Long-Term Effects of Maternal Diabetes on Vascular Reactivity and Renal Function in Rat Male Offspring

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ABSTRACT

Fetal growth impairment can occur in pregnancy complicated by diabetes. Although several studies have focused the effects of nutritional status on intrauterine development, the long-term impact of maternal diabetes on vascular and renal function in the offspring is poorly investigated. In the present study, blood pressure profiles and renal function parameters were investigated in the offspring of diabetic rats (DO). Female rats were made diabetic throughout gestation with a single dose of streptozotocin (STZ) 10 d before mating. After weaning, the offspring had free access to food and water. Arterial pressure was evaluated every 15 d. Functional and morphometric kidney studies were performed in newborn, 3, 6 and 12-mo-old male rats in DO and in controls, C. Although maternal diabetes did not affect nephron number in the young adult rat, glomerular hypertrophy developed from 3 mo on. Glomerular Filtration Rate and Renal Plasma Flow were observed to be significantly decreased in DO when compared with C, from 3 mo on. In DO, hypertension was

observed from 8 wk on and persisted elevated throughout the experimental period (12 mo). Vascular reactivity, evaluated in mesenteric arterial bed showed a decreased endothelium-dependent vasodilatation in 12-mo-old DO animals, while preserved response to sodium nitroprusside was demonstrated. Our data show that exposure to intrauterine diabetes induced by STZ does not affect nephron number in the young offspring but can cause permanent changes in Nitric Oxide (NO)-related vascular response, which, in turn may accelerate the natural age-related nephron loss. (*Pediatr Res* 58: 1274–1279, 2005)

Abbreviations

DO, offspring from diabetic mother
NAE, net acid excretion
NB, newborn,
PAH, para-aminohippurate,
RPF, renal plasma flow

Maternal status can affect several physiologic functions of the newborn. In addition, correlation between fetal growth conditions and susceptibility to a number of adult chronic diseases, including coronary heart disease, stroke and hypertension have been identified (1–4). Recently, we have demonstrated that maternal undernutrition promoted development of adult hypertension, impairment of the renal and endothelium functions, decreased absolute number of the nephrons and hypertrophy of the remaining glomeruli in the adult offspring (5–7). Although several experimental studies have focused the effects of the maternal undernutrition on fetal “programming” of adulthood disease, less attention has been paid to the possible *in utero* late effects of maternal diabetes. In fact, diabetes mellitus can impose several threats both to the mother

and the offspring. Experimental and clinical studies have demonstrated that diabetic pregnancy increases the risk of intrauterine death, prematurity, perinatal mortality and congenital malformations (8–12). Indeed, Chugh *et al.* (13) have demonstrated that a sustained exposure of the fetus to elevated concentration of glucose may result in diabetic embryopathy, which is characterized by a multitude of congenital birth defects, including those of the nervous, cardiovascular, skeletal, and renal systems. These malformations result from defects occurring in early organogenesis including failure of neural tube closure, caudal regression syndrome, and urogenital abnormalities, which can be as severe as renal agenesis (5,14–16). Amri *et al.* (17) have demonstrated that exposure to hyperglycemia *in utero* impairs nephrogenesis in the rat, leading to a reduced number of nephrons. This nephron deficit could be a risk factor for the development of chronic renal disease and hypertension in adulthood. Other studies have shown that the offspring of diabetic mothers have higher incidence of glucose intolerance, obesity, insulin resistance, and hypertension in later life. Manderson *et al.* (18) showed

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that offspring of diabetic mothers might be at an increased risk for the development of vascular disease in later life. Holemans *et al.* (19) have suggested that maternal diabetes may also have lasting adverse consequences on cardiovascular function of the next generation, particularly because offspring of diabetic pregnant rats demonstrate overt insulin resistance in adulthood. On the other hand, aging is associated with loss of renal mass which, by itself, has little impact on overall renal function but may increase the vulnerability of the kidney to other injuries (20). The functional changes include a rise in renal vascular resistance, a decline in GFR, and a rise in filtration fraction (21,22). Age-related increases in renal fibrosis and in reactive oxygen species production were also demonstrated (23,24)

The present experiments were designed to investigate the possible effects of maternal diabetes on renal and vascular function at different ages of the offspring. In addition, renal morphometry and blood pressure profiles were also evaluated. Our data show that glomerular hypertrophy and hypertension developed in an early period of life in the offspring might be related to vascular dysfunction in this experimental model. In the aged diabetic offspring, besides hypertension, intensification of nephron loss was also observed.

