Hyperglycemia-induced teratogenesis is mediated by a functional deficiency of arachidonic acid

(diabetes/embryopathy)

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ABSTRACT   Congenital malformations now represent the largest single cause of mortality in the infant of the diabetic mother. The mechanism by which diabetes exerts its teratogenic effects is not known. This study evaluated whether arachidonic acid might be involved, a possibility raised by the role of arachidonic acid in palatal elevation and fusion, processes analogous to neural tube folding and fusion. This hypothesis was tested in two animal models of diabetic embryopathy, the *in vivo* pregnant diabetic rat and the *in vitro* hyperglycemic mouse embryo culture. The subcutaneous injection of arachidonic acid (200–400 mg/kg per day) into pregnant diabetic rats during the period of organ differentiation (days 6–12) did not alter the maternal glucose concentration, the maternal weight gain, or the weight of the embryos. However, the incidence of neural tube fusion defects was reduced from 11% to 3.8% ($P < 0.005$), the frequency of cleft palate was reduced from 11% to 4% ($P < 0.005$), and the incidence of micrognathia was reduced from 7% to 0.8% ($P < 0.001$). The addition of arachidonic acid to B10.A mouse embryos in culture also resulted in a reversal of hyperglycemia-induced teratogenesis. The teratogenic effect of D-glucose (8 mg/ml) in the medium resulted in normal neural tube fusion in only 32% of the embryos ($P < 0.006$ when compared to controls). Arachidonic acid supplementation (1 or 10 μg/ml) produced a rate of neural tube fusion (67%) that was not significantly different from that observed in controls. The evidence presented indicates that arachidonic acid supplementation exerts a significant protective effect against the teratogenic action of hyperglycemia in both *in vivo* (rat) and *in vitro* (mouse) animal models. These data therefore suggest that the mechanism mediating the teratogenic effect of an increased glucose concentration involves a functional deficiency of arachidonic acid at a critical stage of organogenesis.

Although major advances have been made over the past 20 years in the prognosis of the newborn infant of the diabetic mother, no appreciable improvements have been noted in the rates of malformations seen in these infants (1, 2). Malformations have now become the leading cause of death in infants of diabetic mothers (3). Although the lesion most specific for human maternal diabetes is the caudal regression syndrome (4), spina bifida, hydrocephalus, anencephaly, and other central nervous system defects also occur at a high rate (5). This had led to studies of diabetic embryopathy focused on animal models of failure of neural tube folding and fusion. These defects can be produced by exposing either rat or mouse embryos to high concentrations of glucose *in vitro*, thus demonstrating that increased glucose levels alone are

sufficient to produce malformations (6, 7). However, the mechanism linking hyperglycemia with malformations remains unknown. In this paper, we present evidence that exogenous arachidonic acid exerts a highly significant protective effect against the teratogenic action of hyperglycemia in both *in vivo* and *in vitro* animal models. These data strongly suggest that the mechanism mediating the teratogenic effect of an increased glucose concentration involves a functional deficiency of arachidonic acid at a critical stage of organogenesis.

MATERIALS AND METHODS

In Vivo Studies. Pregnant female Sprague–Dawley rats were used. The animals were designated pregnant on the day on which a vaginal smear showed the presence of spermatozoa after a proestrus female had been caged with a male overnight (day 0). On day 6 of pregnancy, the rats were injected in the tail with streptozotocin (40 mg/kg) that was freshly prepared in cold (4°C) 0.01 M citrate buffer (pH 4.5). This dose of streptozotocin has been reported to produce blood glucose levels in the range of 350 mg/dl and a reproducible incidence of congenital malformations (8, 9). The animals injected with streptozotocin were not considered diabetic unless a fed serum glucose concentration >250 mg/dl had been documented. Blood was collected from a tail vein incision at 8 a.m. daily and analyzed immediately for serum glucose concentration by using a Beckman glucose analyzer. The fetuses were obtained by cesarean section on day 20 of gestation. Maternal glucose concentrations and fetal weights were measured at that time. The fetuses were examined for neural tube fusion defects, cleft palate, and micrognathia. They were then fixed in 95% ethanol and stained with alcian blue and alizarin red S according to the procedure of Inouye (10). This stain allows the differentiation between cartilage and ossified skeleton.

