

Antioxidants Diminish Developmental Damage Induced by High Glucose and Cyclooxygenase Inhibitors in Rat Embryos In Vitro

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Previous studies have suggested that the metabolism of arachidonic acid and radical oxygen species (ROS) are altered in diabetes and that these disturbances may induce severe embryonic dysmorphogenesis in diabetic pregnancy. We tested this hypothesis by studying whether an inhibition of the rate-limiting enzyme of prostaglandin biosynthesis, cyclooxygenase (COX), caused developmental disturbances analogous to those seen in embryos exposed to high glucose concentration. Whether antioxidants could prevent such developmental alterations was also investigated. Whole embryo culture was used in which day-9 embryos were exposed to high concentrations of glucose, arachidonic acid, prostaglandin (PG) E_2 , COX inhibitors, and antioxidants for 48 h. Increased glucose concentration (from 10 to 30 mmol/l) caused embryonic dysmorphogenesis, and addition of either 60 μ mol/l arachidonic acid or 280 nmol/l PGE $_2$ largely protected the embryo from this maldevelopment. Furthermore, exposure to the COX inhibitors indomethacin (200 μ mol/l) or acetylsalicylic acid (700 μ mol/l) in 10 mmol/l glucose concentration yielded embryonic dysmorphogenesis similar to that caused by 30 mmol/l glucose. Supplementation of either arachidonic acid or PGE $_2$ to the culture medium with COX inhibitors in low glucose rectified the embryonic development, and PGE $_2$ supplementation also normalized the development of embryos cultured with COX inhibitors in high glucose concentration. Interestingly, the antioxidants superoxide dismutase (SOD) and *N*-acetylcysteine (NAC) were each able to diminish the dysmorphogenesis induced by the COX inhibitors, at doses previously shown to diminish glucose-induced embryonic damage in the same in vitro culture system. In conclusion, the present study shows that a high glucose concentration disturbs embryonic development and that this disturbance may be partly mediated via altered metabolism of arachidonic acid and ROS in the embryo. *Diabetes* 47:677–684, 1998

Diabetic pregnancy is associated with growth disturbances and malformations in the offspring (1). The malformations occur during the period of embryonic organogenesis and are likely to be induced before the 7th postconception week (2). The cellular mechanisms of the diabetic embryopathy are not completely understood. However, a number of possible teratological processes have been suggested, based mainly on experimental studies. Both disturbed metabolism of arachidonic acid and excess of radical oxygen species (ROS) have been implicated in diabetes-induced embryonic dysmorphogenesis (3).

One conceivable teratologic pathway would be a disturbance of the arachidonic acid cascade in the embryo, which would affect the synthesis and metabolism of prostaglandins. Embryos already have a functioning cyclooxygenase enzyme (COX) and active prostaglandin production during organogenesis. Thus, day-10 rat embryos secrete different arachidonic acid metabolites in which 6-keto-PGF $_1$ (PGI $_2$) is the predominant product (4). Previous studies in vitro have shown that addition of arachidonic acid to the culture medium blocks the dysmorphogenesis elicited by high glucose concentration (5,6). Furthermore, intraperitoneal injection of arachidonic acid to pregnant diabetic rats diminishes the rate of neural tube damage (6), thereby indicating disturbance of the arachidonic acid cascade as a consequence of a diabetic environment. Addition of prostaglandin (PG) E_2 to the culture medium also blocks glucose-induced teratogenicity in vitro (7), as well as maldevelopment of embryos cultured in diabetic serum (8). Measurements of PGE $_2$ have indicated that this prostaglandin is decreased in embryos of diabetic mice during the period of neural tube closure (9). There are, evidently, a number of studies suggesting a role for a disturbed arachidonic acid–prostaglandin metabolism in embryos exposed to a diabetic environment, although the exact metabolic localization of the disturbance has not been determined.

Another possible teratological pathway involves an excess of ROS activity in the embryo exposed to a diabetic environment (10–13), as a consequence of increased production of free oxygen radicals (14), decreased capacity to scavenge ROS (15), or both. This notion is supported by the repeated success in diminishing developmental damage by administering antioxidants to embryos in a diabetic environment, both in vivo (13,16–22) and in vitro (10,11,15,23). Embryos of diabetic rats show decreased vitamin E levels (18) and substantial mitochondrial high-amplitude swelling (24), both of which can be corrected by providing vitamin E supplementation to the mother (Dr. Xiaolin Yang, personal communication).

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ANOVA, analysis of variance; COX, cyclooxygenase; GSH, reduced glutathione; NAC, *N*-acetylcysteine; PG, prostaglandin; PGI $_2$, 6-keto-PGF $_1$; ROS, radical oxygen species; SOD, superoxide dismutase.