METHODS

All procedures used in this study were approved and performed in accordance with guidelines of the Ethics Committee of Biomedical Institute, Federal University of São Paulo and conformed to the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health Publication No.85-23, revised 1996). Wistar rats from our colony (Federal University of São Paulo) were maintained in a room at $22 \pm 1^\circ\text{C}$ with a 12 h light cycle and 60% humidity.

Animals. Diabetes mellitus was induced by streptozotocyn (50 mg/kg), given by a single intraperitoneal injection to female Wistar rats (250–300 g). Control animals were given an equivalent amount of citrate buffer. Diabetic state was confirmed 48 h after, by measuring blood glucose. Blood glucose concentration was evaluated every three days. Only those animals with glycemic levels above 250 mg/dl were considered for mating. The diabetic rats were caged overnight with a male. Vaginal smears were taken in the following morning and a positive smear was considered as day 0 of gestation. All dams were housed and fed individually with the same diet. Maternal body weight gain was measured every 3 d. After birth, each litter, consisting of 6 male rats, was left with the mother for 28 d; when the male number was not enough to complete 6, females were used but discarded at weaning. The offspring were divided into two groups: a) group C—offspring of control mothers; b) group DO—offspring of diabetic mothers. After weaning, the rats were placed in individual cages, with free access to food and water until the time for the experiments. Animals from both C and DO groups were used in a randomized manner for the studies detailed below. Glycemia was measured in newborn rats (NB), 12 h after delivery. In 3, 6 and 12 mo-old rats, glycemia was measured in 12 h-fasted animals. Renal function was evaluated in 3, 6, and 12 mo-old rats. In addition, kidney morphometry was performed at the same mentioned ages and also in NB rats.

Renal function studies. The animals were anaesthetized with sodium thiopental (30 mg/kg) and placed on a heated table to maintain body temperature at 37°C . Tracheotomy was followed by insertion of polyethylene catheters into the jugular vein for infusions, and into the carotid artery for blood sampling. Urine was collected from a catheter inserted into the bladder. After the surgical procedure, one hour stabilization period was allowed before the beginning of three collection periods. The animals were primed with 1 mL of saline containing inulin (300 mg/kg, and sodium para-aminohippurate (PAH, 2 mg/rat) and then submitted to continuous infusion of a saline solution containing inulin (15 g/L) and PAH (4 mg/L) at 0.08 mL/min. Plasma and urine inulin and PAH concentrations were measured by colorimetry for estimation of GFR (GFR) and renal plasma flow (RPF). Blood and urine Na^+ were measured using ion selective electrode (Ciba-Corning 614 Na^+/K^+ analyzer). Net acid excretion (NAE) was calculated using the formula: $\text{NAE} = \text{TA} + \text{NH}_4^+ - \text{HCO}_3^-$, where TA is titratable acidity in urine measured by microtitration with 0.01 M sodium hydroxide, NH_4^+ , excreted amount of ammonium evaluated by

colorimetry and HCO_3^- is the net bicarbonate excretion calculated with a Blood Gas Analyzer (Ciba Corning, model 248). For protein excretion determination, rats were placed in metabolic cages and a 24-h urine collection was performed. Proteinuria was measured from 2 mo on, every 4 wk. Protein concentration was measured by precipitation with 3% sulfosalicylic acid.

All drugs and reagents were purchased from Sigma Chemical Co.-Aldrich, St. Louis Missouri, USA.