Arachidonic acid (free acid, 99% pure; Sigma) was administered subcutaneously according to two different protocols. The first group of pregnant diabetic rats received 200 mg/kg as a single daily dose on days 9–12 of gestation. A second group of diabetic pregnant rats received 200 mg/kg twice daily by the subcutaneous route on days 5–10 of gestation. Neural tube fusion takes place on day 11 in the rat. No insulin was administered to the diabetic rats at any time during the pregnancy.

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Table 1. Effect of arachidonic acid on fetal and maternal weight and maternal glucose levels

Group	Litters, no.	Embryos, no.	Fetal weight, g	Weight gain per embryo, g	Plasma glucose, mg/dl
Streptozotocin on day 6	18	193	3.14 ± 0.06	8.8 ± 1.2	348 ± 15
+ Arachidonic acid, 200 mg/kg per day (one dose), days 9–12	20	212	3.31 ± 0.26	9.4 ± 0.4	328 ± 17
+ Arachidonic acid, 400 mg/kg per day (two doses), days 5–10	4	50	2.84 ± 0.09	8.9 ± 0.2	387 ± 35

Data are presented as mean ± SEM.

***In Vitro* Studies.** Mice of the B10 and B10.A strains were used for the mouse embryo culture studies. The B10.A strain is sensitive to the production of cleft palate by glucocorticoids and phenytoin, whereas the B10 strain is resistant to the teratogenic effect of these agents (11, 12). These strains are congenic and vary genetically only in the H-2 histocompatibility region of chromosome 17.

The mice were maintained on a 12-hr light cycle with the dark phase extending from 5 a.m. to 5 p.m. Females were mated with syngeneic males from 7 a.m. to 11 a.m. or occasionally for longer periods. Embryos were excised from the uterus at 8 1/3 days of gestation (plug day was day 0) using explantation techniques developed by New (13). Each embryo was excised from the uterus, Reichert's membrane was removed, and the visceral yolk sac and ectoplacental cone were left intact. Presomite embryos (late primitive streak–early headfold) with visceral yolk sac widths of 0.6–0.8 mm were selected for culture (14). These specific conditions were chosen because embryos with yolk sac widths of <0.6 mm usually did not complete neural tube closure after 48 hr of culture. On the other hand, embryos with yolk sac widths of >0.8 mm were apparently sufficiently advanced that they were normally unresponsive to glucose-mediated interference with neural tube closure. Embryos were transferred to culture by placing them in 25-ml Erlenmeyer flasks in a medium consisting of immediately centrifuged, heat-inactivated rat serum (13) and a penicillin (50 units/ml)/streptomycin (50 μg/ml) mixture. Glucose (D- or L-, mixed anomers; Sigma) was solubilized in distilled water, sterilized by Millipore filtration, and included in the culture medium as a 3% or 6% addition. The glucose level used as the experimental standard, 8 mg/ml, was chosen since it caused failure of rostral neural tube fusion most of the time. Arachidonic acid (Sigma) was diluted in ethanol and included in the culture medium as a 0.33% addition. Controls received appropriate volumes of distilled water and ethanol. Each flask contained three embryos in 3 ml of medium. The flasks were gassed with a 5% O_2/5% CO_2/90% N_2 mixture and then were stoppered and incubated at 37–38°C on a rotary shaker operating at 70 rpm; the flasks were regassed with the same mixture after 15 hr. At 24 hr, the culture media were changed and the flasks

were gassed with 5% CO_2/95% air and reincubated. The flasks were reexposed to the 5% CO_2/95% air mixture after about 39 hr and the embryos were collected from culture at 48 hr. The visceral yolk sac and the amnion were then removed and the development of the rostral neural tube was evaluated; the embryonic heartbeat was used as a measure of viability. The embryos were photographed and their somite number was determined. The embryos were then fixed for 18 hr at 4°C in 4% formaldehyde/1% $CaCl_2$, pH 7.1, dehydrated, infiltrated, and embedded in glycol methacrylate. Sections (1–2 μm) were prepared and stained with Lee's methylene blue/basic fuchsin.

