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Increased Renal Tubular Reabsorption of Calcium and Magnesium by the Offspring of Diabetic Rat Pregnancy

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ABSTRACT

Diabetic pregnancy has a marked influence on offspring calcium and magnesium homeostasis. Urinary excretion of calcium and magnesium is reduced, yet offspring of diabetic pregnancy exhibit hypomagnesemia and hypocalcemia. The aim of this study was to measure renal hemodynamic and tubular function in the offspring of diabetic (OD) and control, nondiabetic (OC) rats at 4 and 8 wk of age to determine the glomerular and tubular mechanisms through which renal calcium and magnesium handling are programmed *in utero*. The fraction of filtered calcium that was excreted was significantly lower in OD at both 4 and 8 wk of age [8 wk: OC ($n = 6$), 11.8 ± 2.9 *versus* OD ($n = 5$), 4.3 ± 0.6%; $p < 0.05$] and that of magnesium was lower at 8 wk of age [OC ($n = 6$), 42.4 ± 7.5 *versus* OD ($n = 5$), 13.0 ± 1.7%; $p < 0.01$]. This increased reabsorption occurred despite an elevated GFR in OD. These findings clearly indicate that tubular reabsorptive mechanisms for calcium and magnesium are increased markedly in OD. Serum PTH concentration was reduced in 8-wk-old OD [OC ($n = 7$), 539.4 ± 142.1 *versus* OD ($n = 9$), 174.3 ± 69.4 pg/ml; $p < 0.05$], consistent with previous reports in human infants. Taken together, these observations suggest that the basis for the altered renal magnesium and calcium handling in OD involves increased tubular transport activity and possibly increased sensitivity of these mechanisms to PTH. (***Pediatr Res* 57: 890–895, 2005**)

Abbreviations

M6PR, IGF-II/mannose-6-phosphate receptor
OC, offspring of control pregnancy
OD, offspring of diabetic pregnancy
PMCA, plasma membrane Ca^{2+}-ATPase pump
STZ, streptozotocin
UV, urine flow rate

Diabetic pregnancy has a marked impact on maternal calcium and magnesium homeostasis in both humans and rats. Hypomagnesemia has been widely reported in human diabetic pregnancy (1–3), and diabetic pregnant rats display a marked increase in urinary magnesium excretion (4,5). A reduction in third-trimester plasma calcium concentration has been observed in human diabetic pregnancy in one study (3), but the majority of studies have found no difference in calcium concentrations between normal and diabetic pregnancies. However, in the diabetic rat, pregnancy is associated with marked increases in urinary calcium (4,5) and hypocalcemia in some (4) but not all studies (6).

These changes in maternal divalent ion handling have a profound impact on the developing fetus. Human offspring of diabetic pregnancy exhibit hypomagnesemia and hypocalcemia as neonates (2,7–9). In the rat, renal handling of calcium and magnesium is also altered in the offspring of streptozotocin (STZ)-treated diabetic rats (OD). We recently reported that OD retain both calcium and magnesium as neonates and that this continues into adulthood (10). These changes in divalent ion homeostasis also occur in conjunction with changes in bone structure. Human offspring of diabetic pregnancy are susceptible to congenital malformations, including skeletal abnormalities (11), and have lower bone mineral content (12). Reduced bone mineral content and abnormal skeletal development have also been observed in the offspring of STZ-treated diabetic rat mothers (13,14). We recently extended these observations and showed that offspring of STZ-treated diabetic rats have reduced trabecular and higher cortical femoral bone volume that persists into adulthood (15).