Morphometric study. The morphologic evaluation was obtained by the following methodology: both kidneys from rats of each group were dissected out rapidly, cleaned of connective tissue, weighed and fixed in Bouin. Kidneys longitudinally cut were wax embedded and histologic sections (5 μm width) near renal hilus were performed. Sections cut were stained with hematoxylin and eosin to morphologic analysis. Glomerular area and diameters were measured using an image analysis program, Image-Pro PLUS, Media Cybernetica, MD, USA. Images were acquired in microscope (Leica DMLB, Wet-zlar, Germany) connected to a microcomputer by a video camera (Sony-CCD-IRIS). Ten different images (fields) in 10 different slides were analyzed for each group. Each image had an area of 285 000 μm^2 . The number of glomeruli was also evaluated in these same fields. Total glomeruli number was calculated taking into account the glomeruli number per field, the number of studied fields, the glomerular diameter and the section thickness. Two different investigators who were unaware of the origin of the specimens performed this study.

Measurement of systolic blood pressure. Systolic blood pressure was determined in conscious rats from both groups by an indirect tail-cuff method (Harvard Apparatus, UK). Before the effective measurements, rats were adequately trained; briefly, animals were placed in the restrainers for several times. They were covered with a dark tissue; after this step, they remained calm and the pressure records were made after a 15 to 20 min of quietude. The ambient was silent, with constant temperatures and the measures were always performed by the same person. Three consecutive measures were taken and if a great variability was found, all the values were discarded and in the following day, the procedures were repeated. Rats were preheated at 40°C for 5 min, and then three stable consecutive measurements of blood pressure were averaged.

Vascular reactivity in vivo in mesenteric microvessels. At 12 mo of age, males from both C and DO groups were used in a randomized manner for the vascular study. Rats were anesthetized with chloral hydrate (450 mg/kg, s.c), and the mesentery was exteriorized and arranged for microscopic observation *in situ* according to Zweifach (23) with slight modifications by Fortes *et al.* (24). Changes in the diameter of second order arterioles were measured following the application of vasoactive drugs: ACh (300 $\mu\text{g}/\text{mL}$); BK (3.0 $\mu\text{g}/\text{mL}$) and SNP (1.0 mg/mL) in a standard volume of 0.01 mL and were removed by washing out with the warmed Ringer-Locke solution. For each animal, at least three different microscopic fields were observed and the arteriole diameter measured. The rats were killed by excessive anesthetic dose.

Statistical analysis. Statistical analysis was performed by unpaired *t* test, Mann-Whitney U-test or by analysis of variance, followed by the Scheffé test, where appropriate. Values that were not normally distributed were subjected to the Kruskal-Wallis test, followed by Dunn's posttest, when necessary. Statistical significance was defined as $p < 0.05$. The results are reported as means $\pm$ SEM.

RESULTS

Effect of maternal diabetes on body weight, glycemia and blood pressure levels in the adult offspring. As shown in Table 1, in NB, no differences were observed between C and DO concerning body weight. However, from 6 mo on, DO tended to increase the body weight, which at 12 mo, was significantly higher, as shown in Fig. 1. As also shown in Table 1, from NB period until 12 mo, renal weight increased both in C and DO. However, the increment in renal weight assumed different profiles; in C, the maximum weight was attained at 3 mo while after this period a slight decline was observed. In DO, kidney weight continued to increase until 12 mo. Glycemia remained at normal levels in all the studied groups. In DO animals, from 3 mo on, significantly higher pressures were observed when compared with the respective control animals (Fig. 2). Hypertension lasted until the end of the observation period.

Effect of maternal diabetes on renal function. Proteinuria remained unchanged in DO when compared with age-matched controls (data not shown). As shown in Table 2, with age, GFR

Table 1. Effect of maternal diabetes on offspring body and kidney weight

Newborn 6 M 12 M						
	C (26)	DO (20)	C (20)	DO (20)	C (20)	DO (20)
Body weight (g)	5.56 ± 0.13	5.71 ± 0.08	377.6 ± 7.89	407.7 ± 4.19*	410 ± 3.67	471.4 ± 8.3*
Kidney weight (g)	0.042 ± 0.01	0.029 ± 0.001*	1.21 ± 0.03	1.41 ± 0.04	1.42 ± 0.03	1.42 ± 0.03
Kidney weight/Body weight	0.55 ± 0.01	0.503 ± 0.01	0.31 ± 0.005	0.34 ± 0.008	0.34 ± 0.007	0.32 ± 0.008
Blood glucose (mg/dl)	111.4 ± 8.27	91.0 ± 7.88	102 ± 2.59	116.3 ± 6.43	115.1 ± 6.61	114.2 ± 9.57

C are control rats. DO are the offspring of diabetic dams. Data are expressed as mean ± SEM. Numbers in parentheses are the number of animals in each group. Comparisons are made between control and offspring from diabetic mothers at the same age. Significance level is $p < 0.05$ (* C vs. DO).

