0031-3998/91/3002-0150$03.00/0
PEDIATRIC RESEARCH
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Vol. 30, No. 2, 1991
Printed in U.S.A.

Effects of Maternal Diabetes or *In Vitro* Hyperglycemia on Uptake of Palmitic and Arachidonic Acid by Rat Embryos

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ABSTRACT. Altered transfer of nutrients from mother to conceptus may be involved in the pathogenesis of the developmental disturbances in offspring of diabetic mothers. In our study, the embryonic uptake of a saturated (palmitic acid) and a nonsaturated (arachidonic acid) fatty acid was evaluated in a normal and a diabetes-like environment under *in vivo* and *in vitro* conditions that yield growth retardation and somatic malformations in the embryos. The palmitic acid uptake in embryos from diabetic rats and in embryos cultured *in vitro* in 30 mmol/L D-glucose did not differ from the respective controls. Only embryos cultured in the highest D-glucose concentration (60 mmol/L) showed slightly increased uptake, which suggests that alterations in palmitic acid transfer have no role in the processes of embryonic maldevelopment in diabetic pregnancy. In contrast, the results showed that a diabetes-like environment both *in vivo* and *in vitro* causes increased embryonic uptake of arachidonic acid. Consequently, if the teratogenic mechanisms of diabetic pregnancy involve decreased embryonic levels of arachidonic acid, as has been suggested, this would not be the effect of a decreased uptake per se, but rather of an altered intracellular metabolism or decreased extracellular availability of this fatty acid. (*Pediatr Res* 30: 150–153, 1991)

Abbreviations

N, normal, nondiabetic state
MD, manifestly diabetic state (serum glucose >20 mmol/L)

The rate of fetal malformations in diabetic pregnancy is approximately 3-fold higher than in nondiabetic gestation, and the malformations are currently the leading cause of perinatal death in the infants of diabetic mothers (1–3). These infants show other developmental disturbances, such as macrosomia, pancreatic β-cell hypertrophy and hyperplasia, delayed pulmonary maturation (4, 5) and, as has been recently demonstrated, disturbed psychomotor development when tested 4 y after birth (6). The exact pathogenetic mechanisms causing these developmental perturbations are presently unknown.

An undisturbed nutrition of the embryo/fetus *in utero* has been postulated to be necessary for a successful outcome of pregnancy. Because diabetic women show alterations in serum concentrations of all classes of nutrients (4) in addition to decreased blood flow to the placental bed (7), a common pathogenetic mechanism behind the complications in children of diabetic mothers may be a disturbance of the transport of nutrients from the mother to her embryo/fetus. The hypotheses by Pedersen (4) and others (2) imply that the fetus of the diabetic mother is subjected to an increased influx of glucose, in addition to altered placental transfer of lipids and amino acids. The nutritional status of the early embryo of the diabetic mother, however, is unknown. Previous experimental studies in rodents have demonstrated that the major nutritional organ of the early embryo, the yolk sac, shows marked morphologic alterations in diabetic pregnancy (8), as well as after whole embryo culture in elevated glucose concentrations (9, 10). These morphologic changes suggest impaired function of the visceral yolk sac. The present work aimed to study the transport of one important class of nutrients, the lipids, to the offspring in early diabetic pregnancy. This was done by investigating the uptake of fatty acids by embryos previously exposed to maternal diabetes *in vivo* or to a 48-h *in vitro* period in an elevated glucose concentration. We have previously found growth retardation and malformations in embryos subjected to both of these experimental conditions (11). The transfer of the nonsaturated arachidonic acid to the embryos was of special interest, inasmuch as supplementation of this fatty acid decreases the incidence of malformations in pregnant diabetic mice and rodent embryos cultured in elevated glucose concentrations (12, 13). Furthermore, arachidonic acid supplementation also normalizes yolk sac morphology and partially restores the content of different lipids in embryos and membranes cultured in elevated glucose concentrations (14). These findings suggest that a decreased embryonic level of this fatty acid, *e.g.* as a result of decreased uptake, may have considerable developmental consequences.

