Altered calbindin mRNA expression and calcium regulating hormones in rat diabetic pregnancy

K Hamilton\(^1\), M Tein\(^1\), J Glazier\(^1\), E B Mawer\(^2\), J L Berry\(^2\), R J Balmant\(^1\), R D H Boyd\(^3\), H O Garland\(^1\)* and C P Sibley\(^1\)

\(^1\)School of Biological Sciences, Academic Unit of Child Health, University of Manchester, Manchester M13 9PT, UK
\(^2\)Department of Medicine, University of Manchester, Manchester M13 9PT, UK
\(^3\)St George’s Medical School, Cranmer Terrace, London SW17 ORE, UK

(Requests for offprints should be addressed to K Hamilton, Academic Unit of Child Health, University of Manchester, St Mary’s Hospital, Hathersage Road, Manchester M13 OJH, UK; Email: Khamil@js1.cmht.nwest.nhs.uk.)

*(Dr H O Garland has since deceased)*

Abstract

Offspring of rats with diabetes mellitus are at risk of reduced calcium and bone mineral content. Altered expression of the maternal calcium binding proteins, calbindin-D\(_{9K}\) and calbindin-D\(_{28K}\), which are involved in renal and placental calcium transport, may underlie these problems. We have investigated the effect of diabetes on circulating concentrations of regulatory hormones with respect to calbindin-D mRNA concentrations. Three rat groups were studied; control (CP), streptozotocin-induced diabetic (DP), and insulin-treated diabetic (DPI) pregnant rats. Calbindin-D\(_{9K}\) and calbindin-D\(_{28K}\) mRNA abundance in placenta and maternal kidney were measured at days 7, 15, 18 and 21 of gestation, together with serum or plasma concentrations of 1,25 dihydroxy-vitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\)), parathyroid hormone (PTH), PTH-related protein (PTHrP), calcitonin, oestradiol and IGF-I. An increase in placental calbindin-D\(_{9K}\) mRNA abundance between days 18 and 21 in CP and DPI rats was severely blunted in the DP rats. In contrast, renal calbindin-D\(_{28K}\) mRNA abundance was greater at days 7, 15 and 18 in DP compared with CP rats, as was calbindin-D\(_{9K}\) at day 18. Calcitonin concentrations showed no differences between the groups, and both PTH and IGF-I were reduced over the first half of gestation, unlike the calbindins. In contrast, the concentrations of PTHrP and 1,25(OH)\(_2\)D\(_3\) were reduced at term in the DP group compared with the other two groups. Plasma oestadiol concentrations were lower in DP than in CP rats at days 7, 15 and 18, and most striking was the absence in DP rats of the peak of oestadiol seen at day 18 in CP rats. Despite the similarity between changes in placental calbindin mRNA and 1,25(OH)\(_2\)D\(_3\), previous work has shown placental calbindin-D\(_{9K}\) regulation to be vitamin-D-independent. These studies produce suggestive evidence, therefore, that PTHrP and oestadiol may be involved in the altered calbindin-D expression by kidney and placenta in rat diabetic pregnancy.

Journal of Endocrinology (2000) 164, 67–76

Introduction

Significant alterations in calcium homeostasis occur in up to 50% of infants born to insulin-dependent diabetic mothers (Tsang et al. 1972). Such children have an increased incidence of hypocalcaemia (Tsang et al. 1972, Mimouni et al. 1986, 1990) and either a decreased (Lapillone et al. 1997) or an increased (Mimouni et al. 1988) bone mineral content compared with normal infants. A reduced bone mineral content, retarded skeletal development, or both, is found in the offspring of genetically diabetic rats (Verhaeghe et al. 1994) and those rendered diabetic by streptozotocin (STZ; Uriu-Hare et al. 1985, Demignon & Bonneton-Rebut 1988). The reduced bone mineral content in the rat may be attributable in part to a reduced maternofetal placental calcium flux (Husain et al. 1994), associated with an exacerbated maternal renal loss of calcium (Birdsey et al. 1995).

