The effect of diabetes mellitus on urinary calcium excretion in pregnant rats and their offspring

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Abstract

The effect of maternal diabetes mellitus on renal calcium excretion in pregnant rats and their offspring has been examined in order to ascertain the role of the kidney in the disturbed calcium homeostasis of infants born to diabetic mothers. Diabetic pregnant (DP) rats exhibited severe hypercalciuria which greatly exceeded the urinary calcium losses ($U_{CaV}$) in non-diabetic pregnant (CP) or non-pregnant diabetic (D) rats. Means ± s.e.m. for $U_{CaV}$ at day 21 (mmol/24 h) were: DP=1.12 ± 0.09 (n=7); CP=0.06 ± 0.01 (n=7); D=0.66 ± 0.06 (n=7) (P < 0.001 DP vs CP and DP vs D). The profile for urinary calcium excretion in the three groups was different from that of other measured ions. The degree of natriuresis, for example, was comparable in DP and D rats at all stages studied. Although magnesium output was significantly greater in DP than D rats on days 14 and 21, this appeared to result from an additive effect of the magnesium seen when pregnancy and diabetes were studied separately.

The marked renal calcium wasting of diabetic pregnancy will have implications for overall calcium balance in the mother. For example, an enhanced intestinal calcium absorption was seen in DP rats in the second half of gestation. Means ± s.e.m. for day 21 (mmol/24 h) were: DP=3.8 ± 0.8 (n=7); CP=1.4 ± 0.3 (n=7); D=1.6 ± 0.3 (n=7) (P < 0.05 DP vs CP and DP vs D). The hypercalciumia may also contribute to the disturbed calcium homeostasis of the neonate if it reduces the amount of calcium available for transfer to the fetus.

In contrast to their mothers, the offspring of DP rats did not show a raised $U_{CaV}$ compared with CP pups. Means ± s.e.m. at day 1 postpartum (mmol/24 h per pup) were: DP=47.2 ± 15.7 (n=4 litters); CP=72.2 ± 14.1 (n=7 litters) (not significant). Changes in neonatal renal function are therefore unlikely to contribute to their disturbed calcium balance. In fact, their slightly reduced urinary calcium output may be an attempt to compensate for their lowered total body calcium as reported elsewhere.

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Introduction

Significant alterations in calcium homeostasis occur in infants born to insulin-dependent diabetic mothers. Both a reduction in bone mineralization (Mimouni et al 1988) and hypocalcaemia (Craig 1958, Gittleman et al 1959, Tsang et al 1972, 1975, Cruikshank et al 1983, Martinez et al 1991), the latter with associated tetany, convulsions and cardiac arrhythmia (Craig 1958), have been reported to occur. The precise incidence of such events is not known since they are not routinely monitored. Nevertheless several individual studies have reported severe hypocalcaemia in up to 50% of children examined (Tsang et al 1972, Cruikshank et al 1983). The problem is seen even when prematurity and perinatal complications associated with diabetic pregnancy are taken into account. Also unknown, and perhaps of greater consequence, is the long-term effect of an abnormal fetal calcium balance on the offspring's subsequent growth and development. It is becoming increasingly clear, for example, that abnormalities in utero can have profound effects throughout extrauterine life (Barker 1992). Ethical reasons clearly limit the amount of information that can be obtained from pregnant women. However, the offspring of genetically diabetic rats (Verhaeghe et al 1986, 1988) and animals rendered diabetic with streptozotocin (Uriu-Hare et al 1985, Demignon & Rebut-Bonneton 1988) also demonstrate a reduced bone mineral content, thus confirming in an animal model what had previously been seen clinically.