Developmental responses of presomite embryos in the present study using a rotary shaker compare favorably with responses achieved by Sadler and New (14) using a rotator wheel. After 48 hr of culture, cranial folds were fused in 74–77% of controls (see *Results*) and controls developed 23–26 pairs of somites.

Statistical analyses were performed by the χ^2 method. P values > 0.05 were considered not significant.

RESULTS

Table 1 documents that the administration of arachidonic acid *in vivo*, administered according to either protocol, had no significant effect on the diabetic state of the mother or on the weight gain per embryo of the pregnant rat. β-Hydroxybutyrate levels in the pregnant diabetic rats (data not presented here) were generally between 0.1 and 1 mM. The weight gain per embryo in these diabetic animals was not significantly altered by the arachidonic acid treatment (8.8 ± 1.2 versus 9.3 ± 0.3, P = not significant; the figure used for arachidonic acid represents the mean ± SEM of the combined treatment protocols). This weight gain per embryo was significantly different from what was observed in nondiabetic control animals (13.6 ± 1.1, data not shown in Table 1). The number of embryos per litter averaged 10 or 11 in both the nondiabetic control pregnancies and the streptozotocin-diabetic pregnancies. Treatment with arachidonic acid did not appear to affect the weight of the embryos in the diabetic pregnancies (3.22 ± 0.18 versus 3.14 ± 0.06, P = not significant). This embryonic

Table 2. Effect of arachidonic acid on the embryo of the pregnant diabetic rat

Group	Litters, no.	Embryos, no.	Neural tube fusion defect		Micrognathia		Cleft palate	
			No.	%	No.	%	No.	%
Streptozotocin on day 6	18	193	22	11	13	7	21	11
+ Arachidonic acid, 200 mg/kg per day (one dose), days 9–12	20	212	9*	4	1†	0.5	11‡	5
+ Arachidonic acid, 400 mg/kg per day (two doses), days 5–10	4	50	1‡	2	1	2	0§	0
Total arachidonic acid treated	24	262	10*	3.8	2†	0.8	11*	4

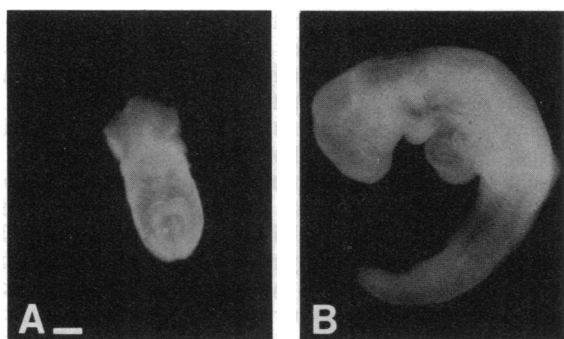*P < 0.005.
†P < 0.001.
‡P < 0.05.
§P < 0.01.

FIG. 1. (*A*) An 8 1/3-day B10.A embryo at explantation. (*B*) Development achieved in a control after 48 hr in culture. The rostral neural tube is now closed. Magnifications for *A* and *B* are the same. (Bar in *A* represents 300 μm.)

Table 3. Glucose inhibition of rostral neural tube closure in culture and reversal by arachidonic acid

Group	B10.A	B10
Control	34/46 (74)	20/26 (77)
D-glucose		
8 mg/ml	6/19 (32)*†	15/26 (58)
16 mg/ml		0/4 (0)‡
L-glucose, 16 mg/ml		3/5 (60)
D-glucose, 8 mg/ml		
+ Arachidonic acid, 1 μg/ml	12/18 (67)	
+ Arachidonic acid, 10 μg/ml	2/3 (67)	

Ratios represent number of embryos with fused neural tubes per total number of embryos. Numbers in parentheses are percentages of embryos with fused neural tubes.
*$P < 0.006$ compared to control.
†$P < 0.03$ compared to D-glucose (8 mg/ml) and arachidonic acid (combined 1 μg/ml and 10 μg/ml experiments).
‡$P < 0.002$ compared to control.

weight is significantly different from that observed in the nondiabetic control group (4.05 ± 0.12, data not shown in Table 1).