Both placental and renal epithelia transport calcium by an energy-dependent transcellular process (Shareghi & Stoner 1978, Stulc & Štulcová 1986). In pregnancy, the expression of the cytosolic calcium binding protein, calbindin-D\(_{9K}\), appears to be rate-limiting to transcellular calcium transport across the rat placenta (Glazier et al. 1992). In diabetic rat pregnancy, the abundance of placental calbindin-D\(_{9K}\) mRNA is markedly reduced near term, in association with a diminished maternofetal calcium flux (Husain et al. 1994). In the kidney, the energy-dependent component of calcium reabsorption occurs mainly in the distal tubule and involves another

Journal of Endocrinology (2000) 164, 67–76

0022–0795/00/0164–0067 © 2000 Society for Endocrinology Printed in Great Britain

Online version via http://www.endocrinology.org

Downloaded from Bioscientifica.com at 01/03/2019 06:47:13AM via free access
cytosolic transporting protein, calbindin-D\textsubscript{28K} (the main renal protein) in addition to calbindin-D\textsubscript{9K} (Thomasset \textit{et al.} 1982). One study has reported that maternal renal concentrations of calbindin-D\textsubscript{9K} and calbindin-D\textsubscript{28K} proteins at term were comparable between control and genetically diabetic rats (Verhaeghe \textit{et al.} 1988b). The regulation of expression of calbindins in these two calcium-transporting epithelia is distinct: whereas renal calbindin expression is dependent on serum vitamin D\textsubscript{3} metabolite concentrations (Thomasset \textit{et al.} 1982), placental calbindin-D\textsubscript{9K} expression is apparently not regulated by this hormone (Glagier \textit{et al.} 1995).

Altered placental and renal calcium handling in rat diabetic pregnancy may, at least in part, reflect an altered production or function of calcium regulatory hormones. However, it is not known when, in the course of pregnancy, the effect of diabetes on placental and renal calbindin expression manifests itself, nor how this relates to pregnancy and to diabetes-dependent changes in circulating hormones. The aim of this study was to address these questions by measuring calbindin-D\textsubscript{9K} and calbindin-D\textsubscript{28K} mRNA expression in placenta and kidney over the course of pregnancy in control, diabetic, and insulin-treated diabetic rats. In the same animals, we also measured maternal serum or plasma concentrations of hormones associated with calcium homeostasis (1,25 dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}), parathyroid hormone (PTH), PTH-related protein (PTHrP) and calcitomin), and those likely to change in diabetes (insulin-like growth factor-I (IGF-I)) or pregnancy (oestradiol). Most of these hormones have also been shown to be putative controllers of calbindin-D expression. It is well known that both renal calbindins are vitamin-D-dependent (Thomasset \textit{et al.} 1982). Both the placental calbindin-D\textsubscript{9K} gene (Darwish \textit{et al.} 1991) and the renal calbindin-D\textsubscript{28K} gene (Gill & Christakos 1995) have been shown to have an oestrogen-responsive element. Although the gene-expression effects of both PTH and PTHrP are unknown, PTH has been shown to increase cytosolic concentration of renal calbindin-D\textsubscript{28K} in vivo (Hemmingsen \textit{et al.} 1996), whereas PTHrP has been shown to increase renal calbindin-D\textsubscript{28K} in the rat by a direct effect, without mediation by 1,25(OH)\textsubscript{2}D\textsubscript{3} or plasma calcium (Hemmingsen \textit{et al.} 1996). We hypothesised that a temporal association between altered calbindin-D expression and an alteration in one or more of the hormones measured would provide clues to the mechanisms responsible for the altered calcium handling by placenta and kidney associated with diabetic pregnancy in the rat.

\section*{Materials and Methods}

\textit{Animals}

All work was performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

\textit{Blood and tissue collection}

Animals were killed by cervical dislocation at day 7, 15, 18 or 21 of gestation. After immediate laparotomy, the terminal blood sample was withdrawn from the abdominal aorta and an aliquot immediately placed in a heparinised
tube for plasma separation. Other blood aliquots were handled appropriately for specific hormone analysis (see below). Both kidneys and all placenta were removed, briefly blotted and immediately frozen in liquid nitrogen. All tissues were stored at \(-80\) °C.