The mechanisms that are responsible for this alteration in calcium and magnesium homeostasis in the offspring of diabetic pregnancy are largely unknown. In whole-kidney homogenates, we have shown that expression of the three key transport proteins that mediate the reabsorption of calcium in the

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distal nephron, namely the apical epithelial calcium channel (16), the intracellular binding protein calbindin-$D_{28\ K}$ (17), and the basolateral plasma membrane Ca^{2+}-ATPase pump (PMCA) (18), is increased in the kidneys of OD rats up to the age of 16 wk (10,15). This suggests that changes in calcium handling by the distal nephron of adult OD rats may be programmed by the *in utero* environment. However, as we measured only 24-h urine output using metabolism cages in our earlier study, we were not able to distinguish between renal hemodynamic and/or tubular effects that are responsible for the altered ion handling. Accordingly, the aim of this study was to assess in more detail renal function in the offspring of diabetic pregnancy to determine the relative contribution of glomerular and tubular mechanisms to the altered renal handling of calcium and magnesium in these animals. We assessed renal function in rats at 4 wk of age, before puberty, and at 8 wk of age, after puberty, to determine whether *in utero* exposure to maternal diabetes exerts a long-term programming effect on offspring kidney function. We also measured serum PTH concentration, as this has been reported to be lower in human offspring of diabetic pregnancy (2,19,20) and may contribute to altered renal calcium and magnesium handling.

METHODS

All experiments described herein were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and received local ethical approval.

Animals. Twenty-five female Sprague-Dawley rats (Charles River UK Limited, Margate, Kent, UK) were used to generate the experimental animals. All animals were housed in individual cages in a room at 22–24°C with a 12-h light:12-h dark cycle. Food (Rat & Mouse Standard Diet; Bantin & Kingman Ltd, Hull, North Humberside) and tap water were provided *ad libitum*.

Diabetes was induced in 11 female rats by the injection of STZ [60 mg/kg i.p. in 0.1 M citrate buffer (pH 4.8)]. Diabetes was confirmed, after 48 h, by the development of glycosuria (Uristix; Ames DVN, Miles Ltd., Slough, UK) and hyperglycemia (blood glucose concentration >15 mM). Blood for the latter was obtained *via* direct needle puncture of the tail vein followed by immediate assay using a blood glucose analyser (Hemocue, Sheffield, UK). Fourteen control animals received an i.p. injection of the vehicle (0.1 M citrate buffer) alone. Urine and blood samples were taken from vehicle-injected animals to confirm that they were not diabetic.

Both control and diabetic female rats were mated with nondiabetic male rats. Pregnancy was confirmed by the appearance of a copulation plug, after which they were returned to individual housing for the remainder of the pregnancy (gestation is 21–22 d in the rat). An additional group of 25 vehicle-injected controls were mated at the same time. At birth, all litters that were born to both diabetic and nondiabetic mothers were fostered to this second group of vehicle-injected control dams, which had given birth on the same day, to eliminate any potential postnatal influence on offspring development. Pups were weaned at 4 wk on to standard rat chow. Male offspring of control (OC) and diabetic (OD) mothers were studied at 4 and 8 wk of age.

Renal function at 4 and 8 wk. Male OC (4 wk, $n = 5$ from five litters; 8 wk, $n = 9$ from six litters) and OD (4 wk, $n = 5$ from five litters; 8 wk, $n = 6$ from five litters) were prepared for renal function study as described previously (21). Briefly, animals were anesthetized with Intraval (100 mg/kg body wt, thiopentone sodium BP; Rhone-Poulenc Rorer Limited, Nenagh, Co Tipperary, Ireland), and cannulae were inserted into an external jugular vein, carotid artery, and the bladder. Euvolemic fluid replacement of spontaneous urine output was achieved using a servocontrolled fluid replacement system, as described previously (21). Briefly, urine flow rate (UV), determined gravimetrically, is transmitted to an adjustable pump *via* a computer. A program developed at the University of Manchester (22) allows the infusion rate of the pump to be automatically adjusted to precisely replace i.v. the volume of fluid lost as urine. A clearance marker (^{3}H inulin in 2.5% dextrose, 4 μCi/h; Amersham International plc, Little Chalfont, Bucks, UK) for the determination of GFR was delivered *via* a second, slow, constant infusion pump (1 mL/h). After surgery, a bolus dose of ^{3}H inulin (4 μCi) was injected *via* the venous cannula and the servocontrolled infusion was initiated.