MATERIALS AND METHODS

Induction of diabetes. The rats used were of a Sprague-Dawley substrain prone to congenital skeletal malformations in diabetic pregnancy (15) and were maintained under outbreeding conditions. They were given standard pelleted food (EWOS AB, Södertälje, Sweden) and tap water *ad libitum* and were subjected to a 12 + 12 h light-dark schedule. Diabetes was induced in female virgin rats (weighing 250–300 g) with a single i.v. injection of streptozotocin (kindly donated by Dr. W. E. Dulin, Upjohn Co., Kalamazoo, MI) at a dose of 40 mg/kg body weight. A serum glucose concentration above 20 mmol/L 1 wk later (Beckman Glucose Analyzer II; Beckman Instruments, Brea, CA) indicated the presence of manifest diabetes (MD). The control female rats (N) of similar weight and age as the MD animals were not injected with streptozotocin. The N and MD female rats were subsequently caged overnight with nondiabetic males and the day sperms were found in a morning vaginal smear was desig-

Received July 30, 1990; accepted March 9, 1991.
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Supported by The Bank of Sweden Tercentenary Foundation, The Swedish Medical Research Council (Grant No. 12X-7475), The "Expressen" Prenatal Research Foundation, The Swedish Diabetes Association, The Nordic Insulin Fund, The Swedish Hoechst Diabetes Fund, and The Juvenile Diabetes Foundation.

nated gestational day 0. The time period between streptozotocin injection and the beginning of mating was 10 d in the MD group.

The pregnancy was allowed to continue up to gestational days 9 or 11, at which time the embryos were dissected out and subjected to either fatty acid uptake experiments (d 11) or to whole embryo culture (d 9) for 2 subsequent days followed by uptake studies (compare Fig. 1).

Direct uptake study. On gestational day 11 between 1100 and 1300 h, embryos from N and MD rats were dissected out from the uterus after cervical dislocation of the mother. The embryos were preincubated for about 5 min in 5 mL RPMI 1640 (Flow Laboratories, Irvine, UK) in 50-mL plastic tubes (Falcon 2070; Becton Dickinson Co., Oxnard, CA) previously gassed with 40% O_2, 5% CO_2, and 55% N_2 (vol/vol/vol). During the preincubation period, the tubes were kept rotating (60 rpm) at 38°C in an incubator. The embryos were then transferred to new Falcon tubes with prepared uptake medium (see below).

Whole embryo culture. On gestational day 9 between 1100 and 1300 h, pregnant N rats were killed by cervical dislocation. The concepti were carefully dissected out and embryos, within intact yolk sacs, were explanted and cultured for 48 h in rotating (60 rpm) plastic tubes (Falcon 2070), containing 75% "immediately centrifuged" rat serum and saline supplemented with D-glucose (BDH Chemicals Ltd., Poole, UK) to a final concentration of 10, 30, or 60 mmol/L (16). The culture medium was also supplemented with 60 μg/mL potassium benzyl penicillinate and 100 μg/mL streptomycin (Flow Laboratories). Each culture tube contained 5 mL medium and three to five concepti. During the culture period, the tubes were kept continuously rotating in an incubator at 38°C. The concepti were initially gassed with 5% O_2, 5% CO_2, and 90% N_2 (vol/vol/vol) for 10 min in the culture tubes. The tubes were then capped and transferred to the incubator. After 24 h, the culture tubes were opened and the concepti were transferred to new tubes with fresh medium, gassed with 20% O_2, 5% CO_2, and 75% N_2 (vol/vol/vol) for 10 min and returned to the incubator. The next morning (*i.e.* after an additional 20 h of culture) the tubes were gassed with 40% O_2, 5% CO_2, and 55% N_2 (vol/vol/vol) (10 min, no change of medium) and, 4 to 6 h later, subjected to fatty acid uptake study (see below).