Plasma was assayed for glucose concentration by the glucose oxidase–peroxidase method (Ames Sera-Pak, Bayer Diagnostics, Basingstoke, Hampshire, UK). Incubated samples were measured on a spectrophotometer (Shimadzu UV-2101PC, Shimadzu Corp., Kyoto, Japan) at a wavelength of 505 nm.

**RNA extraction and Northern/dot blot analysis**

Tissue was used from six to eight rats randomly chosen from each experimental group at each gestational sample time (except for day 7, when the chorioallantoic placenta is not fully formed). Total RNA was extracted from placenta (ten on day 15, four or five on day 18, and three on day 21 from each rat, again using random sampling) and one kidney (left or right, alternate sampling), as described previously (Glazier et al. 1992). The resulting RNA pellet was dissolved in RNase-free water, quantified by measuring absorbance at 260 nm and then stored at \(-80\) °C.

Sets of dot blots of all placental and all kidney RNA were prepared using 20 mg total RNA from each tissue sample, incubated with 10× standard saline citrate (SSC) and 50% formamide at 60 °C for 10 min. The sample was applied to a filter (Hybond N, Amersham International, Bucks, UK), washed twice with 10× SSC and fixed by exposure to u.v. light.

We have previously shown that the calbindin-D\(_{9K}\) cDNA used here detected a single transcript of the expected size (0.6 kb) on Northern blots of placental mRNA (Glazier et al. 1992, 1995). To check the size of the renal calbindin-D\(_{9K}\) and calbindin-D\(_{28K}\) transcripts, a pool of poly-A enriched RNA (Pharmacia Biotech, St Albans, UK) was prepared from kidneys of the CP and DP groups at each gestational age and Northern blots prepared as described previously (Glazier et al. 1992).

Filters were prehybridised, then hybridised with \(^{32}\)P-labelled cDNA for either rat intestinal calbindin-D\(_{9K}\) (placenta and kidney) or calbindin-D\(_{28K}\) (kidney only) as described previously (Thomasset et al. 1982, Lonri et al. 1989), with the addition, at the end of the previous method, of two stringency washes (0-1× SSC, 0-1% SDS at 60 °C for 20 min) for calbindin-D\(_{28K}\) hybridisations.

Replicates of all dot blots were probed with oligo-dT as a control to correct for variations in loading between samples. All blots were analysed and dot blots quantified on an autoimager (Packard Instant Imager, Packard Bioscience, Berks, UK) for varying times, depending on the strength of the signal (2 min for oligo-dT, up to 3 days for the calbindin-D\(_{9K}\) Northern blot). The Instant Imager provides a visual image of the signal and quantitates it by measuring radioactive emission with a wide dynamic range. Northern blots were also autoradiographed at \(-80\) °C.

**Measurements on terminal blood samples**

**1,25(OH)\(_2\)D\(_3\)** A 1 ml aliquot of blood was placed in a lithium-coated tube and the plasma separated by centrifugation. Plasma 1,25(OH)\(_2\)D\(_3\) concentration was measured using a kit (IDS Ltd, Boldon, Tyne and Wear, UK), which involved immunoextraction followed by radioimmunoassay as described previously (Fraser et al. 1997). The inter- and intra-assay variations were 8% and 10% respectively, and the assay sensitivity was 5 pM.

**PTH** To obtain serum, 1 ml blood was allowed to clot and then centrifuged. PTH concentration was determined in duplicate using a Rat PTH Immunoradiometric Assay kit (Nichols Diagnostics, Cambridge, UK). Intra-assay variation was 4–4.3%, inter-assay variation 4.3–4.7% and assay sensitivity was 1 pg/ml (data provided by the suppliers).

**PTHrP** One millilitre of blood was dispensed into an ice-cold PTHrP cocktail tube (Nichols Diagnostics). The sample was centrifuged and the plasma used for determination of PTHrP (in duplicate) using the Allegro PTHrP Immunoassay kit (Nichols Diagnostics). The supplier reported that this assay had a sensitivity of 0.3 pM, an intra-assay variation of 2.9–9.5% and an inter-assay variation of 5.3–5.6%. The validity of using this kit for rat measurements has been confirmed (Benitez-Verguizas & Esbrit 1994, Naranjo et al. 1994).