The infusion protocol differed between the 4-wk and 8-wk groups, as previous experience with young rats suggests that the preparation had reduced stability beyond 4 h. Hence, the 4-wk-old animals were allowed a 2-h equilibration period after which urine samples were collected every 15 min for an additional hour. The 8-wk-old rats were allowed a 3-h equilibration period after which urine samples were collected every 15 min for an additional 3 h. In both age groups, a blood sample (0.5 mL) was taken midway through each hour. Protein-bound and free ionized calcium were separated using Microcon YM-50 centrifugal filters (50,000-Da molecular weight cut-off; Millipore UK Ltd, Watford, UK). Blood glucose concentration was determined using a glucose analyser (Hemocue, Sheffield, UK) in a terminal blood sample at the end of the experiment. The blood glucose concentration ranged between 6 and 9 mM in both OC and OD rats in each age group.

Analysis. Urine and plasma sodium concentrations were measured by flame photometry (Corning 480; Corning Ltd, Halstead, Essex, UK), and calcium and magnesium concentrations were measured by atomic absorption spectrophotometry (model 3100; Perkin Elmer, Beaconsfield, Bucks, UK). ^{3}H inulin was determined using a 1900CA Tri-Carb Liquid Scintillation Analyser β-counter (Canberra Industries, Meriden, CT).

PTH RIA. Serum was collected from a separate group of 8-wk-old rats (OC, $n = 7$; OD, $n = 9$). PTH concentrations were measured using a rat PTH immunoradiometric assay kit (Nichols Diagnostics, Cambridge, UK), as described previously (23).

Calculations and statistical analysis. GFR was calculated as the clearance of inulin [urinary (inulin) × UV/plasma (inulin) ml/min]. The clearance of electrolytes was calculated as urinary (electrolyte) × UV/plasma (electrolyte) ml/min. Fractional excretion of electrolytes was calculated as [urinary (electrolyte) × UV/plasma (electrolyte) × GFR] × 100%. Data are presented as the mean ± SEM, corrected for body weight where appropriate. As the primary manipulation was of the mother rather than the offspring, n represents the number of litters. No more than three rats per litter were used at either age. Statistical analysis of renal data were by repeated measures ANOVA, with $p \leq 0.05$ considered significant (SPSS for Windows, version 10.1.0; SPSS UK Ltd, Surrey, UK). As all measured renal parameters remained stable in both OC and OD groups over the 1-h (4 wk) and 3-h (8 wk) collection periods, data have been combined and are presented as a single mean ± SEM value. Body weights, plasma electrolytes, and serum PTH concentrations were compared using an independent samples t test.

RESULTS

Renal function at 4 wk. Neither body weight [OC ($n = 5$), 96.0 ± 4.8 *versus* OD ($n = 5$), 100.0 ± 8.8 g; $p = 0.7$] nor systolic blood pressure (OC 108 ± 4 *versus* OD 108 ± 7 mm Hg; $p = 1.0$) differed between OC and OD at 4 wk of age. Plasma sodium and magnesium concentrations were also comparable between the two groups. However, the plasma concentration of ionized calcium was significantly higher in OD rats (OC 1.01 ± 0.01 *versus* OD 1.18 ± 0.06 mmol/L; $p = 0.04$).

GFR ($p = 0.001$) and UV ($p = 0.025$) both were significantly higher in OD compared with OC rats (Fig. 1). Urinary sodium excretion ($p = 0.004$; Fig. 1) and sodium clearance [OC ($n = 5$), 47.7 ± 3.6 *versus* OD ($n = 5$), 10.6 ± 0.6 μL · min^{-1} · 100 g body wt^{-1}; $p = 0.004$] were higher in OD animals. However, fractional excretion of sodium did not differ ($p = 0.7$; Fig. 1); thus, the higher rates of sodium excretion reflected the higher GFR of OD rats rather than any changes in tubular reabsorption of filtered sodium.