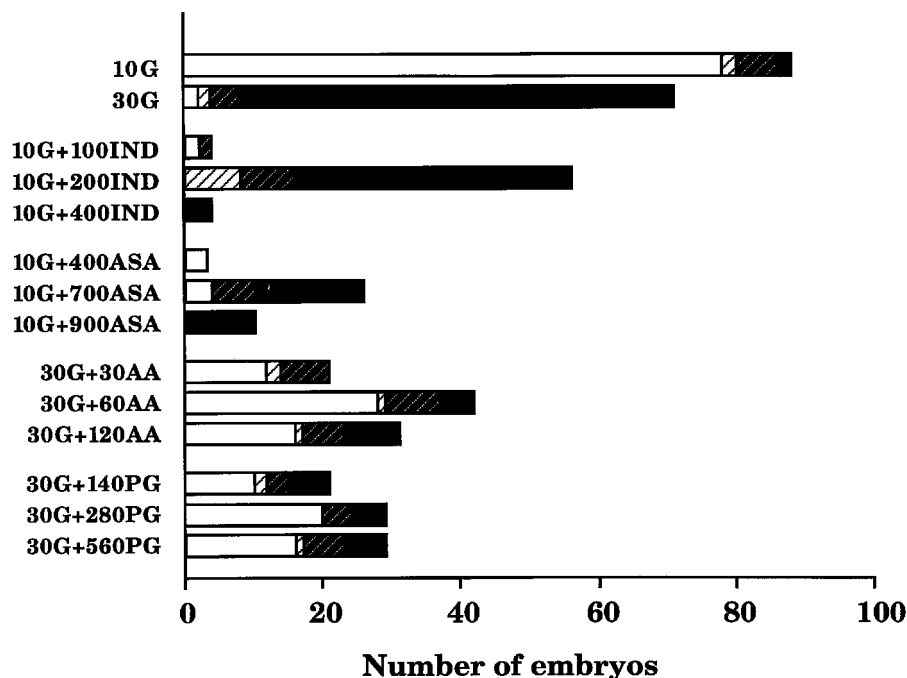


FIG. 1. Morphological outcome in embryos cultured in 10 (10G) or 30 (30G) mmol/l glucose, with the addition to 10G of 100, 200, or 400 $\mu\text{mol/l}$ indomethacin (IND), the addition to 10G of 400, 700, or 900 $\mu\text{mol/l}$ acetylsalicylic acid (ASA), the addition to 30G of 30, 60, or 120 $\mu\text{mol/l}$ arachidonic acid (AA), or the addition to 30G of 140, 280, or 560 nmol/l PGE₂. The embryos showed normal morphology (□), minor malformation (▨), slight malformation (▩), or major malformation (■).

In the present study, we investigated in detail the relationships among diabetic environment, metabolism of prostaglandins and ROS, and embryonic dysmorphogenesis. In particular, we aimed to characterize a possible association between disturbances of the metabolism of prostaglandins and ROS. For this purpose, we used whole rat embryo culture and exposed the embryos to high glucose concentrations with or without arachidonic acid or PGE₂, to COX inhibitors (indomethacin, acetylsalicylic acid), and to antioxidants (superoxide dismutase [SOD], *N*-acetylcysteine [NAC]). After a 48-h culture, we examined the effects of the different substances on embryonic growth and malformation rate and related the outcome to the *in vitro* environment.

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 (25). All rats were fed a commercial pelleted diet (AB Analyser, 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.

Preparation of culture serum. Male retired breeders weighing 400–450 g were used for serum preparation. The serum donors were anesthetized with ether and laparotomized, and the blood was collected from the abdominal aorta and centrifuged immediately (26). The resulting serum was supplemented with sodium benzylpenicillin and streptomycin to give a final concentration of 60 mg/l 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. Female and male rats were caged together during the night, and the following morning was designated gestational day 0 if a positive vaginal smear was retrieved from the female. 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 48 h, as previously described (27). Briefly, the conceptuses were explanted, and culture of whole embryos was performed according to New (26). The embryos, within their intact yolk sacs, were maintained in polypropylene tubes (Falcon 2070; Becton Dickinson Labware, Lincoln Park, NJ) in a roller incubator at 38°C and 60 r/min. Each tube contained 4–5 conceptuses in 5 ml culture medium consisting of 80% rat serum from normal rats (see below) and 20% saline. The glucose concentration of culture media was adjusted to 10 or 30 mmol/l 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, arachidonic acid (sodium salt; Sigma, St. Louis, MO), PGE₂ (Cayman Chemical, Ann Arbor, MI), indomethacin (Sigma), acetylsalicylic acid (Sigma), SOD (Sigma), or NAC (Sigma) was added from sterile stock solutions to final concentrations of 30–120 $\mu\text{mol/l}$, 140–560 nmol/l, 100–400 $\mu\text{mol/l}$, 0.4–0.9 mmol/l, 4,800 U/ml, and 0.5 mmol/l, respectively. In a pilot study, different concentrations of the four first agents were tested in embryo culture to detect suitable concentrations for further work, concentrations that either blocked glucose-induced dysmorphogenesis (arachidonic acid and PGE₂) or caused altered development similar to that induced by high glucose concentrations (indomethacin and acetylsalicylic acid) (Fig. 1). In the main series of experiments, embryos from 5–10 pregnant rats were isolated simultaneously and subsequently cultured in one batch of 10–20 culture tubes with 5–10 different test conditions. In each culture batch, 1–2 control tubes were always included that contained embryos exposed to 10 mmol/l glucose with no further addition. If the control tubes showed major dysmorphogenesis, the whole culture batch was discarded (this happened only once during the experimental series). We also included 1–2 high-glucose tubes with 30 mmol/l glucose in culture batches in which the outcome of exposure to 30 mmol/l glucose with further additive (i.e., arachidonic acid, PGE₂, COX inhibitors, or antioxidants) was evaluated. Because the embryonic outcome in the 10 mmol/l and 30 mmol/l glucose culture tubes did not differ considerably between batches in the experimental series, these data were combined into the 10G and 30G groups for the statistical analysis. The compiled data from all groups were then compared with the 10G and 30G groups; this is graphically illustrated in Figs. 1 and 3–5.