The precise cause of an altered calcium homeostasis in the offspring of diabetic mothers is not known. However, it is likely to be multifactorial, involving defective fetal skeletal mineralization, abnormal function and/or production of calcium-regulating hormones, together with an altered calcium handling by the major ionoregulatory organs such as the placenta and kidney. We have recently shown that maternofetal placental calcium flux is lower in diabetic rats than in control or insulin-treated diabetic
animals (Husain et al. 1994). The present study looks specifically at the role of the maternal and neonatal kidney by comparing renal calcium excretion in pregnant diabetic rats and their offspring with that in control pregnant animals. Since we have previously shown that experimental diabetes increases renal calcium excretion in non-pregnant animals (Anwana & Garland 1990) we have also included such a group in the present study to see whether diabetes has an enhanced renal effect during pregnancy. The present study also includes some preliminary data for magnesium and sodium. Magnesium is of interest in the light of reports of a disturbed magnesium homeostasis in diabetes (Garland 1992). More specifically, Tsang et al. (1976) have reported hypomagnesaemia to occur in infants born to diabetic mothers. Urinary sodium values were included to see whether any observed renal changes were specific for divalent ions. Some of the data have been presented in abstract form (Birdsey et al. 1993).

Materials and Methods

Animals

Experiments were performed using female Sprague–Dawley rats (Charles River Laboratories, Wilmington, Kent, UK), aged 8 weeks at the time of injection (see below). Body weights are detailed in the Results. Animals were maintained under a constant 12-h light photoperiod (lights on at 0800 h) and an environmental temperature of 21–23 °C. Rats had free access to food (CRM Labsure, Poole, Dorset, UK) and deionized water throughout the study. Food composition was assessed on nine samples from three food batches as described in the section on Analyses. Average values (mmol/g) were: Na, 0·113±0·003; Ca, 0·203±0·009; Mg, 0·075±0·005. Three animal groups were studied.

Diabetic non-pregnant rats (D) Rats were rendered diabetic with streptozotocin (STZ; Sigma, Poole, Dorset, UK; 60 mg/kg). The drug was freshly dissolved in citrate buffer (pH=4·8) and maintained on ice prior to use (injection volume 1 ml/kg i.p.). At present, STZ is the preferred diabetogenic agent; short-term renal changes resulting from STZ treatment in rats have been shown to result from the experimental diabetes itself rather than any toxic effect of the drug (Evan et al. 1984). Diabetes was confirmed by the development of glycosuria (Labstix; Ames, Slough, Berks, UK) within 36 h of drug administration.

Diabetic pregnant rats (DP) Rats were rendered diabetic as described above and mated 5 days after drug injection. For mating, each female was housed with a male rat, copulation occurring within 2±2 days of cohabitation. The day on which a vaginal copulation plug was found was designated day 1 of gestation (Sprague–Dawley rats usually deliver on days 22/23).

Control pregnant rats (CP) Control animals received an i.p. injection of citrate buffer alone and were mated as described above.

Metabolic study

The study was performed on animals housed individually in glass metabolism cages (Metabowls; Jencons Scientific Ltd, Hemel Hempstead, Herts, UK). An acclimatization period of 14 days allowed the animals to become accustomed to the cages. An initial (control) 24-h collection was then made (the preinjection collection), noting fluid and food intake and urinary and faecal excretion. On completion of this sample, animals were treated with either STZ or citrate buffer as described above. A 24-h post-injection collection was taken 5 days after drug or vehicle administration. Animals which were to form the CP and DP groups were then mated. Twenty-four hour samples from mated animals were collected on days 7, 14 and 21 of gestation; simultaneous sampling from D and DP groups ensured that the duration and severity of diabetes was comparable in the two drug-treated groups.

Neonatal study

On completion of maternal sampling, pregnant rats were placed into hard bottomed cages provided with nesting material and allowed to give birth. Urine samples were collected from the offspring of these pregnancies on day 1 postpartum using the method of Kavlock & Gray (1982). This method utilizes the fact that neonatal rats cannot micturate voluntarily but require an external stimulus to void their bladders. The four heaviest males and females from each litter were temporarily isolated from the dam to allow for sampling. The pups' bladders were then voided by stimulating the perineal region. Two hours later the bladders were voided again and the urine produced by each pup was collected. Urine samples collected from members of the same litter were pooled to produce a sufficient volume for analysis.

Ten days postpartum, terminal blood samples were taken for glucose determination from sodium amytal (100 mg/kg i.p.)-anaesthetized lactating dams and diabetic non-pregnant females via the abdominal aorta. Animals were subsequently killed by cervical dislocation. All blood was collected into heparinized tubes and centrifuged immediately to separate the plasma.