In contrast, in the face of the higher filtered load of ions in OD, urinary calcium excretion was similar in the two groups. Fractional excretion of calcium was significantly lower ($p = 0.014$) in the OD rats (Fig. 2), indicating substantially increased tubular reabsorption of calcium. Calcium clearance, accordingly, was comparable between OC and OD animals [OC ($n = 5$), 45.9 ± 3.3 *versus* OD ($n = 5$), 39.8 ± 6.1 μL · min^{-1} · 100 g body wt^{-1}; $p = 0.4$]. Urinary magnesium excretion ($p = 0.67$; Fig. 2) and magnesium clearance [OC ($n = 5$), 184.8 ± 41.9 *versus* OD ($n = 5$), 194.1 ± 54.6 μL ·

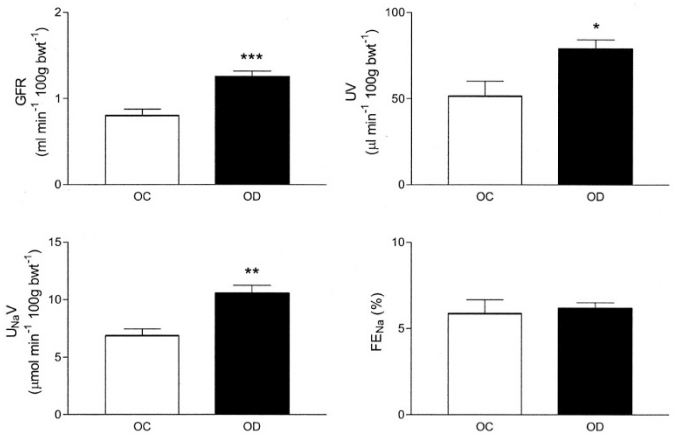

Figure 1. GFR, UV, sodium excretion ($U_{Na}V$), and fractional sodium excretion (FE_{Na}) in 4-wk-old OC (□; $n = 5$ from five litters) and OD (■; $n = 5$ from five litters). Statistical comparisons were by repeated measures ANOVA. As all measured renal parameters remained stable in both OC and OD groups over the 1-h experimental period, data have been combined and are presented as a single mean ± SEM value. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ OC *vs* OD.

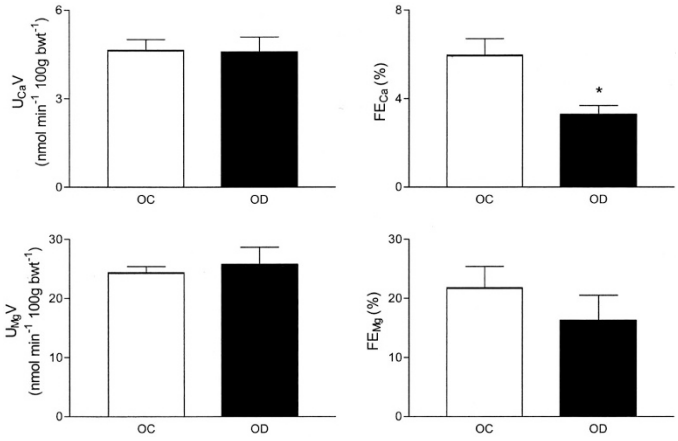

Figure 2. Calcium excretion ($U_{Ca}V$), fractional calcium excretion (FE_{Ca}), magnesium excretion ($U_{Mg}V$), and fractional magnesium excretion (FE_{Mg}) in 4-wk-old OC (□; $n = 5$ from 5 litters) and OD (■; $n = 5$ from five litters). Statistical comparisons were by repeated measures ANOVA. As all measured renal parameters remained stable in both OC and OD groups over the 1-h experimental period, data have been combined and are presented as a single mean ± SEM value. *$p < 0.05$ OC *vs* OD.

$min^{-1} \cdot 100$ g body wt^{-1}; $p = 0.89$] did not differ between the groups. Again, the higher filtered load of magnesium seems to have been compensated by increased tubular reabsorption, although the lower fractional magnesium excretion was not statistically different from OC ($p = 0.35$; Fig. 2).

Renal function at 8 wk. Body weight [OC ($n = 6$), 403.9 ± 6.0 *versus* OD ($n = 5$), 365.8 ± 19.1 g; $p = 0.1$] and systolic blood pressure (OC 123 ± 9 *versus* OD 124 ± 4 mm Hg; $p = 0.92$) remained comparable between OC and OD at 8 wk of age. Plasma sodium, calcium, and magnesium concentrations did not differ between the two groups.