The culture tubes were gassed with different mixtures of N₂, O₂, and CO₂ gas, as previously described (10), at the start and after 24 and 40 h of culture. The embryos were harvested, dissected from their yolk sacs, and morphologically evaluated after 48 h of culture. 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) (Fig. 2) were determined by one examiner who was not aware of the culture conditions to which the embryos had been exposed. Subsequently, the embryos were homogenized and hydrolysed in 1 ml 0.5 mol/l NaOH. The protein content of the homogenates was determined by the method used by Lowry et al. (28), using bovine serum albumin as a standard, and DNA was measured as described by Kissane and Robins (29) and Hinegardner (30).

Statistical analysis. Differences between means were evaluated by one-way analysis of variance (ANOVA), where the applied test was Fisher's protected least significant difference at the 95% significance level (31), or with χ^2 analysis (with Yates' correction), depending on which method was applicable (32). The comparisons between different experimental groups were based on individual embryos, except for the evaluation of malformation score, for which the Fisher's exact χ^2 test for 2 × 2 tables was used, and we therefore combined the scores (one group with scores 0 and 1, another group with scores 5 and 10).

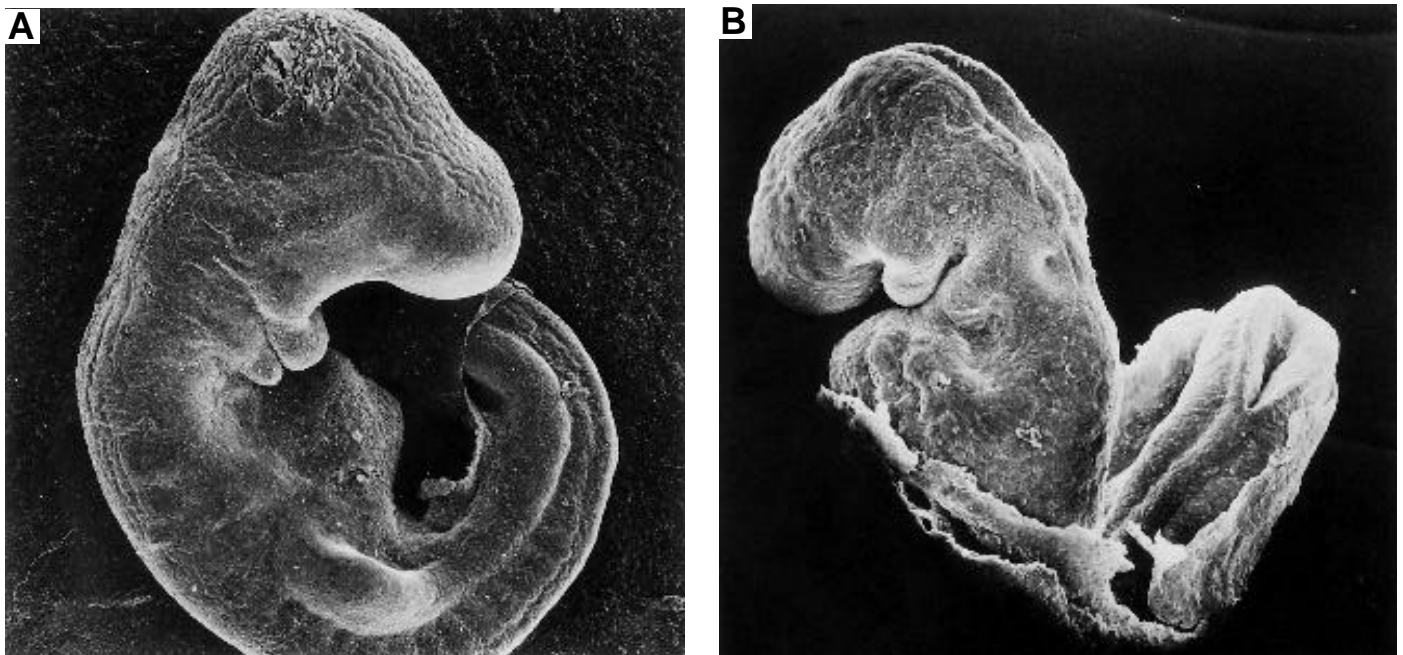


FIG. 2. Electron micrographs of rat embryos after 48 h in vitro either in 10 mmol/l glucose (A) or after exposure to 30 mmol/l glucose (B), with malformation scores 0 and 10, respectively. The embryo in A is completely normal, whereas the embryo in B is malrotated and shows a neural tube that is completely open in the head region, as well as in several areas along the dorsal aspects of the tube.