Analyses

Adult and neonatal urine was analysed for calcium and magnesium by atomic absorption spectrophotometry (Baird α3; Baird Atomic Ltd, Brantree, Essex, UK).
TABLE 1. Body weight, food consumption and fluid intake, prior to and during gestation (or non-pregnant equivalent) in control pregnant (CP), diabetic pregnant (DP) and diabetic non-pregnant (D) animals. Values are means ± s.e.m., n=7 in each group.

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<th>Injection</th>
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<tr>
<td></td>
<td>Body wt (g)</td>
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<tr>
<td></td>
<td>CP</td>
<td>210.5±4.9</td>
<td>224.7±6.1</td>
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<td></td>
<td>DP</td>
<td>212.5±8.2</td>
<td>211.2±7.9</td>
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<td></td>
<td>D</td>
<td>218.3±7.4</td>
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<td>Food intake (g/24 h)</td>
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<td>CP</td>
<td>20.5±1.3</td>
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<td>DP</td>
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<td>28.8±4.6***</td>
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<td></td>
<td>D</td>
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<td>Fluid intake (ml/24 h)</td>
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<td></td>
<td>CP</td>
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<td></td>
<td>D</td>
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<td></td>
<td>Day 7</td>
<td>273.9±6.1</td>
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<td></td>
<td>Day 14</td>
<td>255.0±8.7</td>
<td>258.8±8.7***</td>
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<td></td>
<td>Day 21</td>
<td>246.4±6.3</td>
<td>250.9±7.6</td>
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***P<0.001 vs CP; †P<0.05 vs D; ††P<0.01 vs D (one-way analysis of variance plus Scheffe’s procedure (OAV)).

Flame photometry (Corning EE1 model 450, Scientific and Medical Products Ltd, Manchester, UK) was used to determine the sodium concentration of adult urine. The glucose concentration of adult terminal plasma was assessed using the glucose oxidase method (Sera Pak; Ames, Slough, Berks, UK). Food and dried faecal samples were also analysed to allow calculation of intestinal ion absorption. For this, approximately 0.2 g of the ground sample was mixed with 10 ml concentrated nitric acid. The mixture was then incubated in an oven for 24 h at 88 °C, dried down and reconstituted with 2 M nitric acid. Calcium, magnesium and sodium contents were determined as described above.

Calculations
Solute absorption across the intestine was calculated as the difference between solute intake and faecal solute excretion.

Statistical analyses
Data are presented as means±s.e.m. Statistical analysis of the data was performed using the SPSS-PC software package. For adult data, a repeated measures analysis of variance (MANOVA) was performed to test for differences between groups over the five sample periods. Where overall group differences were apparent, a one-way analysis of variance in conjunction with a Scheffe’s multiple comparisons procedure (OAV), was used to detect differences between groups CP and DP and groups CP and D at individual time-points. Pup data were analysed using a Student’s t-test.

Results
Metabolic study
Table 1 presents data for body weight, food consumption and fluid intake in the three experimental groups. Rats were of comparable weight at the start of the experiment. CP animals subsequently showed a greater increase in body weight than DP rats as gestation proceeded (P<0.001; MANOVA). Body weight was similar in DP and D groups until day 21 when D animals were significantly lighter (P<0.001; OAV). The three groups also showed different patterns of food consumption and fluid intake over the five sampling periods (P<0.001; MANOVA). Food intake was similar in all animals prior to drug or vehicle administration, as was fluid intake. As expected, STZ-treated groups demonstrated significant hyperphagia and polydipsia (P<0.001; OAV). When compared with CP rats, DP animals were significantly polydipsic and hyperphagic at all stages of gestation studied (P<0.001; OAV). The quantity of food consumed by DP animals was also greater than that of D animals on days 14 and 21 of pregnancy (P<0.05; OAV). Fluid intake differed significantly between these two groups (P<0.05; OAV) only on day 21. Terminal plasma taken from the three groups 10 days postpartum (or non-pregnant equivalent) showed both STZ-treated groups to have an elevated plasma glucose concentration compared with controls. Values (mmol/l) were: CP=8.45±0.76 (n=7); DP=47.26±2.39 (n=3); D=41.24±1.35 (n=6).