Figure 1. Effect of maternal diabetes on body weight in the offspring. Maternal diabetes promoted a significant increase in BW of aged rats. Comparisons were made among animals of the same age. Number of animals: C (▲, $n = 30$) and DO (◆, $n = 26$). Significance level: $p < 0.05$ (* C vs DO)

Figure 2. Effect of maternal diabetes on blood pressure levels in the offspring. Maternal diabetes promoted a significant increase in the offspring BP from 3 mo on. Comparisons were made among animals of the same age. Number of measurements: C (■, $n = 20$) and DO (▲, $n = 18$). Significance level: $p < 0.05$ (* C vs. DO);

and RPF tended to decrease in both C and DO. However, in DO, mean values for these parameters were significantly decreased in all the studied periods, when compared with age matched controls. As also shown in this table, absolute net acid excretion (NAE) decreased in DO while fractional excretion of Na^+ increased in both 6 and 12-mo-old DO rats, suggesting impairment in Na^+ conservation. As seen in Fig. 3, when NAE values were calculated as a function of GFR, in C, with age,

values tended to increase while in DO they were accentuatedly decreased, suggesting an impairment in tubular acid excretion. Taken together these results suggest that maternal diabetes may accentuate the natural age-related decay, acting as a negative adjuvant in the physiopathology of the aging process.

Effect of maternal diabetes on renal morphology. As expected, from newborn period until 3 mo after birth, increased glomerular diameters were observed both in C and DO groups. However, a significant increase in glomerular area was observed in 3 mo-old DO rats, when compared with respective age-matched control, which persisted until the end of the experimental observation; these data suggest that glomerular hypertrophy was an early event in this DO model.

As shown in Fig. 4, glomeruli number determined 3 mo after birth was the same for C and DO but in 12 mo-old rats, DO had about 20% fewer.

Effect of maternal diabetes on vascular function—Responses to Acetylcholine, Bradykinin and Sodium Nitroprusside. There were no differences in the baseline diameter between the control and restricted offspring (18.65 ± 0.57 versus. $19.91 \pm 0.65 \mu\text{m}$). The magnitude of ACh (Fig. 5A) and BK (Fig. 5B) responses was significantly less in DO compared with control group. In contrast to the effects observed with these agents, SNP produced similar vasodilatation in the control and DO groups (Fig. 5C).

DISCUSSION

Clinical and experimental studies point to the importance of intra-uterine environment in determining the metabolic, cardiovascular and endocrine disorders in the offspring. Maternal diabetes is recognized as an important cause of congenital anomalies, progeny hyper or hypoglycemic syndromes, with all the consequences of these metabolic disturbances (9,14,15).

In this study we have demonstrated that gestational diabetes mellitus promotes remarkable changes in both kidney function and vascular reactivity in the mature offspring. Our data concerning DO model point to an interesting model in which, although a normal nephron number is observed in the young rat, two important factors, which contribute to the progression of renal disease, systemic hypertension and glomerular hypertrophy are present. In our model, although decreased nephron number was not observed in young animals, a significant decrease in nephron number was observed in 12 mo-old DO. These findings suggest that maternal diabetes not only represent a new component to the growing list of causes that contribute to the fetal origins of adult-onset diseases but also can accelerate the aging process of nephron loss. In a previous