RESULTS

Addition of 100–400 $\mu\text{mol/l}$ indomethacin or 0.4–0.9 mmol/l acetylsalicylic acid to 10 mmol/l glucose medium showed that 200 $\mu\text{mol/l}$ indomethacin or 0.7 mmol/l acetylsalicylic acid induces a teratogenic outcome comparable to that of 30 mmol/l glucose alone (Fig. 1). The comparative experiments with different concentrations of arachidonic acid (30–120 $\mu\text{mol/l}$) and PGE_2 (140–560 nmol/l) yielded 60 $\mu\text{mol/l}$ and 280 nmol/l, respectively, as optimal inhibitors of high glucose-induced teratogenesis (Fig. 1).

Increasing the glucose concentration from 10 to 30 mmol/l in the culture medium caused major disturbances in embryonic development, as evidenced by decreased protein and DNA content and decreased somite number and crown-rump length, as well as an increased malformation score from 0.5 to 9.1 (Table 1) corresponding to an increase in major malformation of from 1 to 87% of the embryos (Fig. 3). Supplementation of arachidonic acid or PGE_2 to the low-glucose

medium did not change any of the embryonic parameters, whereas addition of these compounds to high-glucose culture medium markedly decreased the incidence of dysmorphogenesis induced by glucose (Table 1). Thus, the protein and DNA content were almost completely normalized, whereas the somite number and crown-rump length remained slightly decreased (Table 1). Likewise, the malformation score was improved in the high-glucose cultures with addition of arachidonic acid and PGE_2 to 2.2–2.4, compared with 9.1 (12–17% major malformation, compared with 87%) (Fig. 3), but remained increased compared with culture in 10G (Table 1).

Indomethacin and acetylsalicylic acid at concentrations of 200 and 700 $\mu\text{mol/l}$, respectively, caused embryonic malformations at the same level as did high glucose concentration alone. Thus, the embryos showed decreased protein and DNA content, decreased somite number and crown-rump length (Table 2, upper panel), and increased malformation score

TABLE 1
Outcome of embryo culture with added glucose, arachidonic acid, and PGE_2

Type of serum	<i>n</i>	Protein content ($\mu\text{g}/\text{embryo}$)	DNA content ($\mu\text{g}/\text{embryo}$)	Somites	Crown-rump length (mm)	Malformation score
10G	91	301 \pm 7	34 \pm 1	28.5 \pm 0.2	3.7 \pm 0.04	0.5
10G + 60AA	15	287 \pm 13	38 \pm 4	28.3 \pm 0.8	3.7 \pm 0.1	0.8
10G + 280 PGE_2	16	272 \pm 17	35 \pm 4	27.6 \pm 1.0	3.6 \pm 0.1	0.3
30G	76	156 \pm 8*	16 \pm 1*	14.9 \pm 0.6*	2.7 \pm 0.1*	9.1*
30G + 60AA	42	280 \pm 9†	38 \pm 2†	26.4 \pm 0.5*†	3.4 \pm 0.1*†	2.2*†
30G + 280 PGE_2	29	269 \pm 8*†	36 \pm 2†	26.7 \pm 0.6*†	3.3 \pm 0.1*†	2.4*†

Data are means \pm SE. Embryos were cultured in 10 (10G) or 30 (30G) mmol/l glucose with 60 $\mu\text{mol/l}$ arachidonic acid (AA) or 280 nmol/l PGE_2 . * P < 0.05 vs. 10G; † P < 0.05 vs. 30G. All P values were determined by ANOVA or χ^2 analysis with Yates' correction.

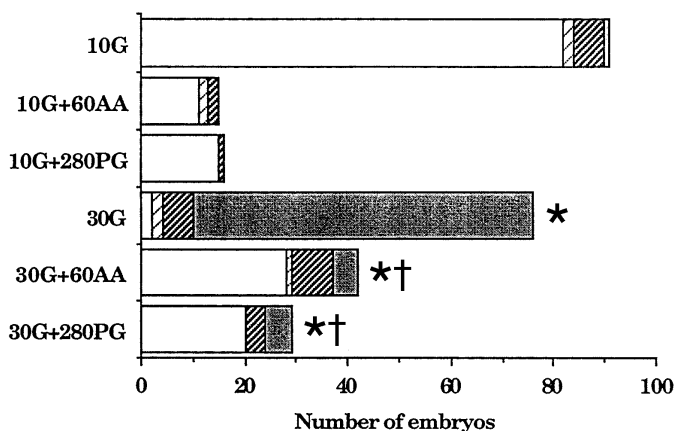


FIG. 3. Morphological outcome in embryos cultured in 10 (10G) or 30 (30G) mmol/l glucose with the addition of 60 μmol/l arachidonic acid (AA) or 280 nmol/l PGE₂. The embryos showed normal morphology (□), minor malformation (▨), slight malformation (▩), or major malformation (■). **P* < 0.05 vs. 10G; †*P* < 0.05 vs. 30G (χ² analysis with Yates' correction).

(from 0.5 to 7.7–7.9, corresponding to an increase in major malformations from 1 to 67–82% of the embryos, Fig. 4A).

Addition of arachidonic acid or PGE₂ to the culture medium containing indomethacin or acetylsalicylic acid in 10 mmol/l glucose reversed the embryonic maldevelopment caused by the COX inhibitors (Table 2, upper panel, and Fig. 4A). The protein content was normalized in all groups, and all other embryonic parameters were improved, although not completely normalized (Table 2, upper panel). The malformation score in the COX inhibitor groups (7.7–7.9) was decreased by arachidonic acid and PGE₂ addition to scores of 1.2–2.5, corresponding to only 8–17% major malformation (Fig. 4A). Supplementation with arachidonic acid appeared to be slightly more beneficial to the embryos, since the mal-

formation score in these groups did not differ from the 10G embryos (1.2–1.8 vs. 0.5) (Table 2, upper panel).