Figure 1 presents data for maternal urinary calcium output. This variable differed significantly between the groups throughout the study (P<0.001; MANOVA). Daily calcium excretion was comparable in all three groups prior to drug or vehicle administration. However, once diabetes had been induced, 24-h calcium excretion was enhanced (P<0.01; OAV). When compared with CP values, calcium excretion was significantly (P<0.001; OAV) elevated in DP animals at all stages of gestation. Urinary excretion of this cation was comparable in DP and D groups on day 7. However on days 14 and 21 of gestation, the hypercalciuria of DP rats was significantly (P<0.001; OAV) greater than that of D animals.

Table 2 shows data for maternal urine volume, urinary sodium and urinary magnesium excretion, the latter two included for comparison with calcium. The three groups

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Table 2 shows data for maternal urine volume, urinary sodium and urinary magnesium excretion, the latter two included for comparison with calcium. The three groups
had a significantly different excretory pattern for each of these variables over the five sampling periods (P<0.001; MANOVA). Prior to drug or vehicle administration, the three animal groups showed comparable levels of urine output, sodium excretion and magnesium excretion. Induction of diabetes initiated a rise in all three variables when compared with vehicle-treated animals (P<0.001; OAV). Throughout gestation, DP animals demonstrated an elevated 24-h urine output and sodium and magnesium excretion, compared with CP values (P<0.001; OAV, for each variable at all stages studied). DP and D groups showed a comparable rise in urine output and urinary sodium excretion at all stages of gestation or non-pregnant equivalent. However, when compared with D animals, the DP group excreted significantly greater amounts of magnesium in their urine on days 14 (P<0.01; OAV) and 21 (P<0.05; OAV) of pregnancy.

Metabolic studies allow for an assessment of solute absorption across the intestine, calculated as the difference between solute intake and faecal solute excretion. Figure 2 presents data for maternal intestinal calcium absorption. The three animal groups showed different patterns of absorption.
absorption over the sampling period (P<0.001; MANOVA). Prior to drug or vehicle administration, calcium absorption was comparable in all three groups. Induction of diabetes initiated a slight but non-significant rise in absorption of this cation. When compared with CP values, intestinal calcium absorption was significantly (P<0.05; OAV) elevated in the DP group on days 14 and 21 of pregnancy. Values for DP animals significantly exceeded those of D animals only on day 21 (P<0.05; OAV).

Table 3 shows data for maternal intestinal absorption of sodium and magnesium. The three groups showed a different pattern of absorption for both ions (P<0.01; MANOVA). Before drug or vehicle injection, the three groups had comparable levels of sodium and magnesium uptake. Induction of diabetes initiated a significant (P<0.001; OAV) increase in sodium absorption across the gut. However, a similar enhancement of absorption was not observed for magnesium at this stage. Nevertheless, when compared with CP values, DP animals showed an enhanced intestinal absorption of both sodium (P<0.001; OAV) and magnesium (P<0.05; OAV) at all stages of gestation. Moreover, sodium absorption was significantly increased in DP animals compared with D animals on days 14 (P<0.05; OAV) and 21 (P<0.01; OAV) of pregnancy. Intestinal absorption of magnesium by DP animals significantly exceeded that of the D group only on day 14 (P<0.05; OAV).

**Neonatal study**

DP rats produced smaller litters with lighter pups when compared with CP animals. Mean litter sizes were: CP=15±0.4; DP=10±1.6 (P<0.01). Mean pup weights

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**Figure 2.** Intestinal calcium absorption (a) prior to and (b) during gestation (or non-pregnant equivalent) in control pregnant (CP, solid bars), diabetic pregnant (DP, cross-hatched bars) and diabetic non-pregnant (D, lined bars) animals. All values are means ± s.e.m., n=7 in each group. *P<0.05 vs CP; †P<0.05 vs D (one-way analysis of variance plus Scheffé’s procedure (OAV)).