**Calcitonin** Calcitonin concentration was determined in duplicate serum samples using a Calcitonin Chemiluminescence Immunoassay kit (Nichols Diagnostics). Intra-assay variation was 6.7–8.3%, inter-assay variation was 6.6–11.6%, and assay sensitivity was 3–5 pg/ml as reported by the suppliers. The validity of using this kit for rat measurements has been confirmed (Kalu et al. 1988).

**Oestradiol** Oestradiol concentration in duplicate 100 µl serum samples was determined using a Coat-A Count Oestradiol radioimmunoassay kit (DPC Limited, Gwynedd, UK). Intra-assay variation was 4–7%, inter-assay variation 4.2–8.1% and assay sensitivity 8 pg/ml as reported by the suppliers.

**IGF-I** IGF-I was measured in duplicate 200 µl serum samples using an IGF-I Immunoassay kit (Nichols Diagnostics). Assay sensitivity was 0.1 ng/ml as reported by the suppliers. Intra-assay variability was 2.4–3.3%, the inter-assay coefficient of variation was 5.2–8.4%.

Journal of Endocrinology (2000) 164, 67–76
compared with DPI (Mann–et al means ** 8 ), DPI (taken at days 7, 15, 18 and 21 of gestation in CP (76) Journal of Endocrinology cDNA gives a single transcript of 6 kb, as expected (Huang & Christakos 1988, Li & Christakos 1991). Quantitation of renal calbindin-D9K mRNA in dot blots, normalised to oligo-dT, is shown in Fig. 2b. In CP animals, calbindin-D9K mRNA levels remained steady until day 18 of gestation, after which there was a sharp increase (P<0.01, comparing days 18 and 21 by t-test). In contrast to the pattern seen in the placenta, renal calbindin-D9K mRNA in diabetic animals was higher than in either the CP or DPI groups throughout gestation. However, this achieved statistical significance only at day 18 (P<0.001 DP compared with CP; P<0.001 DP compared with DPI, one-way ANOVA). At term, no differences existed between the three groups. This was due primarily to the increase in calbindin-D9K mRNA in the CP group, rather than a decrease in the values in DP animals.

Northern hybridisation of rat renal poly-A-enriched RNA (from CP and DP rats) with calbindin-D9K cDNA revealed a single transcript at 0.6 kb in both the CP and DPI groups (Fig. 3a), consistent with previous observations (Huang et al. 1989, Li & Christakos 1991). Quantitation of renal calbindin-D9K mRNA in dot blots, normalised to oligo-dT, is shown in Fig. 2b. In CP animals, calbindin-D9K mRNA levels remained steady until day 18 of gestation, after which there was a sharp increase (P<0.01, comparing days 18 and 21 by t-test). In contrast to the pattern seen in the placenta, renal calbindin-D9K mRNA in diabetic animals was higher than in either the CP or DPI groups throughout gestation. However, this achieved statistical significance only at day 18 (P<0.001 DP compared with CP; P<0.001 DP compared with DPI, one-way ANOVA). At term, no differences existed between the three groups. This was due primarily to the increase in calbindin-D9K mRNA in the CP group, rather than a decrease in the values in DP animals.

Northern hybridisation of renal poly-A-enriched RNA with calbindin-D28K cDNA (Fig. 3b) revealed three transcripts, at 3.3, 2.8 and 1.9 kb, which agrees well with previous reports (Huang & Christakos 1988, Huang et al. 1989). All three transcripts on the Northern blot appeared to change similarly over gestation and to have greater expression in the DP group than in the CP group. This was confirmed when quantification of the dot blot was performed (Fig. 2f). In CP kidneys, there was a trend in calbindin-D28K mRNA expression similar to that seen for calbindin-D9K, with mRNA levels remaining relatively low until day 18, after which a sharp increase occurred to day 21 (P<0.05, comparing days 18 and 21 by t-test). Again, calbindin-D28K mRNA levels in DP kidneys were greater (P<0.01, Mann–Whitney U-test) than in those of the CP animals throughout gestation until day 21. As for

Statistics

All data are presented as means ± s.e.m. and n=number of animals. A two-way analysis of variance (ANOVA) was first performed, including the Bartlett–Box test for the homogeneity of variances between the groups. If variances were not significantly different, a one-way ANOVA with Scheffé’s post hoc test was performed at each gestational time point, to distinguish differences between the three groups. If variances were significantly different, the non-parametric Kruskall–Wallis test was used, followed by paired Mann–Whitney U-tests for specific differences between the groups. Any further specific post hoc tests (a decrease or increase in values) were performed using an unpaired Student’s t-test.