GFR ($p = 0.001$) and UV ($p = 0.005$) remained higher in OD at 8 wk of age (Fig. 3). In contrast to the pattern at 4 wk, urinary sodium excretion (Fig. 3) and sodium clearance [OC ($n = 6$), 13.1 ± 4.9 *versus* OD ($n = 5$), 18.9 ± 1.8 $\mu L \cdot min^{-1}$

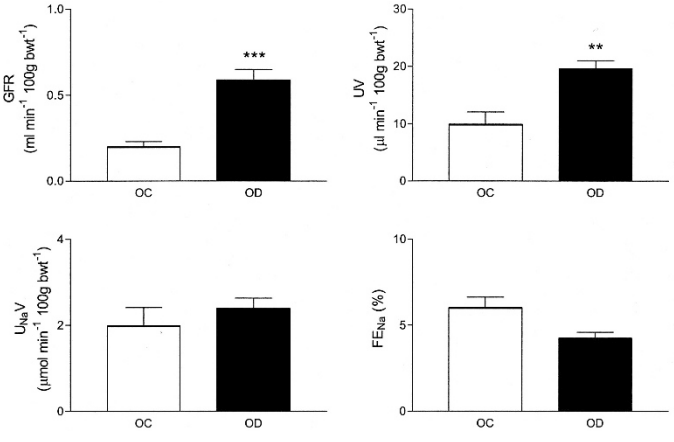

Figure 3. GFR, UV, $U_{Na}V$, and FE_{Na} in 8-wk-old OC (□; $n = 9$ from six litters) and OD (■; $n = 6$ from five litters). Statistical comparisons were by repeated measures ANOVA. As all measured renal parameters remained stable in both OC and OD groups over the 3-h experimental period, data have been combined and are presented as a single mean ± SEM value. **$p < 0.01$, ***$p < 0.001$ OC *vs* OD.

$\cdot$ 100 g body wt^{-1}; $p = 0.31$] by OD rats were no longer different from those of OC rats. Fractional excretion of sodium, although lower in OD rats, did not differ significantly between groups at 8 wk of age (Fig. 3).

Renal calcium handling by 8-wk-old OD rats was similar to that seen at 4 wk. Urinary calcium excretion did not differ between OD and OC rats (Fig. 4), and calcium clearance was comparable between the two groups [OC ($n = 6$), 14.7 ± 2.0 *versus* OD ($n = 5$), 19.3 ± 2.3 $\mu L \cdot min^{-1} \cdot 100$ g body wt^{-1}; $p = 0.154$]. Again, this reflected increased tubular reabsorption such that the fractional excretion of calcium by OD rats was 63% lower than that of OC rats ($p = 0.035$; Fig. 4). Magnesium handling followed the same pattern. Magnesium excretion did not differ (Fig. 4), and magnesium clearance was comparable between the two groups [OC ($n = 6$), 74.1 ± 12.9 *versus* OD ($n = 5$), 59.7 ± 5.8 $\mu L \cdot min^{-1} \cdot 100$ g body wt^{-1}; $p = 0.4$]. This resulted from enhanced tubular reabsorption;

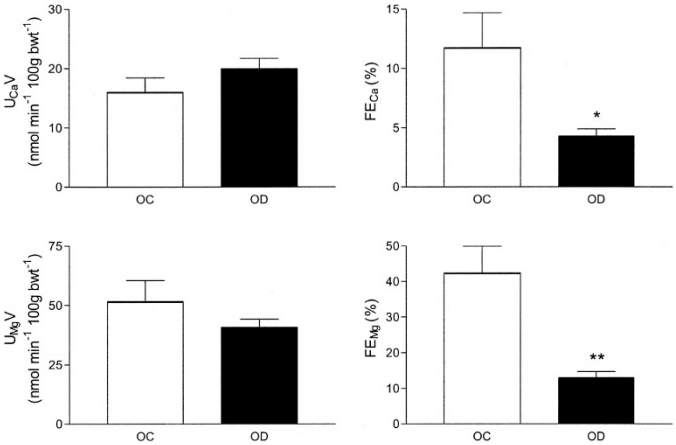

Figure 4. $U_{Ca}V$, FE_{Ca}, $U_{Mg}V$, and FE_{Mg} in 8-wk-old OC (□; $n = 9$ from six litters) and OD (■; $n = 6$ from five litters). Statistical comparisons were by repeated measures ANOVA. As all measured renal parameters remained stable in both OC and OD groups over the 3-h experimental period, data have been combined and are presented as a single mean ± SEM value. *$p < 0.05$, **$p < 0.01$ OC *vs* OD.