When the embryos were exposed to the combined dysmorphic effects of high glucose and either indomethacin or acetylsalicylic acid, the arachidonic acid addition did not manage to reverse this maldevelopment, since protein content, DNA content, somite number, and crown-rump length remained decreased despite arachidonic acid addition (Table 2, lower panel). In addition, the malformation score of the 30G embryos (9.1) remained high in the two arachidonic acid-supplemented groups with COX inhibitors (9.5–9.6), corresponding to major malformation in the range of 92–93%.

In contrast, addition of PGE₂ managed to reverse the disturbed development in the 30G group in regard to most parameters, the exceptions being DNA content and crown-rump length in the 30G + 280PGE₂ + 700ASA group (Table 2, lower panel, and Fig. 4B). The malformation score was reduced from that of the 30G embryos (9.1) to 1.8–5.0, representing 4–47% major malformation in the PGE₂-supplemented groups with COX inhibitors (Fig. 4B).

The teratogenic action of COX inhibitors was also diminished by addition of the antioxidative compounds SOD and NAC to the culture medium (Table 3). Thus, both SOD and NAC managed to improve most embryonic parameters toward 10G values, with the nonaltered somite number and crown-rump length of the 10G + 700ASA + SOD group as exceptions.

Complete normalization of the protein content and crown-rump length was seen only in the 10G + 200IND + NAC group. The malformation score was markedly improved from 7.7–7.9 in the COX inhibitor groups (67–82% major malformation) to 2.2–3.6 in the antioxidant-treated groups (12–28% major malformation) (Fig. 5).

DISCUSSION

One important finding in the present study was that high glucose concentration and COX inhibitors cause similar types of

TABLE 2
Outcome of embryo culture with added glucose, indomethacin, acetylsalicylic acid, arachidonic acid, and PGE₂

Type of serum	<i>n</i>	Protein content (μg/embryo)	DNA content (μg/embryo)	Somites	Crown-rump length (mm)	Malformation score
10G	91	301 ± 7	34 ± 1	28.5 ± 0.2	3.7 ± 0.04	0.5
10G + 200IND	56	165 ± 10*	15 ± 1*	18.5 ± 0.8*	2.8 ± 0.1*	7.9*
10G + 200 IND + 60AA	25	299 ± 9†	29 ± 1*†	27.4 ± 0.5†	3.5 ± 0.1†	1.2†
10G + 200IND + 280PGE ₂	32	278 ± 10†	28 ± 1*†	24.9 ± 1.1*†	3.4 ± 0.1*†	2.2*†
10G + 700ASA	30	179 ± 11*	16 ± 1*	20.3 ± 0.7*	2.9 ± 0.1*	7.7*
10G + 700ASA + 60AA	12	277 ± 18‡	28 ± 2*‡	24.0 ± 1.4*‡	3.2 ± 0.1*‡	1.8‡
10G + 700ASA + 280PGE ₂	12	281 ± 20‡	27 ± 2*‡	25.4 ± 1.1*‡	3.4 ± 0.1*‡	2.5*‡
30G	76	156 ± 8	16 ± 1	14.9 ± 0.6	2.7 ± 0.1	9.1
30G + 60AA	42	280 ± 9§	38 ± 2§	26.4 ± 0.5§	3.4 ± 0.1§	2.2§
30G + 60AA + 200IND	30	176 ± 9	17 ± 1	14.7 ± 1.0	2.8 ± 0.1	9.5
30G + 60AA + 700ASA	13	165 ± 11	14 ± 1	13.1 ± 1.2	2.4 ± 0.1§	9.6
30G + 280PGE ₂	29	269 ± 8§	36 ± 2§	26.7 ± 0.6§	3.3 ± 0.1§	2.4§
30G + 280PGE ₂ + 200IND	28	252 ± 9§	27 ± 1§¶	25.6 ± 0.4§	3.1 ± 0.04§	1.8§
30G + 280PGE ₂ + 700ASA	15	206 ± 23§¶	20 ± 2¶	20.0 ± 1.7§¶	2.7 ± 0.2¶	5.0§

Data are means ± SE. Embryos were cultured in 10 (10G) or 30 (30G) mmol/l glucose with 200 μmol/l indomethacin (IND) or 700 μmol/l acetylsalicylic acid (ASA) and with or without 60 μmol/l arachidonic acid (AA) or 280 nmol/l PGE₂. **P* < 0.05 vs. 10G; †*P* < 0.05 vs. 10G + 200IND; ‡*P* < 0.05 vs. 10G + 700ASA; §*P* < 0.05 vs. 30G; ||*P* < 0.05 vs. 30G + 60AA; ¶*P* < 0.05 vs. 30G + 280 PGE₂. All *P* values were determined by ANOVA or χ² analysis with Yates' correction.