Table 2. Parameters of renal function in the offspring of diabetic rats

Group	N		GFR ml/min/kg	RPF ml/min/kg	FENa%	U/P	FF%	NAE μ Eq/min/kg
3 M	C	24	7.17 $\pm$ 0.42	19.97 $\pm$ 1.87	0.45 $\pm$ 0.07	52.93 $\pm$ 2.09	35.75 $\pm$.048	6.34 $\pm$ 0.14
	D	20	4.67 $\pm$ 0.17*	15.86 $\pm$ 0.98	0.41 $\pm$ 0.04	40.37 $\pm$ 1.14	31.97 $\pm$ 2.53	4.78 $\pm$ 0.27
6 M	C	32	4.66 $\pm$ 0.27†	11.58 $\pm$ 0.67†	0.45 $\pm$ 0.03	51.75 $\pm$ 3.36	40.86 $\pm$ 1.51	5.25 $\pm$ 0.36
	D	28	2.83 $\pm$ 0.22*†	12.19 $\pm$ 1.43	1.07 $\pm$ 0.31®	39.32 $\pm$ 2.28	31.72 $\pm$ 3.21*	2.59 $\pm$ 0.15
12 M	C	16	4.70 $\pm$ 0.33§	14.90 $\pm$ 0.71§	0.42 $\pm$ 0.07	52.91 $\pm$ 3.38	31.58 $\pm$ 1.38	6.48 $\pm$ 0.47
	D	13	2.08 $\pm$ 0.15*‡§	6.18 $\pm$ 1.25*‡§	1.38 $\pm$ 0.37®#	43.97 $\pm$ 9.10	42.05 $\pm$ 4.11	1.06 $\pm$ 0.17

Data expressed as mean $\pm$ SEM. N are the number of experiments. Significance level is $p < 0.05$.

Comparisons are made between control and offspring from diabetic mothers at the same age and between group at different ages.

* $p < 0.05$ for C and DO at the same ages.

† 3 vs 6 months for the same experimental groups.

‡ 6 vs 12 months for the same experimental group.

§ 3 vs 12 months for the same experimental group.

Figure 3. Net Acid excretion expressed per milliliter of GFR. Maternal diabetes caused a significant decrease in net acid excretion (NAE) from 6 mo on. Data from control (C: ■, $n = 24$) and from diabetic offspring (DO: ▲, $n = 24$) rats. Significance level: $p < 0.05$ (* C vs. DO).

Figure 4. Numbers of glomeruli in the offspring from control (□) and diabetic mothers (■). Maternal diabetes caused a significant decrease in glomeruli number in 12 mo-old DO rats. Number of measurements: 3 mo-old, C ($n = 49$) and DO ($n = 50$). 12 mo-old C ($n = 50$) and DO ($n = 50$). Significance level: $p < 0.05$ (* C vs. DO).

study, Amri *et al.* (17) suggested that hyperglycemic environment is able to impair *in vitro* metanephrons development. The authors showed a decreased nephron number in the offspring of STZ-treated dams, induced to diabetes at the beginning of pregnancy. In our model, diabetes was induced before preg-

Figure 5. Percentage of change in vascular diameter of mesenteric arterial bed. Maternal diabetes caused an impaired response to endothelium-dependent agents Bar graphs shows the response of the mesenteric microvessels to: (A) acetylcholine (300 μ g/mL); (B) bradykinin (3.0 μ g/mL) and (C) sodium nitroprusside (1.0 mg/mL) in control (□, $n = 06$) and DO (■; $n = 09$) groups. Values are expressed as mean $\pm$ SEM * $p < 0.05$ compared with control group.

nancy, in a time were STZ is expected not to affect, directly, pancreatic development. On the other hand, in Amri studies, (17) the nephron count was performed by using glomeruli isolation. In our study, we have used a methodology in which glomeruli number is counted in 40 fields of both kidneys and glomerular diameter and area are calculated by using a computer program. Changes in renal function were also shown by Nelson *et al.* (25) in Pima Indians children, exposed to intra-uterine diabetes. The authors showed high levels of urinary albumin excretion in that population, suggesting increased risk of renal disease. Although proteinuria was not observed in DO group, significant decrease in GFR, RPF and urinary flux suggest that the natural age-related decline in renal function, which was also present in C group, was accelerated in these rats; the increase in glomerular area shown in DO characterizes glomerular hypertrophy known to be the early event in the progression of glomerular damage (26–28).