**Table 3.** Intestinal sodium and magnesium absorption prior to and during gestation (or non-pregnant equivalent) in control pregnant (CP), diabetic pregnant (DP) and diabetic non-pregnant (D) rats. Values are means ± s.e.m., n=7 in each group

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<th>Group</th>
<th>Injection</th>
<th>Gestation</th>
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<td>Pre</td>
<td>Post</td>
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<tr>
<td>Intestinal sodium absorption (mmol/24 h)</td>
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<tr>
<td>CP</td>
<td>2.2±0.2</td>
<td>2.0±0.2</td>
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<tr>
<td>DP</td>
<td>2.2±0.1</td>
<td>3.5±0.2***</td>
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<tr>
<td>D</td>
<td>2.1±0.1</td>
<td>3.3±0.2</td>
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<td>Intestinal magnesium absorption (mmol/24 h)</td>
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<tr>
<td>CP</td>
<td>0.7±0.1</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>DP</td>
<td>0.6±0.1</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>D</td>
<td>0.5±0.03</td>
<td>0.8±0.1</td>
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*P<0.05 vs CP; †P<0.05 vs D; ††P<0.01 vs D (one-way analysis of variance plus Scheffé’s procedure (OAV)).
on day 1 postpartum (g) were: CP=6.37±0.05; DP=5.16±0.09 (P<0.001). In contrast to their mothers, the offspring of DP rats did not show an enhanced urinary excretion of calcium or magnesium compared with the CP group. Means ± s.e.m. were: for calcium (nmol/2 h per pup), CP=72.2±14.1 (n=7 litters); DP=47.2±15.7 (n=4 litters); for magnesium (nmol/2 h per pup), CP=212.6±49.5 (n=7 litters); DP=128.5±15.3 (n=4 litters). In neither case were differences between CP and DP groups significant.

Discussion

Metabolic study

The marked hypercalciuria, hyperphagia, polydipsia and polyuria seen in non-pregnant diabetic animals in this study have been well documented elsewhere (Hough et al. 1982, Wen & McSherry 1982, Wood et al. 1984, Hebden et al. 1986, Guruprakash et al. 1988, Anwana & Garland 1990, Garland et al. 1991). There are also some, albeit limited, reports of the more modest hypercalciuria and polydipsia noted in our control pregnant rats (Atherton et al. 1982, Green & Hatton 1987). In contrast, our study is the first to document changes in renal calcium handling throughout gestation when pregnancy is superimposed on experimental diabetes. The magnitude of the urine calcium loss in DP rats was dramatic and far exceeded that seen in diabetes or pregnancy alone. It was not simply an additive effect of the two conditions. For example, term DP animals showed a 42-fold increase in urinary calcium output compared with preinjection values. For term CP animals the increase was just 2-fold; for D rats the increase was 20-fold. The profile for urine calcium excretion in the three groups was also strikingly different from that of sodium or magnesium. The degree of naturessis, for example, was comparable in DP and D groups at all stages studied. Although magnesium output was significantly greater in DP than D animals on days 14 and 21, this appeared to result from an additive effect of the magnesiumuresis seen when pregnancy and diabetes were studied separately. There are no previous studies of renal sodium or magnesium excretion in diabetic pregnancy. Nevertheless, the enhanced urinary magnesium losses seen here may be especially relevant as they may contribute to the overall magnesium deficit of diabetes (Garland 1992). Such a deficit has recently been linked to the development of a number of diabetic complications and the adverse fetal outcome of some diabetic pregnancies (Mimouni et al. 1987, Miodownik et al. 1988).

The precise cause of the dramatic hypercalciuria of diabetic pregnancy is not known. We did not assess maternal plasma calcium concentrations in our animals during gestation. However, in other studies term pregnant diabetic rats and humans generally show normal or reduced plasma calcium concentrations (Tsang et al. 1975, Cruikshank et al. 1980, 1983, Verhaeghe et al. 1986, Mimouni et al. 1989, Husain et al. 1994). An increased glomerular filtration rate (GFR) is a well-known characteristic of early human and experimental diabetes (Mogensen 1971, Puig et al. 1981, O'Donnell et al. 1988, Fujihara et al. 1992). Hyperfiltration is also seen in pregnant women and rats (Atherton & Pirie 1981, Davison & Noble 1981, Garland & Green 1982, Dafnis & Sabatini 1992). To our knowledge, GFR has not been assessed in pregnant diabetics. Nevertheless, from the above reports it is likely that it will be raised. An increased filtered load of calcium may therefore contribute to the hypercalciuria seen in our DP rats. However, glomerular changes alone would be unlikely to account for the marked differences between ions seen in the present study. Assuming plasma concentrations to be similar in the two groups, an increased GFR would enhance the filtered load of calcium, sodium and magnesium to a similar extent. However, the increased output of these ions in DP rats varied considerably. For example, term DP rats showed a 42-fold increase in urinary calcium excretion compared with preinjection values. The increased outputs for sodium and magnesium were just twofold and ninelfold respectively. The implications are, therefore, for a marked depression in tubular calcium reabsorption in our DP animals. Of particular relevance here may be recent reports (Guruprakash et al. 1988, Garland et al. 1991) of a calcium reabsorptive defect (most probably in the loop of Henle and distal nephron) in non-pregnant diabetic rats. Clearly, further studies are required to see whether such a lesion is exacerbated in diabetic pregnant animals.