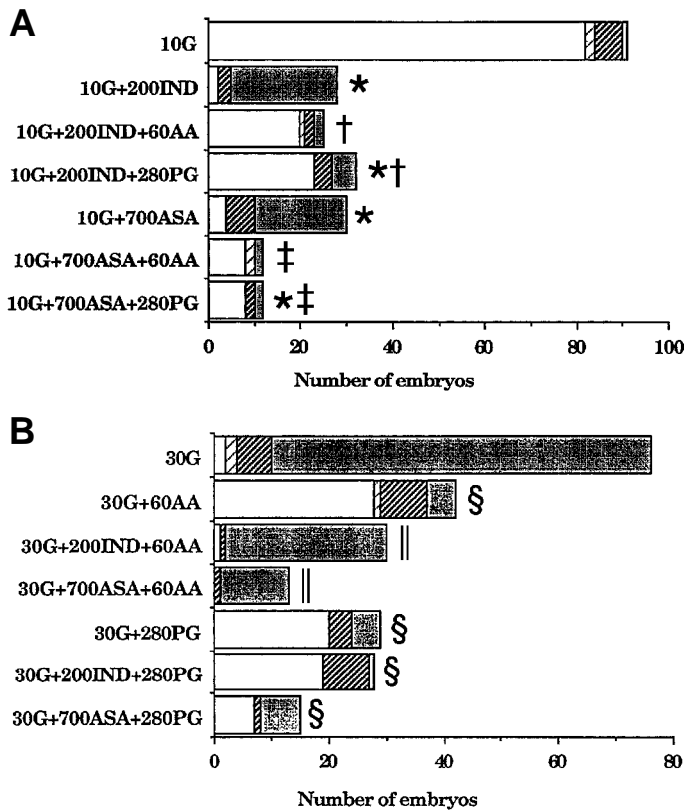


FIG. 4. Morphological outcome in embryos cultured in 10 mmol/l glucose (10G) (A) or in 30 mmol/l glucose (30G) (B), with the addition of 200 μ mol/l indomethacin (IND) or 700 μ mol/l acetylsalicylic acid (ASA) with or without the further addition of either 60 μ mol/l arachidonic acid (AA) or 280 nmol/l PGE₂. The embryos showed normal morphology (□), minor malformation (▨), slight malformation (▩), or major malformation (■). * $P < 0.05$ vs. 10G; † $P < 0.05$ vs. 10G + 200IND; ‡ $P < 0.05$ vs. 10G + 700ASA; § $P < 0.05$ vs. 30G; || $P < 0.05$ vs. 30G + 60AA (all χ^2 analysis with Yates' correction).

embryonic dysmorphogenesis in vitro, as evidenced by retarded growth and the presence of similar somatic malformations in both series of cultured embryos. Both the glucose-induced dysmorphogenesis and the COX inhibitor-induced embryonic dysmorphogenesis were diminished by adminis-

tration of either arachidonic acid or PGE₂ to the culture medium. These results suggest that high glucose concentration and COX inhibition share common teratogenic pathways.

A second major finding of the study was the diminishing effect exerted by SOD and NAC on the COX inhibitor-induced dysmorphogenesis, analogous to the effect of the antioxidants on glucose-induced embryonic maldevelopment (23). This result, together with the finding of diminished glucose-induced embryopathy by addition of arachidonic acid and PGE₂, suggests a cross-talk between teratogenic effects caused by decreased prostaglandin synthesis and ROS excess in embryos subjected to a diabetic environment.

We found a marked difference in the anti-teratogenic potency of arachidonic acid versus PGE₂. Thus, the molar concentration of arachidonic acid (60 μ mol/l) needed to block both the glucose-induced and COX inhibitor-induced teratogenicity was ~200 times larger than that of the optimal PGE₂ concentration (280 nmol/l). Furthermore, when the cultured embryos were exposed to the combined teratogenic influence of high glucose concentration and a COX inhibitor, the arachidonic acid supplementation was largely ineffective in blocking embryonic dysmorphogenesis, whereas the PGE₂ addition had a clear anti-teratogenic effect. This finding suggests that high glucose concentration in the embryo may specifically decrease the activity of COX and/or subsequent enzymes involved in the synthesis of PGE₂.

In previous studies, it has been shown that high glucose concentration is teratogenic in vitro (10,33,34) and in vivo (35). It has also been suggested from clinical studies that high glucose concentration is not the only teratogen present in a diabetic environment (36). This notion is supported in experiments where serum from insulin-treated diabetic rats was found to be teratogenic in vitro, despite normalized glucose concentration (27,37), and in addition, several other teratogens have been identified in vitro (3). The exact mechanism of the teratogenic effect of high glucose is not known, but a number of anti-teratogenic processes have been identified in experimental studies (3). For instance, the addition of compounds with oxygen-radical scavenging capacity has been shown to diminish the glucose-induced teratogenicity in vitro (10,11,38), as well as in vivo (13,16–22). Therefore, a role for antioxidative therapy in the prevention of diabetic malformations has been suggested. In this context, it is of interest to note that normal COX function involves the production

TABLE 3

Outcome of embryo culture with added glucose, indomethacin, acetylsalicylic acid, SOD, and NAC