thus, fractional excretion of magnesium was significantly lower in OD rats ($p = 0.005$; Fig. 4). The serum concentration of PTH was significantly lower in OD compared with OC rats [OC ($n = 7$), 539.4 ± 142.1 *versus* OD ($n = 9$), 174.3 ± 69.4 pg/ml; $p = 0.026$) at 8 wk of age.

DISCUSSION

Hypomagnesemia and hypocalcemia have been widely reported in the neonatal offspring of human diabetic pregnancy (2,7–9), despite recent observations that show a reduction in urinary calcium and magnesium excretion by children who are born to diabetic mothers (24). This study has demonstrated that offspring that are born to mothers with diabetes maintain altered renal function, in particular enhanced tubular reabsorption of calcium and magnesium, under the conditions of renal clearance analysis used herein. This was associated with a reduction in serum PTH concentration. These observations extend our previous report of a reduction in 24-h calcium and magnesium output in metabolism cage studies in OD at 0, 8, 12, and 16 wk of age (5,10,15). In the current study, the reductions in fractional excretion of calcium and magnesium occurred despite a concurrent increase in GFR in OD rats. Renal hyperfiltration is widely reported in diabetic rats; suggested mechanisms include glucosuria-mediated osmotic diuresis (25), a reduction in tubuloglomerular feedback (26), increased glomerular arginine uptake (27), increased nitric oxide generation (28), and renal sympathetic nerve activity (29). These mechanisms have not been explored in OD rats, so the reason(s) for the increase in GFR observed in OD in the current study remains unclear.

An increase in GFR leads to an increase in the filtered load of ions, but despite this, reabsorption of calcium and magnesium remained enhanced. In contrast, UVs were elevated in both age groups and sodium excretion was increased in 4-wk-old OD rats. This suggests that there was no tubular compensation in the handling of water and sodium, whereas markedly enhanced calcium and magnesium tubular reabsorptive transport is a characteristic feature in OD. Hence, the observed reduction in fractional excretion of calcium and magnesium seems to reflect specific alterations in renal tubular handling of these ions by OD animals.

Calcium reabsorption occurs both in the proximal tubule, *via* a passive paracellular pathway, and in the distal nephron, *via* a hormone-regulated transcellular route (30). We demonstrated recently that expression of all three key distal tubular transport proteins, epithelial calcium channel, calbindin-$D_{28\ K}$, and PMCA, is increased in homogenates of whole kidneys from both neonatal and adult rats that are born to diabetic mothers (10,15), which is consistent with the reduction in the fractional excretion of calcium reported herein (31). Taken together with our previous observations of reduced calcium excretion in OD from neonates to 16 wk of age (5,10,15), these data suggest that expression of renal calcium transporter protein and tubular calcium reabsorption are programmed *in utero* in the OD rat to exceed that of the normal animal.

Like humans, diabetic pregnancy in the rat is associated with hypercalciuria (5) and hypocalcemia of the mother (4). Placental calcium content is reduced in pregnant diabetic rats (32), and maternofetal calcium flux across the placenta is also reduced (6). This probably occurs as a result of a reduction in the placental calcium binding protein calbindin$_{9\ K}$ (6,33), which translocates calcium across the trophoblast cytosol (34), resulting in fetal calcium deficiency *in utero*. This background, accordingly, may promote a calcium retention strategy by the developing fetal kidney that then seems to persist post partum.