The marked renal calcium wasting of diabetic pregnancy will have implications for overall calcium homeostasis in the mother. Our study has demonstrated an enhanced intestinal calcium absorption in DP animals. Interestingly, differences between DP and CP animals for this variable do not become significant until day 14 of pregnancy. This contrasts with the hypercalciuria which was significant in the DP group by day 7. This may suggest that the enhanced gut uptake is a secondary effect compensating for the urinary calcium losses. Some of the enhanced intestinal uptake of calcium in DP mothers will be destined for the fetus. However, our recent study (Husain et al. 1994) reported a significantly decreased placental calcium transport in diabetic pregnant rats compared with controls. Perhaps this is also a result of the mother trying to maintain her calcium homeostasis. The excessive renal calcium wasting by the diabetic mother seen in the present study may, therefore, be one of the factors responsible for reducing the amount of calcium available for transfer to the fetus. A reduced placental calcium transfer in turn may be responsible for some of the alterations in calcium homeostasis seen in the offspring of diabetic mothers (viz hypocalcaemia and reduced bone mineralization: Gittleman et al. 1959, Tsang et al. 1972, 1975, Cruikshank et al. 1983, Uriu-Hare et al. 1991).
Neonatal study

The reduced body weight of rat pups born to our diabetic mothers has been documented in previous studies (Verhaeghe et al. 1986, 1988, Demignon & Rebut-Bonneton 1988). This contrasts with the situation in human diabetes where fetal macrosomia is often evident (Brudenell & Dodridge 1989). The smaller litter size of our DP rats had not been noted before, although an increased number of fetal resorptions had been seen in one previous study (Verhaeghe et al. 1986). The severity of diabetes in the present study (as reflected by plasma glucose concentrations in excess of 40 mmol/l) may have contributed to the poorer reproductive performance of our females in general. We did not assess the plasma calcium concentrations of our neonates. However, the recent study of Husain et al. (1994) demonstrated a reduced total fetal calcium content in the offspring of DP rats when compared with CP animals. This would concur with several clinical reports of hypocalcaemia and a reduced bone mineral content in children born to diabetic mothers as outlined in the Introduction.

The present study has shown that, in contrast to their mothers, the offspring of diabetic pregnant rats did not show a significantly increased urinary calcium output when compared with the offspring of control pregnant animals. Indeed, urinary calcium (and magnesium) excretion was slightly (although not significantly) lower in this group at day 1 postpartum. It would appear, therefore, that changes in neonatal renal function are unlikely to be responsible for the altered calcium homeostasis seen in infants born to insulin-dependent diabetic mothers. Indeed, their slightly reduced urinary calcium output may represent an attempt to compensate for their lowered total body calcium as reported elsewhere (Husain et al. 1994). The precise mechanisms responsible for this are not known. Finally, in non-pregnant diabetic rats, hypercalciuria may be prevented by insulin replacement commencing upon diagnosis of the disease (Hough et al. 1982, Anwana & Garland 1990). However, delayed insulin treatment is ineffective in restoring calcium transport to normal (Hoskins & Scott 1984, Garland et al. 1991), suggestive of an irreversible change in the transporting system in diabetes. This may be particularly relevant clinically where insulin replacement is inevitably started some time after the onset of the disease. Clearly this needs to be investigated in diabetic pregnancy.

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