Preparation of uptake medium. A solution of 2.78 mg palmitic acid (sodium salt, Sigma Chemical Co., St. Louis, MO) and 100 μL [9,10(n)-^{3}H]-palmitic acid (40–60 Ci/mmol; Amersham International plc, Amersham, Buckinghamshire, UK) were thoroughly mixed with 400 μL 95% ethanol. Thereafter, 200 μL 0.5 M NaOH was added and the solution was centrifuged (600 rpm for 10 min) and dried overnight at 60°C. The powder was then dissolved in 2 mL warm RPMI 1640 (Flow Laboratories) and diluted with RPMI 1640 containing 1% bovine albumin (essentially fatty acid free, Sigma Chemical Co.) to reach the end concentrations of 0.25, 0.5, or 1.0 mmol/L palmitic acid. The medium was placed on a shaker overnight, distributed to uptake tubes (Falcon 2070) the next morning, and gassed with 40% O_2, 5% CO_2, and 55% N_2 for 5 min before addition of the concepti.

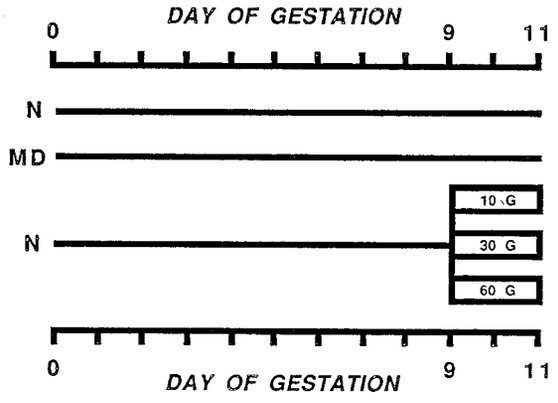

Fig. 1. Schematic outline of the different experimental groups subjected to fatty acid uptake experiments. *10G*, *30G*, and *60G* denote embryos from 48-h cultures in 10, 30 or 60 mmol/L D-glucose.

A shorter procedure was used to make the uptake medium for arachidonic acid, where the fatty acid (sodium salt, Sigma Chemical Co.) was directly dissolved in RPMI 1640 (containing 1% essentially fatty acid-free bovine albumin) to yield end concentrations of 0.25, 0.5, or 1.0 mmol/L. To each uptake tube (Falcon 2070) containing 5 mL medium, 50 μL [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (80–135 Ci/mmol, Amersham) was added, and the tubes were gassed with 40% O_2, 5% CO_2, and 55% N_2 for 5 min.

Incubation of concepti. To each uptake tube (containing 5 mL medium), three to five concepti were transferred, from either preincubation tubes (N or MD d-11 embryos) or culture tubes (48-h cultured d-9 embryos from N rats). The uptake tubes were capped and transferred to the incubator where the tubes were left rotating (60 rpm) for 30 min. Aliquots of 25 μL uptake medium were drawn before and after the incubation for estimation of added radioactivity.

After the incubation, the concepti were quickly removed, washed in cold saline, and inspected with the aid of a stereo microscope. Concepti with ruptured yolk sacs were discarded. The undamaged concepti were dissected, and the embryos and yolk sac membranes were separately placed in 0.5 mL 0.5 M NaOH, disrupted ultrasonically (20 kHz, 60 W; Ultrasonic, MSE, London, UK), and left at 4°C overnight. DNA was measured fluorometrically by the method of Kissane and Robins (17) as modified by Hinegardner (18). After addition of 4 mL of a scintillator (Supersolve X; Scintvaruhuset AB, Uppsala, Sweden), the radioactivity in medium and sonicates was determined in a liquid scintillation counter (no. 300-C; Packard AB, Stockholm, Sweden) and converted to dpm by external standardization.

Ten concepti from N rats were incubated for 30 s in uptake medium with added tracer (five in palmitic acid, five in arachidonic acid) to yield blank values of label uptake in concepti. Calculation of fatty acid uptake was made using the sp act of the uptake medium (dpm/amount fatty acid) and the radioactivity counts of the sonicates (minus the blank values). The signal/blank ratio was always >3.5 in the sonicates.

Statistics. Statistical comparisons between the different groups were made with the aid of a two-tailed *t* test (19). MD concepti were compared with N concepti, whereas concepti cultured in 10 mmol/L glucose were used as the standard for comparisons within the *in vitro* group.

