culture day; +P < 0.05 vs. 10G+I on same gestational day (ANOVA).

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ates an embryonic ROS excess either from increased ROS production (16) or from diminished antioxidant defense capacity (14). Such an excess of ROS may be rather small and restricted to particular cell populations, and may vary with gestational time and nutritional status. For all of these reasons, direct ROS estimations are difficult; nevertheless, a relative ROS excess should subsequently yield an accumulation of lipid peroxides in the embryonic cells, as detected by the F_2-isoprostane assay.

The beneficial effect of NAC addition to the high glucose culture was clearly not dependent on a change in COX-2 expression but may rather be due to an enhancement of the fraction of PGH_2 transformed into PGE_2, since GSH is a cofactor for this particular prostaglandin pathway (Fig. 3). This is supported by the present results and those of previous studies of antioxidant-mediated diminution of COX-inhibitor–induced teratogenicity and lowered PGE_2 concentration (Fig. 2). This notion is also supported by previous findings of lowered PGE_2 levels in endothelial cells (49) and fibroblasts (50) exposed to glutathione depletion and oxidative stress, respectively, thereby suggesting a specific sensitivity of the PGE_2 concentration to the oxidative status of the cell.

The finding of a hampered COX-2 expression in embryos and membranes subjected to high glucose in vitro and diabetes in vivo clarifies several earlier findings in the diabetic embryopathy research field. Previous studies in vitro have shown that addition of arachidonic acid to the culture medium diminishes the dysmorphogenesis elicited by high glucose concentration (7,24,25). Furthermore, intraperitoneal injections of arachidonic acid to pregnant diabetic rats lower the rate of neural tube damage (24), thereby indicating a disturbance of the arachidonic cascade as a teratologic consequence of a diabetic environment. Addition of PGE_2 to the culture medium also blocks glucose-induced teratogenicity in vitro (7,26), as well as maldevelopment of embryos cultured in diabetic serum (27). A disturbed arachidonic acid–prostaglandin metabolism in embryos exposed to

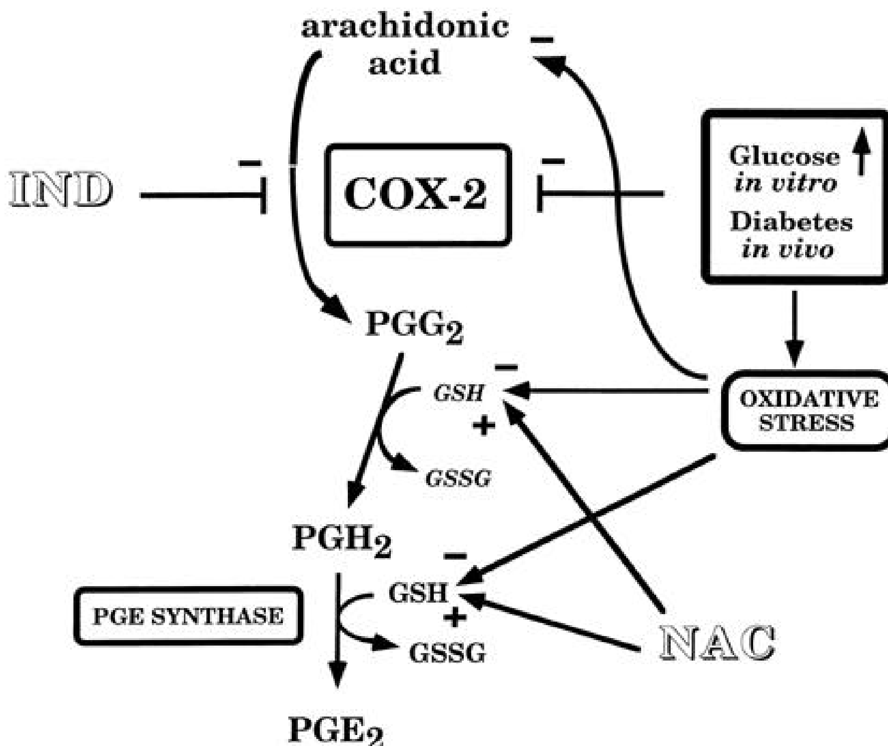

FIG. 3. Schematic and speculative outline of the relationships between arachidonic acid, COX-2 activity, the COX-2 inhibitor indomethacin (IND), and the effect of addition of the GSH precursor NAC in embryos on gestational days 9–10. To the left is the prostaglandin biosynthesis depicted, from arachidonic acid, via PGG_2, PGH_2 to PGE_2. Note the GSH-dependence of the PGE synthase enzyme, diverting the PGH_2 precursor to the resulting PGE_2. The main results of this study, the decreased expression (and presumably decreased activity) of the COX-2 enzyme (Table 3) caused by either high glucose concentration in vitro or (maternal) diabetes in vivo, and the beneficial effect on the PGE_2 production of adding the GSH-precursor NAC, as well as the increased rate of lipid peroxidation in the embryos (Fig. 1), are depicted here. GSSG, oxidized glutathione.

a diabetes-like environment was also inferred from studies failing to demonstrate a deficient embryonic uptake of arachidonic acid (51) or a decreased cellular concentration of arachidonic acid (52) under diabetes-like conditions.

We have chosen to regard both the COX-2 downregulation and the oxidative stress to be contributing to the teratogenic effect of hyperglycemia in vitro and diabetes in vivo. This choice is supported by results from a recent study during which we exposed rat embryos to the combined teratogenic effect of 30 mmol/l glucose and a COX inhibitor (indomethacin or acetylsalicylic acid) and attempted to normalize embryonic development by adding arachidonic acid or PGE_2 to the culture medium at concentrations that diminish dysmorphogenesis caused by either agent alone (7). Arachidonic acid was mainly ineffective, but PGE_2 had a clear antiteratogenic effect, which would support the notion that high glucose concentration disturbs both the COX enzyme and some other aspect of prostaglandin (in particular PGE_2) production, different from COX action (7).

We propose that the previously postulated disturbance of the arachidonic acid–PGE_2 pathway (24) in embryos exposed to high glucose in vitro or diabetes in vivo is decreased COX-2 expression on gestational days 9–10, leading to diminished PGE_2 concentration during a developmentally sensitive embryonic period (7,26–28), corresponding to postconception weeks 3–4 of human pregnancy. We also suggest that hyperglycemia/diabetes–induced oxidative stress aggravates the effect of decreased COX-2 activity by diminishing the GSH-dependent conversion of PGH_2 to PGE_2 in the embryo and, furthermore, decreases the available precursor pool through extensive arachidonic acid peroxidation (Fig. 3 shows a tentative outline of the etiologic mechanisms). We face the challenging task of investigating the possible associations between the present findings and a presumed inositol deficiency (53,54), a recently demonstrated 3-deoxyglucosone excess (55), an increased DNA mutation rate (56), and a reduced Pax-3 gene expression (57) in embryos exposed to hyperglycemia in vitro and diabetes in vivo.

ACKNOWLEDGMENTS

This study was supported by the Ernfors Family Fund, the Swedish Diabetes Association, the Juvenile Diabetes Foundation International, the Novo Nordisk Foundation, and the Swedish Medical Research Council (grants 12X-7475, 12X-11564, 12X-109).

The authors wish to express their gratitude to Lisbeth Sagulin for serum collection.

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