This may be exacerbated by the effects of hyperglycemia on the developing kidney. Glucose readily crosses the placenta down a maternofetal concentration gradient; hence, the fetuses of diabetic rats have elevated plasma glucose concentrations as a result of their mother's hyperglycemia (35). Exposure of the developing metanephros to high glucose concentrations, either *in vitro* or *in vivo* by STZ-induced maternal diabetes or maternal glucose infusion, inhibits nephrogenesis (36). As a result, the offspring of diabetic pregnancy have 10–35% fewer nephrons (36). The mechanisms involved are not fully understood but seem to involve up-regulation of the IGF-II/mannose-6-phosphate receptor (M6PR) (37). IGF-II, which plays an important role in renal organogenesis (38), is internalized and degraded by M6PR (39). M6PR expression is increased in the metanephroi of fetuses that are exposed to diabetes *in utero* (37), remaining higher than control animals up to day 20 of gestation (40). It has been suggested that this contributes to the impaired nephrogenesis in fetuses of diabetic mothers (40). We did not determine nephron number in the current study, but it is reasonable to assume that nephrogenesis was impaired in OD animals. Because whole-kidney GFR was increased in OD, single-nephron GFR must be markedly higher in these animals, resulting in higher filtered loads of electrolytes. An increase in the tubular delivery of calcium may also contribute to the up-regulation in calcium transport proteins observed in our earlier studies (10,15).

Magnesium is reabsorbed primarily in the thick ascending limb *via* a paracellular route, with some reabsorption in the proximal tubule and hormone-mediated uptake in the distal tubule (41). Mammalian magnesium transporters have yet to be cloned, but the transport characteristics of the distal nephron have been described. Apical uptake seems to be passive, through a putative magnesium channel (41), whereas movement across the basolateral membrane is against both electrical and concentration gradients. There is evidence for a Na^+/Mg^{2+} exchanger (42) and a Mg^{2+}-ATPase pump (41), which may be linked to the activity of the Ca^{2+}-ATPase pump (PMCA) (43). Distal tubular magnesium reabsorption is also load dependent; increased delivery of magnesium to the distal tubule results in an increase in reabsorption (44). Thus, it is possible that the increase in fractional magnesium reabsorption observed in the current study reflects an increase in distal tubular flow, arising from the associated increase in GFR. However, this may be aggravated by maternal magnesium depletion associated with pregnancy and exacerbated by diabetes (45). Human studies reveal a reduction in amniotic fluid magnesium concentration in diabetic pregnancy (46), which is associated with hypomag-

nesemia in the infant (9). The situation in the rat seems to be slightly different, as plasma magnesium concentrations were not altered in OD in the current study at 4 and 8 wk of age. However, we have shown previously that maternofetal magnesium flux across the placenta is reduced in pregnant diabetic rats (6), suggesting that the rat fetus is magnesium deficient. This could program the fetal kidney to retain magnesium as well as calcium. This hypothesis warrants further investigation once the molecular identity of the renal magnesium transporters has been described.

The serum PTH concentration of 8-wk-old OD was lower than that of OC rats in the current study, which is in accordance with observations in human neonates (2,19,20). A number of hypotheses have been proposed to account for the apparent paradox of hypocalcemia and low PTH concentration. Bergman *et al.* (19) suggested that the fall in glucose and consequent increase in glucagon that arises in the neonate post partum stimulates an increase in calcitonin secretion. This inhibits bone resorption and results in hypocalcemia despite low PTH concentrations. Loughead *et al.* (47) suggested that hypomagnesemia blunts the parathyroid secretory response to hypocalcemia, leading to an inappropriately low PTH concentration. However, these proposed mechanisms do not account for the observations made in the current study. At 8 wk of age, serum PTH was lower in OD, yet both plasma magnesium and calcium concentrations were no different from OC rats. Fractional excretion of magnesium and calcium was considerably lower in OD rats in the face of this reduction in PTH, which is known to stimulate both magnesium and calcium reabsorption (48). These observations suggest that not only the renal tubular reabsorptive mechanism(s) for magnesium and calcium transport but also their sensitivity to PTH action may be programmed *in utero*. Preliminary data suggest that there may be similar effects on renal function in children who are born to women with diabetes in pregnancy (24). The mechanisms by which maternal diabetes induces these alterations in renal divalent ion handling now require attention.

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