RESULTS

The changes in DNA content (μg) induced by maternal diabetes or high glucose culture were parallel in the palmitic acid and arachidonic acid groups (Table 1). Thus, both MD embryos/membranes and 60-mM glucose-cultured embryos/membranes had less DNA than their N and 10-mM glucose-cultured counterparts, whereas 10- and 30-mM glucose-cultured embryos/membranes did not differ in DNA content (Table 1). Furthermore, all groups of *in vitro* cultured embryos and membranes (with the exception of 10-mM glucose-cultured membranes subjected to arachidonic acid uptake) showed less DNA than their corresponding N embryos/membranes ($p < 0.05$).

There were no differences in palmitic acid uptake (estimated as pmol/min and DNA) between the N and MD embryos (Fig. 2) and membranes (Fig. 3) at any concentration of the fatty acid. Furthermore, the palmitic acid uptake of embryos and membranes cultured *in vitro* in 10 and 30 mmol/L glucose only differed marginally from the corresponding N and MD embryos/membranes (Figs. 2 and 3). In contrast, the 60-mM glucose-cultured concepti displayed increased uptake of the fatty acid in both embryos (at 0.5 and 1.0 mmol; compare Fig. 2) and membranes (Fig. 3).

The arachidonic acid uptake (pmol/min and DNA) was in-

Table 1. *DNA content (μg) in embryos and membranes from offspring of N and MD rats on gestational day 11 and from d-9 N concepti cultured for 48 h in presence of 10, 30, or 60 mmol/L glucose (10G, 30G, or 60G)**

	Embryos				Membranes			
	n	PALM	*n*	ARAC	*n*	PALM	*n*	ARAC
N	35	37.5 ± 1.4	90	38.0 ± 1.2	36	9.1 ± 0.5	95	9.0 ± 0.2
MD	37	20.5 ± 1.6†	84	21.5 ± 1.0†	30	6.5 ± 0.5†	78	6.0 ± 0.4†
10G	22	22.5 ± 1.4	28	28.4 ± 1.9	22	6.6 ± 0.5	28	8.0 ± 0.6
30G	20	23.8 ± 1.5	44	26.8 ± 1.4	23	7.4 ± 0.6	44	7.1 ± 0.3
60G	18	15.7 ± 2.2‡	47	18.4 ± 1.4§	17	4.9 ± 0.5‖	36	5.1 ± 0.3§

* The concepti were subjected to uptake experiments of either palmitic acid (PALM) or arachidonic acid (ARAC). Values are mean ± SEM.
† $p < 0.001$ *vs* N embryos/membranes.
‡ $p < 0.01$ *vs* 10G embryos.
§ $p < 0.001$ *vs* 10G embryos/membranes.
‖ $p < 0.05$ *vs* 10G membranes.

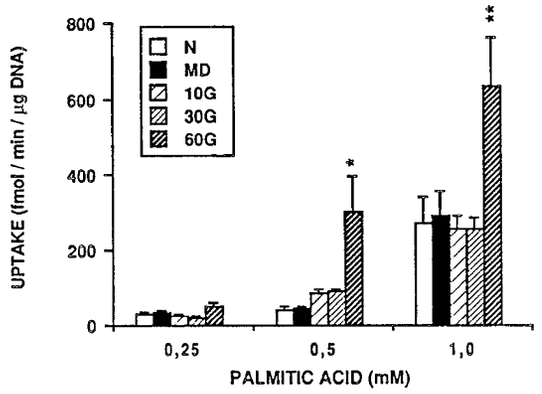

Fig. 2. Uptake of palmitic acid by day-11 embryos from N and MD rats and by day-9 embryos of N rats cultured for 48 h *in vitro* in 10, 30, or 60 mmol/L glucose (*10G*, *30G*, or *60G*) ($8 \leq n \leq 37$, mean + SEM). MD embryos are compared to N embryos; 30G and 60G embryos are compared to 10G embryos. Significances: *, $p < 0.05$; and **, $p < 0.01$.