Type of serum	<i>n</i>	Protein content (μ g/embryo)	DNA content (μ g/embryo)	Somites	Crown-rump length (mm)	Malformation score
10G	91	301 \pm 7	34 \pm 1	28.5 \pm 0.2	3.7 \pm 0.04	0.5
10G + 200IND	56	165 \pm 10*	15 \pm 1*	18.5 \pm 0.8*	2.8 \pm 0.1*	7.9*
10G + 200IND + SOD	33	269 \pm 14*†	26 \pm 1*†	25.5 \pm 0.8*†	3.4 \pm 0.1*†	2.2*†
10G + 200IND + NAC	36	285 \pm 12†	27 \pm 1*†	26.4 \pm 0.8*†	3.5 \pm 0.1†	2.7*†
10G + 700ASA	30	179 \pm 11*	16 \pm 1*	20.3 \pm 0.7*	2.9 \pm 0.1*	7.7*
10G + 700ASA + SOD	18	246 \pm 15*‡	24 \pm 2*‡	22.8 \pm 1.5*	3.0 \pm 0.1*	3.6*‡
10G + 700ASA + NAC	24	265 \pm 18*‡	25 \pm 2*‡	25.1 \pm 1.1*‡	3.3 \pm 0.1*‡	3.3*‡

Data are means \pm SE. Embryos were cultured in 10 (10G) mmol/l glucose with 200 μ mol/l indomethacin (IND) or 700 μ mol/l acetylsalicylic acid (ASA) and with or without SOD or NAC. * $P < 0.05$ vs. 10G; † $P < 0.05$ vs. 10G + 200IND; ‡ $P < 0.05$ vs. 10G + 700ASA. All P values were determined by ANOVA or χ^2 analysis with Yates' correction.

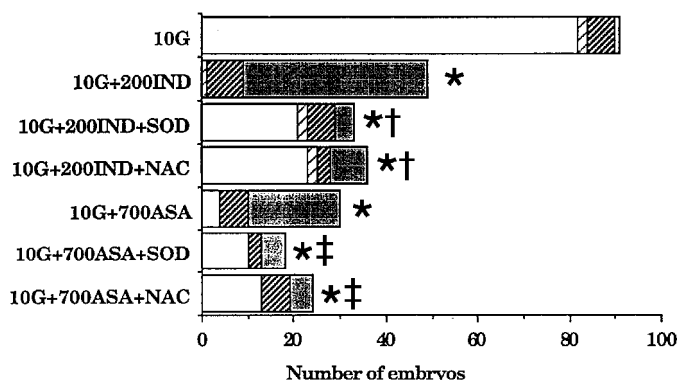


FIG. 5. Morphological outcome in embryos cultured in 10 mmol/l glucose (10G) with the addition of 200 μ mol/l indomethacin (IND) or 700 μ mol/l acetylsalicylic acid (ASA) with or without the further addition of either SOD or NAC. The embryos showed normal morphology (\square), minor malformation (\square), slight malformation (\square), or major malformation (\blacksquare). * $P < 0.05$ vs. 10G; † $P < 0.05$ vs. 10G + 200IND; ‡ $P < 0.05$ vs. 10G + 700ASA (χ^2 analysis with Yates' correction).

of peroxyl radicals and lipid peroxides, processes that are likely to affect the balance of oxidative metabolites in the cell.

A direct teratogenic effect of COX inhibitors has not been described before. On the other hand, from earlier in vivo and in vitro studies, it may be inferred that low activity of embryonic COX can disturb embryonic development (5–8). When indomethacin was added to medium containing teratogenic levels of glucose and protective concentrations of myo-inositol, it reversed the protective effect of myo-inositol, resulting in disturbed development of the cultured mouse embryos (7). Addition of arachidonic acid to in vitro cultured embryos blocked the teratogenic effect of high glucose (5,6). Also, addition of PGE₂ yielded diminished maldevelopment in embryos subjected to high glucose (7) and diabetic serum (8). Intraperitoneal injection of arachidonic acid to pregnant diabetic rats diminished the rate of congenital malformations (5), and the embryos of diabetic mice have been shown to contain less PGE₂ than control embryos during the neurulation process (9). In the experimental literature, therefore, there is evidence supporting that COX inhibition, particularly in a diabetic environment, may be teratogenic: decreased cellular concentration of PGE₂ may play a central role in distorted embryonic development.

The possibility that inhibition of COX and/or subsequent PGE₂-producing enzymes is a major component of the diabetic teratogenic process is evident from the above reasoning. Biochemical pathways by which altered glucose concentration may affect the embryonic synthesis of prostaglandins are unknown, however. Previous investigations have shown evidence in support of both a decreased (9,39,42) and an increased (40) prostaglandin production in fetal and uterine tissue exposed to high concentration of glucose.

Glucose may cause changes in the enzyme activity involved in prostaglandin synthesis. Thus, a reduction in PGI₂ production in umbilical vessels in IDDM pregnancies has been reported (39). Fowden et al. (41) have found that decreased glucose concentration in late pregnancy in ewes causes increased prostaglandin production, and that increased glucose levels in the mother, achieved either by infu-