Results

Plasma glucose

Plasma glucose concentrations in terminal blood samples for the DP group were significantly greater than those in the CP and DPI groups at each gestational time point (Fig. 1). Plasma glucose concentrations in the DPI rats were comparable to those in the CP group, except at day 7, when DPI values were slightly but significantly (P<0.01) lower.

Gestational changes in placental and renal calbindins

We have previously shown, by Northern blotting, that probing of placental mRNA with the calbindin-D9K cDNA gives a single transcript of 0.6 kb, as expected (Glazier et al. 1992, 1995, Husain et al. 1994). Figure 2a

*Figure 1* Plasma glucose concentrations from the terminal sample taken at days 7, 15, 18 and 21 of gestation in CP (●, n=15, 9, 8), DP (■, n=14, 9, 8, 14) and DPI (▲, n=11, 9, 7, 9) rats. **P<0.01, ***P<0.001 compared with CP and DPI; ++P<0.01 compared with DPI (Mann–Whitney U-tests). Values are means ± S.E.M.
calbindin-D_{9K}, the DPI kidneys were intermediate between the other two groups, and showed a gestational pattern in mRNA expression similar to that seen in the CP group.

**Gestational changes in maternal hormone concentrations**

1,25(OH)_{2}D_{3} (Fig. 4a) Maternal plasma concentrations of 1,25(OH)_{2}D_{3} remained relatively stable in both the CP and DPI groups until day 18 of gestation, when there was a marked, significant increase (P<0.01 for both groups, Mann–Whitney U-test). In the diabetic animals the concentration of 1,25(OH)_{2}D_{3} was significantly (P<0.05) lower than that in the CP and DPI animals at day 15, and failed to show the increase seen in the other two groups towards term.

PTH (Fig. 4b) Maternal serum PTH concentrations differed between the three groups early in gestation. In particular, values for the DP group were significantly (P<0.001) lower than the CP group at day 7. Toward the end of gestation (days 18 and 21), no significant differences were apparent between the groups, and there was a consistent increase over the last 3 days of gestation in all groups.

PTHRP (Fig. 4c) DP animals showed a steady decrease in PTHrP concentrations from day 7 of pregnancy and, as a result, both the CP and DPI groups had significantly greater serum PTHrP concentrations than the DP animals at days 18 and 21 of gestation. This contrasts with the data for PTH, for which no significant differences were seen between groups at these stages. The value in the DPI group was significantly greater than those in the other two groups at day 15.

Calcitonin There was a decrease in calcitonin concentrations in each group as gestation proceeded, but no statistically significant differences were apparent between the three rat groups at any stage of gestation (data not shown).

Oestradiol (Fig. 4d) Maternal serum oestradiol concentrations in CP animals were greatest at day 18 of gestation, with a sharp decrease towards term. In contrast, in the DP group oestradiol concentrations decreased steadily throughout gestation and were lower than in controls until term, although this achieved significance only at day 18 (P<0.01 CP compared with DP, Mann–Whitney U-test). In the DPI rats, oestradiol concentrations were also greater than in the DP group until day 18, when values decreased sharply, to become lower than in either of the other groups. It is striking that both the DP and DPI groups failed to show the late gestational peak in oestradiol concentration that was evident at day 18 in CP rats.
IGF-I (Fig. 4) Maternal serum IGF-I concentrations showed a marked gestational decrease in all groups. The DP animals had a greatly reduced serum IGF-I concentration compared with both CP and DPI rats early in pregnancy ($P < 0.001$ at days 7 and 15, Mann–Whitney U-test). Towards term, hormone concentrations in the three groups of animals began to converge, and by day 21 there was no significant difference between the groups.