Table 2 presents the congenital malformations observed in the embryos of the diabetic rats. Eleven percent of the control diabetic animals were observed to manifest neural tube fusion defects. Seven percent of the diabetic embryos were affected with micrognathia, and cleft palate was observed in 11% of the control diabetic embryos. A significant protective effect on the incidence of malformations was seen following the administration of arachidonic acid at both dose levels. If one takes all of the arachidonic acid-treated embryos as a group of 24 litters comprising 262 embryos and compares this with the 193 embryos in the 18 litters of the diabetic control animals, it can be seen that the neural tube fusion defect was significantly decreased from 11% to 3.8% ($P < 0.005$). The incidence of micrognathia was reduced from 7% to 0.8% ($P < 0.001$), and the incidence of cleft palate was reduced quite significantly ($P < 0.005$).

The results of the *in vitro* studies in the mouse embryo culture system paralleled those obtained *in vivo*. At explantation (Fig. 1*A*), the cranial neural folds are only slightly elevated. After 48 hr in culture, control embryos complete the process of rostral neural tube closure (Fig. 1*B*). Fig. 2*A* shows a section of a control embryo with a clearly fused rostral neural tube. Fig. 2*B* is a section of an embryo that had been cultured for 48 hr in the presence of 8 mg of D-glucose per ml. The neural folds are widely separated and the appearance is characteristic of the inhibition of neural fold fusion seen with hyperglycemia. The example shown was not

chosen because of the severity of the lesion but rather because it is representative of what was observed. A section of an embryo that had been cultured in the presence of D-glucose (8 mg/ml) and arachidonic acid (1 μg/ml) is shown in Fig. 2*C*. The neural folds have completely fused, as in the control (Fig. 2*A*).

The overall data on the B10.A strain are presented in Table 3. Control embryos demonstrated complete neural tube fusion in 34 of 46 instances. This 74% rate is comparable to that seen by Sadler and New (14). The presence of D-glucose at a concentration of 8 mg/ml was associated with a significant inhibition ($P < 0.006$) of neural tube fusion; only 6 of the 19 embryos (32%) incubated in this concentration of glucose demonstrated fused rostral neural tubes. Arachidonic acid supplementation of the medium was protective against the teratogenic action of the elevated levels of D-glucose. Twelve of 18 (67%) embryos incubated in the presence of D-glucose (8 mg/ml) and arachidonic acid (1 μg/ml) demonstrated fused rostral neural tubes. Similarly, the presence of arachidonic acid at 10 μg/ml resulted in a neural tube fusion in 2 of 3 embryos (67%) incubated in the presence of D-glucose (8 mg/ml). Thus, neural tube fusion was seen in 14 of 21 embryos cultured in the hyperglycemic (8 mg/ml) medium to which arachidonic acid had been added. This 67% rate of neural tube fusion was similar to what was observed in the control embryos (P = not significant) but was significantly different ($P < 0.03$) from what was seen in the embryos incubated in D-glucose (8 mg/dl) in the absence of arachidonic acid.
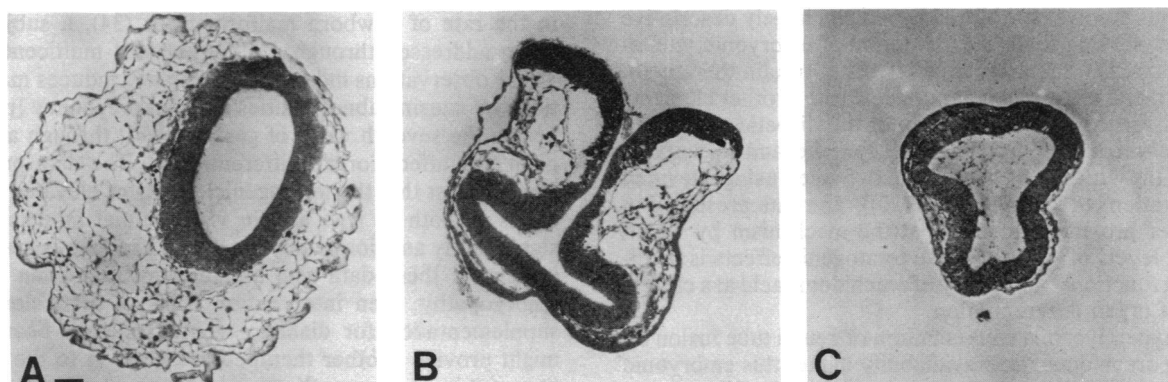