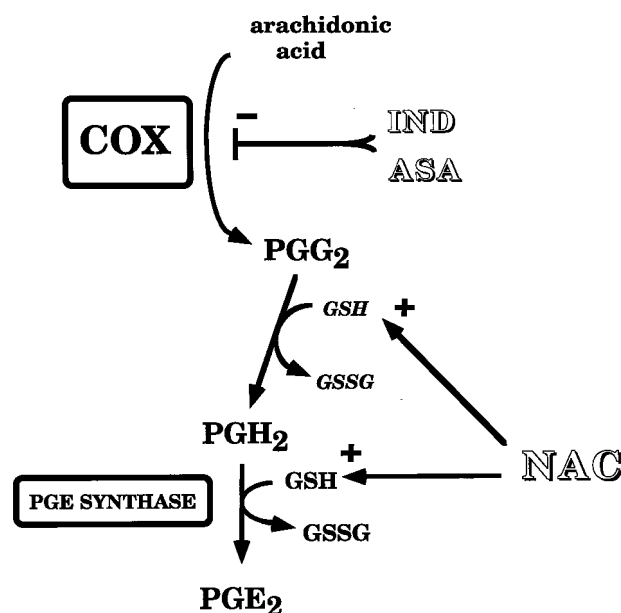


FIG. 6. Schematic outline of the relationships among arachidonic acid, COX activity, the COX inhibitors indomethacin (IND) and acetylsalicylic acid (ASA), and the putative positive effect of addition of the reduced glutathione (GSH) precursor NAC. GSSG, oxidized glutathione.

sion or feeding, leads to decreased prostaglandin production. On the other hand, other investigators have found increased concentration of arachidonic acid, as well as decreased PGI₂ production and an increased thromboxane B₂ production, in placentas from IDDM pregnancies (40). Of specific interest in this context are the measurements by Schoenfeld et al. (42) of PGE₂ in the yolk sacs of early human pregnancy at gestational weeks 8–10. They reported that normal yolk sac fluid contained 3,605 pg/ml PGE₂, whereas the yolk sacs of diabetic women did not have detectable levels of this prostaglandin. Taken together with the decreased PGE₂ levels in embryos of diabetic mice (9), these findings suggest that alterations in glucose concentration may cause changes in COX activity, leading to imbalances among the different classes of produced prostaglandins and especially to a decreased PGE₂ production.

An alternative explanation for our findings would be a restriction of the principal substrate for the prostaglandin pathway, arachidonic acid, by a diabetes-like environment. This may implicate an inhibition of the rate-limiting enzyme, phospholipase A₂, or a decreased uptake or an inhibition of earlier biosynthetic steps in the production of this polyunsaturated fatty acid. Our previous studies have shown, however, that the uptake of arachidonic acid by embryonic yolk sacs is increased in a hyperglycemic environment (43). This finding would preclude an outright deficiency of arachidonic acid in the conceptus of diabetic pregnancy, a result supported by the demonstration of similar concentration of arachidonic acid in high-glucose cultured embryos in vitro (44). In all, the changes in embryogenesis demonstrated in the present study do not seem to depend on a decreased availability of arachidonic acid.

There may be another way of understanding the findings of the present study. We have previously suggested that

high glucose or a diabetic environment may cause ROS excess in the embryonic tissues and thereby yield a decrease in scavenging capacity of the cells (10,13). Mitochondrial morphological changes and evidence of superoxide production in neuroepithelial tissue provide support for embryonic ROS generation (24,25). The notion of decreased scavenging capacity is supported by low embryonic vitamin E concentrations during organogenesis (18) and also by the blocking of dysmorphogenesis in embryos cultured in high glucose or diabetic serum by addition of the reduced glutathione (GSH) precursor NAC (23), or GSH ester (15). Exposure of rat embryos to high glucose in vitro has also been found to decrease the activity of the rate-limiting GSH-synthesizing enzyme, γ -glutamyl-cysteine-synthetase (γ -GCS) (15), and a high glucose concentration may conceivably decrease cellular NADPH content, and thereby deplete the GSH pool, by high sorbitol shunt activity (45,46). This notion should be combined with the fact that complete COX inhibition is seldom achieved by exogenous COX inhibitors. In the present study, we are likely to have inhibited the COX activity by 30–40% with the indomethacin and acetylsalicylic acid additions. Furthermore, several steps in the production of PGE₂ from arachidonic acid are influenced by the cellular GSH concentration. In the conversion of PGG₂ to PGH₂, i.e., reduction of the 15-hydroperoxide group in PGG₂, GSH is used as an electron donor. Also, in the conversion of PGH₂ to PGE₂ by PGE synthase, GSH is needed as a cofactor by the enzyme (47,48). A diabetes-induced decrease in GSH availability would therefore specifically diminish the production of PGE₂ in the embryos, and thereby yield disturbed development (Fig. 6). And consequently, restoration of GSH levels by antioxidant treatment may also normalize the PGE₂ levels in the embryo and rectify embryonic development despite the presence of high ambient glucose concentration, as found in the present work. This line of reasoning is supported by the beneficial effects of antioxidative treatment on COX inhibitor-induced embryonic dysmorphogenesis, demonstrated in the present work, which may reflect an enhanced activity of the PGH-PGE isomerase, yielding higher production of PGE₂ from the available PGH₂ and, therefore, restored embryo development.

In conclusion, we suggest that prostaglandin biosynthesis is decreased in embryos cultured in high glucose or COX inhibitors, which leads to an imbalance among the different classes of arachidonic acid metabolites. The results of the present study suggest that disturbance of the arachidonic acid pathway and ROS metabolism are major components of the glucose-induced effects on embryonic metabolism, subsequently leading to embryonic dysmorphogenesis.

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