Discussion

Gestational changes in placental and renal calbindins

Over the last third of normal pregnancy, the rat fetus accumulates more than 99% of its body calcium (Comar 1956). During this time, the unidirectional materno-fetal placental calcium transfer increases markedly and approaches net flux (Glazier et al. 1992, Sulk & Sulková 1986). This is normally matched by a gestational increase in placental calbindin-D$_{9K}$ mRNA expression (current data and Glazier et al. 1992, Krisinger et al. 1992), suggesting that this protein is rate-limiting to placental calcium transport in the rat. Here, we report a progressively blunted gestational increase in placental calbindin-D$_{9K}$ mRNA expression in the diabetic pregnant rat, complementing previous data showing a reduction at term (Husain et al. 1994, Verhaeghe et al. 1988b). The diminished increase in placental calbindin-D$_{9K}$ mRNA expression in diabetic animals that we observed from day 18 of pregnancy correlates with the diminished materno-fetal placental calcium flux and lower fetal calcium content previously observed in these animals (Husain et al. 1994).

In normal pregnancy, the increased fetal requirement for calcium as gestation proceeds places an increasing demand on maternal calcium homeostasis, although maternal plasma calcium concentrations are maintained in the rat towards term (Green & Hatton 1988, Husain et al. 1994) and bone calcium stores are not depleted (Miller et al. 1986). One possible mechanism whereby the non-diabetic mother might increase dietary calcium availability for the fetus is to limit renal losses by increasing renal calcium reabsorption. Indeed, previous reports from our laboratory have shown that there is a slight decrease in maternal renal calcium excretion towards the end of pregnancy (Birdsey et al. 1995, Garland et al. 1997). This may result, at least partially, from the sharp increase we have observed here in the expression of both calbindin-D species in the maternal kidney over days 18–21 of gestation. Such an increase would serve to enhance maternal renal calcium reabsorption in the distal tubule, thereby making more calcium available to the mother.

The novel longitudinal design of our study showed that renal calbindin-D mRNA expression was greatly increased in diabetic pregnant rats, compared with control values, up to day 18 of gestation. Our findings, at term, that maternal renal abundance of calbindin-D$_{9K}$ and calbindin-D$_{28K}$ mRNA were comparable between control and diabetic rats agree well with previous observations (Verhaeghe et al. 1988b). The fact that renal calbindin mRNA abundance is high at the start of diabetic pregnancy may be interpreted as an adaptive response to the extremely high renal calcium output that occurs immediately after the onset of experimental diabetes (Anwana 1989, Birdsey et al. 1995). It is, perhaps, surprising that this is not further enhanced as the increased calcium demands
Figure 4 (a) Maternal plasma 1,25(OH)₂D₃ at days 7, 15, 18 and 21 of gestation in CP (●, n=12, 7, 7, 9), DP (■, n=11, 9, 10, 10) and DPI (▲, n=8, 8, 8, 8) rats. **P<0.01 compared with DPI; ***P<0.001 compared with CP and DPI. (b) Maternal serum PTH at days 7, 15, 18 and 21 of gestation in CP (●, n=9, 10, 9, 9), DP (■, n=10, 10, 12, 12) and DPI (▲, n=7, 8, 7, 8) rats. *P<0.05, **P<0.01 compared with CP; *P<0.05 compared with CP and DPI (Mann–Whitney U-tests). Values are means ± S.E.M. (c) Maternal serum PTHrP at days 7, 15, 18 and 21 of gestation in CP (●, n=8, 7, 6, 7), DP (■, n=9, 11, 9, 10) and DPI (▲, n=10, 8, 7, 8) rats. +P<0.05, ++P<0.01 compared with CP; ^P<0.05, +++P<0.001 compared with DPI (Mann–Whitney U-tests at day 15, one-way ANOVA at days 18 and 21). Values are means ± S.E.M. (d) Maternal serum oestradiol at days 7, 15, 18 and 21 of gestation in CP (●, n=7, 8, 9, 7), DP (■, n=9, 7, 7, 10) and DPI (▲, n=8, 8, 7, 5) rats. +P<0.05, ++P<0.01 compared with CP; *P<0.05, **P<0.01 compared with DPI (Mann–Whitney U-tests). Values are means ± S.E.M. (e) Maternal serum IGF-I at days 7, 15, 18 and 21 of gestation in CP (●, n=9, 9, 8, 8), DP (■, n=11, 11, 11, 12) and DPI (▲, n=6, 8, 7, 6) rats. +++P<0.001 compared with CP; *P<0.05, **P<0.01 compared with CP (Mann–Whitney U-tests). Values are means ± S.E.M.
of pregnancy ensue, and this may contribute to the reduced calcium of diabetic pregnant offspring.