FIG. 2. Sections through the rostral neural tube of B10.A embryos after culture for 48 hr showing a closed neural tube in a control (*A*), open neural folds in a glucose-treated (8 mg/ml) embryo (*B*), and reversal to a fused neural tube in an embryo exposed to glucose (8 mg/ml) and arachidonic acid (1 μg/ml) (*C*). The different shapes of the rostral neural tube in *A–C* reflect slightly different planes of section. Magnifications for *A–C* are the same. (Bar in *A* represents 50 μm.)

Table 3 also suggests that the B10 strain of mouse may be resistant to the teratogenic effect of glucose on neural tube closure in a manner similar to its resistance to phenytoin- or glucocorticoid-induced cleft palate. In this study, the control B10 embryos demonstrated a fusion rate of 77% (20 of 26 embryos). In the presence of D-glucose at a concentration of 8 mg/ml, 58% of the B10 embryos (15 of 26) demonstrated fused rostral neural tubes, a figure not significantly different from what was seen in the control animals. However, in preliminary studies, a teratogenic effect of D-glucose was observed when the concentration was raised to 16 mg/ml. None of 4 embryos demonstrated a fused rostral neural tube, a difference which is significant at the $P < 0.002$ level. Experiments utilizing L-glucose at the same concentration of 16 mg/ml resulted in a value not significantly different from the control rate of fusion of the rostral neural tube.

DISCUSSION

The incidence of failure of neural tube fusion found in the control pregnant diabetic rats in this study was 11%. In our previous publication, the malformation rate for neural tube fusion was 10% (8). These figures are quite similar to what was observed by Eriksson *et al.* (9), who found neural tube fusion defects in 10% of the fetuses born to rats that had been made diabetic prior to pregnancy. This rate of neural tube fusion defects in the control diabetic rats was markedly altered by the administration of arachidonic acid during the period of organogenesis. In this large series of litters and embryos, the incidence of neural tube fusion defects was reduced from the 11% level to 3.8%, a highly significant ($P < 0.005$) reduction. In addition, the incidence of micrognathia was reduced from 7% [Eriksson *et al.* (9) found 9%] to 0.8%, again highly significant ($P < 0.001$). The incidence of cleft palate was similarly reduced. Although cleft palate has not usually been considered a manifestation of diabetic embryopathy, this malformation is seen in excess in infants of diabetic mothers (5, 15), and this malformation is also seen in the mouse animal model of diabetic embryopathy (16). The observations *in vivo* have been strengthened by parallel studies of the protective effect of arachidonic acid on the whole mouse embryo cultured in the presence of elevated levels of glucose. The teratogenic effects of excess glucose on the rate of neural tube fusion in the mouse embryo culture reported here are similar to those found in the reports of Sadler (7), Garnham *et al.* (17), and Cockcroft and Coppola [in rats, (6)]. The protection provided by arachidonic acid supplementation is seen in the improvement in the rate of neural tube fusion (67%) to the control level (74%) from the low rate of neural tube fusion seen in the embryo cultured in the presence of D-glucose alone (32%).