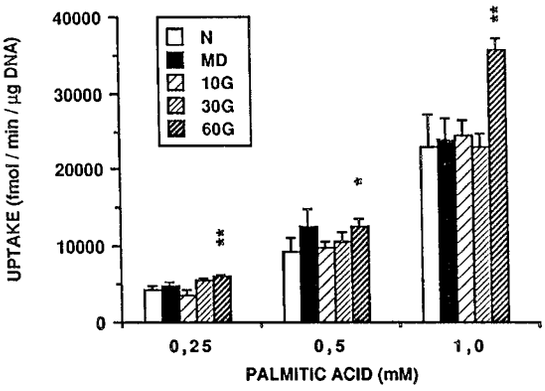

Fig. 3. Uptake of palmitic acid by day-11 membranes from N and MD rats and by day-9 membranes of N rats cultured for 48 h *in vitro* in 10, 30, or 60 mmol/L glucose (*10G*, *30G*, or *60G*) ($5 \leq n \leq 36$, mean + SEM). MD membranes are compared to N membranes; 30G and 60G membranes are compared to 10G membranes. Significances: *, $p < 0.05$; and **, $p < 0.01$.

creased in MD embryos compared with N embryos at 1.0 mM of the fatty acid, whereas at lower concentrations a similar difference failed to become significant ($0.05 < p < 0.1$, Fig. 4).

There were no differences in arachidonic acid uptake (pmol/min and DNA) by N and MD membranes (Fig. 5). The *in vitro* cultured concepti showed a pattern of increased uptake of arachidonic acid with increased glucose concentration during culture. This was significant in embryos at 0.5 and 1.0 mmol/L arachidonic acid (Fig. 4) and in membranes at 1.0 mmol/L (Fig. 5), and a similar trend (where the numerical difference failed to reach statistical significance) could be found in embryos at 0.25 mmol/L and membranes at 0.5 mmol/L of the fatty acid (Figs. 4 and 5).

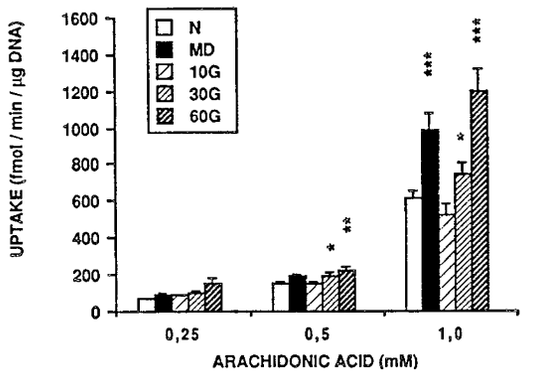

Fig. 4. Uptake of arachidonic acid by day-11 embryos from N and MD rats and by day-9 embryos of N rats cultured for 48 h *in vitro* in 10, 30, or 60 mmol/L glucose (*10G*, *30G*, or *60G*) ($11 \leq n \leq 90$, mean + SEM). MD embryos are compared to N embryos; 30G and 60G embryos are compared to 10G embryos. Significances: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

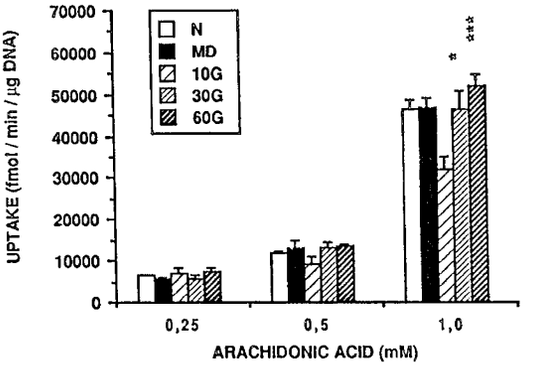

Fig. 5. Uptake of arachidonic acid by day-11 membranes from N and MD rats and by day-9 membranes of N rats cultured for 48 h *in vitro* in 10, 30, or 60 mmol/L glucose (*10G*, *30G*, or *60G*) ($5 \leq n \leq 95$, mean + SEM). MD membranes are compared to N membranes; 30 G and 60G membranes are compared to 10G membranes. Significances: *, $p < 0.05$; and ***, $p < 0.001$.

When the total uptake (pmol/min) was compared in embryos and membranes at 0.5 mmol/L of each fatty acid, only a few differences emerged within the groups (Table 2). The palmitic acid uptake in the 30-mM glucose-cultured group of membranes was higher than in the 10-mM glucose group, and the arachidonic acid uptake was lower in both MD embryos and MD membranes compared with N embryos/membranes (Table 2).