**Gestational changes in maternal hormone concentrations**

1,25(OH)₂D₃ The observed increase in maternal plasma 1,25(OH)₂D₃ concentration over the last third of normal pregnancy confirms the findings of previous investigations in the rat (Halloran et al. 1979, Verhaeghe et al. 1988b, Paulson et al. 1990). As the expression of both renal calbindins has previously been shown to be vitamin-D-dependent (Thomasset et al. 1982), this increase is likely to stimulate gene expression and may account for the increase in calbindin-D mRNA levels in the kidney that we have demonstrated here. It is difficult, however, to reconcile the severely reduced 1,25(OH)₂D₃ concentration seen in late gestation in our DP rats from day 15 and also reported by others at term (Demignon & Bonneton-Rebut 1988, Mimouni et al. 1988, Verhaeghe et al. 1999) with the very high renal calbindin-D mRNA levels seen in the same group at the same time.

In contrast to the findings in kidney, the gestational profiles for placental calbindin-D₉K and 1,25(OH)₂D₃ concentration did mirror each other for each of our experimental groups. However, despite the 1,25(OH)₂D₃-responsive element in the calbindin-D₉K gene (Darwish & DeLuca 1992), placental calbindin-D₀K expression is not dependent on this hormone (Glazier et al. 1995). It seems likely, therefore, that factors other than vitamin D₃ metabolites are involved in activating the renal and placental calbindin genes in diabetic pregnancy.

PTH Several studies have reported an increase in PTH concentrations during the last few days of gestation in the rat (e.g. Bourdeau et al. 1990), consistent with the data reported here. One study has reported lower PTH concentrations in DP than in CP rats (Verhaeghe et al. 1999). However, clinical studies have found similar (Cruickshank et al. 1980, Mimouni et al. 1989) or reduced (Cruickshank et al. 1994) serum PTH concentrations in diabetic pregnant women compared with controls. In the non-pregnant diabetic rat, PTH concentrations have been found to be slightly lower than those in controls (Takeshita et al. 1994), which agrees with the early gestational measures in our study. However, no significant differences existed between the three groups over days 18 and 21. Although PTH has been shown to increase cytosolic concentration of renal calbindin-D₂₈K in vivo (Hemmingsen et al. 1996), the PTH profile for our diabetic rats throughout gestation does not concur with that for the calbindins. It seems unlikely, therefore, that PTH is responsible for the altered calbindin-D mRNA expression patterns seen in placenta and kidney of diabetic pregnant animals.

PTHrP It is well documented that PTHrP acts mainly as an autocrine factor in the adult (Philbrick et al. 1996). In the fetal sheep, it has been shown to act on the placenta to regulate calcium transport (Rodda et al. 1988). Although this does not appear to happen in the rat (Shaw et al. 1991), absence of the PTHrP receptor in the mouse diminishes calcium acquisition by the fetus (Kovacs et al. 1996). The similarity between our PTHrP data and the placental calbindin-D₀K mRNA towards term (significantly lower in DP rats, compared with increasing concentrations in CP rats) suggests that PTHrP might thus be a regulatory factor for placental calbindin-D₀K mRNA. PTHrP has been shown to increase renal calbindin-D₂₈K in the rat by a direct effect (Hemmingsen et al. 1996), but the changing pattern of maternal PTHrP seen in the CP and DP rats in this study did not parallel the renal calbindin-D profile for the two groups.