Previous studies in this area have been largely descriptive in nature, serving to define the period of embryonic vulnerability (8, 9, 17), the protective effects of insulin therapy (8, 9, 18, 19), and the possible teratogenic role of other elements of the disturbed metabolic milieu of the diabetic pregnancy [the "fuel-mediated" hypothesis of Freinkel and co-workers (1) and the studies of Horton and Sadler using elevated concentrations of ketone bodies (20)]. The data presented in this paper provide evidence that the mechanism by which elevated levels of glucose cause teratogenic effects is mediated by a functional deficiency of arachidonic acid at a critical period of organ differentiation.

That hyperglycemia causes failure of neural tube fusion by affecting arachidonic acid availability makes this embryonic malformation similar to what has been described for experimentally produced cleft palate. Neural tube fusion and palatal shelf fusion are analogous in that both require embryonic cell movements and apposition of the two advancing layers (21, 22), with cell death at that point leading to fusion

and closure (23, 24). In the malformation model of glucocorticoid- and phenytoin-induced cleft palate, this sequence of events has been experimentally delineated (11, 25). Glucocorticoids or phenytoin interact with a cytosolic receptor and the receptor complex induces phospholipase A_2-inhibiting proteins (PLIP) (26, 27). Phospholipase A_2 catalyzes the release of arachidonic acid from its position on the number 2 carbon of phospholipids; an increase in PLIP therefore sharply decreases the availability of arachidonic acid. This sequence has been verified by experiments that demonstrate that specific blockade of the cytosolic glucocorticoid receptor or provision of exogenous arachidonic acid will act to prevent the malformation (28–30). In this system, arachidonic acid availability for prostaglandin synthesis appears to be of major importance, since indomethacin will reverse the protective effects of exogenous arachidonic acid (28, 29). Whether this is also true in hyperglycemia-induced failure of neural tube fusion remains to be clarified. Preliminary studies in experimental models of failure of penile fusion also implicate the arachidonic acid and prostaglandin pathways,[||] thus raising the possibility that this system may be a major mechanism mediating many embryonic events that involve cellular movements and fusion.

Another intriguing parallelism between glucocorticoid- or phenytoin-induced cleft palate and hyperglycemia-induced failure of neural tube fusion is the possible difference in susceptibility to these lesions in mice of the B10.A and B10 strains. These congenic strains differ only in the H-2 alleles of chromosome 17 and could therefore provide a model to map the genetic susceptibility to these malformations. Differences in susceptibility to cleft palate have been explained by the influence on glucocorticoid and phenytoin receptor levels of genes in the H-2 region (11, 25, 31). Further study is needed to define the mechanism by which hyperglycemia-induced effects are influenced by genes in this region. Studies of this model could provide a basis for the clinical impression that there are genetic influences on the susceptibility to diabetic embryopathy (such as the repeat occurrence of malformations in certain families) and provide a genetic basis for the differences in rates of skeletal malformation noted by Eriksson *et al.* (32) in embryos of diabetic rats of the Uppsala and Hannover substrains of Sprague–Dawley rats.

Studies in human diabetic embryopathy have been consistent with the observations in experimental animals. Diabetic women with elevated levels of glycosylated hemoglobin at the time of their initial prenatal evaluation in the first trimester are at much higher risk for having offspring with malformations than women whose metabolic control has resulted in lower levels of hemoglobin A_1C (33). Meticulous diabetic control during the period of conception and the first weeks of pregnancy appears to result in a major improvement in the rate of newborn malformations (34), a subject also being addressed through a U.S. national multicenter trial. These observations indicate that diabetes induces malformations by causing abnormalities early in pregnancy [probably before the seventh week of gestation (4)] through a mechanism that affects organ differentiation. The data presented here suggest that the teratogenic effects of elevated glucose levels in both *in vivo* and *in vitro* animal models can be significantly ameliorated by providing exogenous arachidonic acid. If these data also are relevant to human diabetic embryopathy, then intervention trials of arachidonic acid supplementation for diabetic women of child-bearing age might provide another therapeutic approach to the prevention of this major complication of pregnancy in the diabetic woman.

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