Both experimental and clinical studies have indicated a decreased nephron number as a common denominator of hypertensive states (29–31). In some models of reduced nephron numbers, systemic hypertension and glomeruli hypertrophy are interpreted as compensatory mechanism to maintain renal function in adequate levels (32). In a model of renal ablation performed in newborn rats, Woods *et al.* (31) showed that hypertension developed from 8 wk on and proteinuria was significantly higher in the 20-wk-old group, suggesting that hypertension preceded glomerular damage, which was significant only in the older group. Our previous work in the aged offspring of food restricted mothers showed that glomerular involvement occurred independently of systemic hypertension, which was prevented by L-arg administration (33), similar to

the findings from Floege *et al.*, in Milan normotensive rats, that showed that an early podocyte damage was observed, preceding glomerulosclerosis, in the absence of systemic hypertension, hyperglycemia and glomerular hypertension (34). On the other hand, hypertension by itself plays an important role in the progression of renal disease (29,30). A persistent elevation in blood pressure can be transmitted to glomeruli leading to progressive injury, which in turn, can aggravate systemic hypertension. In the aging spontaneously hypertensive rats (SHR), Tolbert *et al.* (35), showed that at 9 mo of age, glomerular hypertension developed because of a small increase in systemic blood pressure and a decline in preglomerular vascular resistance, allowing transmission of elevated systemic pressure to the glomerular capillaries. In the present study, hypertension was an early event in DO suggesting that it could be the initial trigger factor of renal function impairment, which is reflected by glomerular hypertrophy and changes in renal function parameters. However, other hypertrophic stimuli may be present in this model. For example, TGF β is known to be increased in glomeruli of human and experimental diabetes (36). The possibility of transference of mother's phenotypic characteristics was demonstrated by Malathi *et al.* (37) that showed that offspring originated from hyperglycemic mothers had also several metabolic disturbances similar to the hyperglycemic dams. In their study, they concluded that a dietary modification in the early postnatal life of the mothers sets up a vicious cycle of spontaneous transfer of the phenotype to its progeny.

To investigate alterations of vascular reactivity in the adult offspring from diabetic mothers, responses to acetylcholine, bradykinin and sodium nitroprusside were investigated. Whereas the responses induced by endothelium-dependent vasodilators were decreased in mesenteric microvessels, no differences could be detected to sodium nitroprusside. Therefore, we may suggest that it is not the smooth muscle vasodilating capability that is reduced in mesenteric bed from offspring of diabetic rats, but some function related to the endothelium. Corroborating our *in vivo* data, *in vitro* studies have demonstrated that severe maternal diabetes can lead to vascular dysfunction in adult offspring, characterized by enhanced sensitivity to noradrenaline and abnormal endothelium-dependent relaxation to acetylcholine and bradykinin in small mesenteric arteries (38). The origin of the vascular dysfunction induced in these offspring cannot be directly inferred from the present study. However, it seems that these animals had acquired an endothelial defect similar to that observed in arteries of diabetic adult rats (39) and in patients with diabetes, (40–43) suggesting that these disturbances, which were already described in diabetes, could be transferred to the fetus, changing blood pressure regulation and interfering in the developing organs.

The exact mechanism by which maternal diabetes impairs the endothelial function is not fully elucidated. Considering that nitric oxide (NO) is the main agonist responsible for the endothelium-dependent relaxation induced by acetylcholine or bradykinin, maternal diabetes may cause a reduction in NO synthesis and/or bioavailability, decreasing the endothelium-dependent vasodilation in the adult offspring. However, studies

are necessary to evaluate the mechanisms involved in this alteration of the vascular function induced by maternal diabetes.

Although our data point to systemic hypertension as the primary cause of both glomerular hypertrophy and renal function impairment in DO model, the role of the kidney in perpetuating vascular alterations is a possibility that cannot be ruled out and need to be addressed in further investigations. To date, no study has specifically examined the late consequences of maternal diabetes on offspring blood pressure regulation and kidney function. Most observations have been found directly in the presence of diabetic status or have focused the disturbances in the hormonal control of glycemia in the offspring of diabetic mothers. Our data suggest that maternal diabetes can represent an important cause of hypertension and renal dysfunction later in life, reinforcing the concept of fetal programming of adult diseases.

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