Calcitonin The lack of differences in calcitonin concentrations between our three groups throughout gestation compares well with previous human data for calcitonin (Cruickshank et al. 1980, 1983). Thus it seems unlikely that calcitonin affects calbindin-D expression in the placenta or kidney.

Oestradiol In the DP rats, oestradiol concentrations were lower than those in controls until day 21, and failed to show the sharp peak found at day 18 in the CP group; this peak was also absent in the DPI group. Several previous studies have reported a similar peak in maternal oestradiol concentrations at or around day 18 of normal rat pregnancy (e.g. Garland et al. 1987). As the calbindin-D₀K gene is known to have an oestrogen-responsive element (Darwish et al. 1991), it is tempting to speculate that there might be a causal link between this oestradiol peak and the coincident marked gestational increase in placental expression of calbindin-D₀K mRNA in the CP group. This proposal is supported by the blunted increase in placental calbindin-D₀K mRNA expression and the absence of a peak in oestradiol in the diabetic pregnant rats. The lack of increase in oestradiol in the DPI group with a concurrent increase in calbindin-D₀K mRNA expression is, however, not consistent with this hypothesis.

Nevertheless, oestradiol concentrations were lower in the diabetic pregnant group than in either the insulin-treated diabetic or control groups. Thus, if oestradiol does have a role in activating the placental calbindin-D₀K gene, these reduced oestradiol concentrations may be the key to explaining the lower placental calbindin-D₀K mRNA of diabetic rats seen in our study. Oestradiol is apparently involved in control of the uterine calbindin-D₀K gene (L’Horset et al. 1990), and uterine calbindin-D₀K mRNA changes profoundly throughout pregnancy and lactation in the rat (Krisinger et al. 1992). Oestrogen can also inhibit uterine calbindin-D₂₈K in the mouse (Opperman et al. 1992), perhaps explaining the greater renal calbindin mRNA levels in the DP rats in the face of lower oestradiol concentrations.
IGF-I  The sharp decline in maternal serum IGF-I concentrations from approximately half-way through normal pregnancy is in accordance with earlier observations in the rat (e.g. Gargosky et al. 1990). Reduced serum IGF-I concentrations in diabetic pregnancy, as here in the rat, have previously been reported in humans (Whittaker et al. 1990) and rabbits (D’Ercole et al. 1984). As the gestational profiles for IGF-I and calbindin-D mRNA levels were so dissimilar, it seems unlikely that IGF-I contributes to the altered calbindin-D mRNA expression of DP rats.

Calbindin mRNA expression, diabetes and hormonal control
This study underscores the likely importance of interplay between regulatory factors of gene expression for calbindin-D_{28K} and calbindin-D_{9K} in the kidney and placenta in response to maternal diabetes. Our data pinpoint gestational day 18 as a likely time of control of calbindin-D expression in both kidney and placenta, and its alteration in diabetes. The current study, with previous work, points to an effect of diabetic pregnancy on oestradiol and PTHrP concentrations as being key to the altered renal and placental expression of calbindin mRNA and, thus, the derangement in calcium handling by these organs in this condition.

Acknowledgements
The calbindin-D_{28K} probe was generously donated by Dr M Thomasset, Institut National de la Santé et de la Recherche Médicale, Paris, France. We thank Dr David Owen and Dr Ian Lang for performing one of the oestradiol assays, and Dr Val Hillier for clear statistical advice. The authors acknowledge the support of the Sir Jules Thorn Charitable Trust.

References
Glazier JD, Mawer EB & Sibley CP 1995 Calbindin-D_{9K} gene expression in rat chorioallantoic placenta is not regulated by 1,25-dihydroxyvitamin D_{3}. Pediatric Research 37 720–725.
Green R & Hatton TM 1988 Calcium handling by the kidney during pregnancy in the anaesthetised rat. Journal of Physiology 403 17P.
(PTHrP) regulates fetal–placental calcium transport through a receptor distinct from the PTH/PTHrP receptor. *Proceedings of the National Academy of Sciences of the USA* 15233–15238.


Received 2 March 1999

Revised manuscript received 29 July 1999

Accepted 21 August 1999