Among the embryos, in particular those from the MD and 60-mM glucose-cultured groups, were some that showed severe morphologic alterations (rotation defects, open neural tube, enlarged heart) in addition to diminished size. The uptake of fatty acids of these embryos (and their corresponding membranes) did not differ in any consistent way from the controls (N and 10-mM glucose-cultured concepti).

DISCUSSION

In human diabetic pregnancy, the teratogenic insult has been estimated to occur before the 7th week of gestation (20). This estimation has been supported by experimental studies. In diabetic rat pregnancy, congenital malformations of the skeleton

Table 2. *Uptake of fatty acids (pmol/min) in embryos and membranes from offspring of N and MD rats on gestational day 11 and from d-9 N concepti cultured for 48 h in presence of 10, 30, or 60 mmol/L glucose (10G, 30G, or 60G)**

	Embryos				Membranes			
	n	PALM	*n*	ARAC	*n*	PALM	*n*	ARAC
N	14	1.6 ± 0.3	25	5.6 ± 0.6	13	81 ± 15	28	108 ± 8
MD	8	0.9 ± 0.1	27	3.9 ± 0.3†	11	93 ± 14	21	70 ± 13‡
10G	9	1.9 ± 0.1	11	3.9 ± 0.5	10	57 ± 6	11	74 ± 11
30G	8	2.2 ± 0.2	18	5.2 ± 0.6	9	83 ± 6§	18	87 ± 8
60G	10	2.0 ± 0.3	15	4.2 ± 0.5	5	55 ± 11	5	92 ± 6

* The concepti were subjected to uptake experiments of either 0.5 mmol/L palmitic acid (PALM) or 0.5 mmol/L arachidonic acid (ARAC). Values are mean ± SEM.

† $p < 0.05$ *vs* N embryos.

‡ $p < 0.01$ *vs* N membranes.

§ $p < 0.01$ *vs* 10G membranes.

and neural tube are likely to be induced during the organogenetic period (21–23). Both D-glucose and β-hydroxybutyric acid are able to induce malformations in rodent embryos *in vitro*, particularly in young embryos with no or few somites (24–26).

The available data thus indicate that the teratogenic period(s) in diabetic pregnancy occur(s) before the establishment of the chorioallantoic placenta, *i.e.* at a time when the embryo is dependent on the yolk sac placenta for its nutrition. Previous experimental studies in rodents have demonstrated that the yolk sac shows marked morphologic alterations in a diabetic environment, both *in vivo* and *in vitro*. The thickness of the endodermal cell layer of the yolk sac is decreased and contains fewer vitelline vessels than in normal pregnancy. The endodermal cells also show fewer microvilli and a decreased amount of rough endoplasmic reticulum, as well as lower numbers of ribosomes, mitochondria, and lipid droplets in the cytoplasm (8–10). These changes suggest impaired function of the visceral yolk sac.

In our study, we have demonstrated, that the uptake of palmitic acid and arachidonic acid at physiologic concentrations was largely dose-dependent. The yolk sac membranes' uptake of the fatty acids exceeded that of the embryos at all concentrations of the fatty acids. The uptake of arachidonic acid was slightly greater on a molar basis than that of palmitic acid, both in embryos and membranes. Embryos from manifestly diabetic rats showed a marginal increase in arachidonic acid uptake compared with controls, whereas diabetes in the mother did not seem to affect palmitic acid uptake at all. In contrast, culture in elevated glucose levels exerted more profound effects, inasmuch as both embryos and membranes of the 60 mmol/L glucose group showed increased uptake of both palmitic and arachidonic acid.

The results of our study suggest that a diabetes-like environment both *in vivo* and *in vitro* causes increased uptake of arachidonic acid. Consequently, if the teratogenic mechanisms of diabetic pregnancy involve decreased embryonic levels of arachidonic acid, as has been suggested by several previous authors (12, 13), this would not be the effect of a decreased uptake per se, but rather of an altered intracellular metabolism or a decreased extracellular availability of this fatty acid. Changes in palmitic acid uptake seem to have no role in the processes of embryonic maldevelopment in diabetic pregnancy.

Acknowledgments. The authors thank Parri Wentzel and Birgitta Bodin for excellent technical assistance.

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