

Impaired mammary function and parathyroid hormone-related protein during lactation in growth-restricted spontaneously hypertensive rats

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Abstract

Evidence implicates pivotal roles for parathyroid hormone-related protein (PTHrP) during lactation, including stimulation of mammary and pup growth. As spontaneously hypertensive rat (SHR) pups are growth restricted compared with the control Wistar Kyoto (WKY), we examined the relative roles of pup suckling and maternal lactational environment on pup growth, mammary PTHrP, and milk PTHrP and calcium concentrations. SHR pups were lighter compared with the control from 6 days. SHR mammary PTHrP content and milk PTHrP were lower but maternal plasma PTHrP was raised compared with WKY. SHR mammary morphological development was also impaired compared with control. Cross fostering growth-restricted pups onto WKY mothers in-

creased pup weight in association with normal mammary function and higher milk PTHrP and calcium. Control pups suckling on an SHR mother had reduced body weight. Both cross fostering groups were associated with increased maternal and milk PTHrP concentrations, indicating the importance of suckling, together with a functional mammary gland. The results suggested that impaired SHR mammary function and milk PTHrP are associated with a reduced SHR postnatal growth. Our data also indicated that milk and mammary PTHrP are regulated by different mechanisms but that they are influenced by the maternal lactational environment and the suckling pup.

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Introduction

Mothers' milk is the only source of nutrition for the suckling pup and is ideally suited to ensure growth and development during the neonatal period. Parathyroid hormone-related protein (PTHrP) is present in mammary epithelia, the maternal circulation during lactation and in the mothers' milk of various species (Moseley & Gillespie 1995, Philbrick *et al.* 1996). Milk contains PTHrP at many times the concentrations of plasma (Budayr *et al.* 1989, Philbrick *et al.* 1996). Milk-derived PTHrP may be absorbed by the gastrointestinal tract and systemically influences neonatal calcium metabolism and growth (Philbrick *et al.* 1996). The proposed functional roles of PTHrP during lactation include: stimulation of mammary and neonatal cell growth and differentiation, increasing calcium transport from blood to milk, influencing mammary blood flow and myoepithelial cell tone and regulation of maternal and neonatal calcium homeostasis (Philbrick *et al.* 1996). Both PTHrP and its parathyroid hormone (PTH)/PTHrP receptor are essential for fetal and hence postnatal development. PTHrP-deficient mice, generated

by partial PTHrP gene deletion and homologous recombination, die at birth due to severe skeletal dysplasia and have a reduced maternal–fetal calcium gradient (Karaplis *et al.* 1994, Kovacs *et al.* 1996). Furthermore, mice with homologous deletion of the PTH/PTHrP receptor gene are growth restricted and die mid-gestation (Lanske *et al.* 1996). Recently, overexpression of PTHrP has been shown to disrupt branching morphogenesis during mammary gland development (Wysolmerski & Stewart 1998). In the mammary gland, PTHrP is produced in alveolar epithelial cells and the PTH/PTHrP receptor is expressed in adjacent stromal cells (Dunbar *et al.* 1998, Wojcik *et al.* 1999). Indeed targeted overexpression and knockout studies have revealed the critical importance of PTHrP in normal branching morphogenesis and mammary epithelial development. PTHrP mRNA and protein expression in rat mammary tissue is dependent upon the suckling-induced rise in prolactin (Thiede 1989). Milk-derived PTHrP, but not PTH, vitamin D or calcitonin, is thought to act as a maternal endocrine factor to mobilise calcium from bone for lactation (Philbrick *et al.* 1996). PTHrP is present in myoepithelial cells and may play a role in

modulating milk ejection (Philbrick *et al.* 1996, Wojcik *et al.* 1999), thus potentially influencing milk production and delivery to the neonate. PTHrP also may increase calcium concentrations in the milk of lactating goats, implicating a role for PTHrP in stimulating active transport of calcium from blood to milk by mechanisms similar to those in the placenta (Barlet *et al.* 1991).

PTHrP is proposed to be a previously unrecognised factor in the development of perinatal growth restriction. A deficiency in PTHrP may impair fetal and postnatal growth, alter milk composition and reduce calcium transfer to the fetus and into milk. Intrauterine growth restriction, which affects 5% of human fetuses, contributes significantly to perinatal morbidity and mortality. Recent studies suggest that a predisposition to adult diseases including cardiovascular disease, hypertension and diabetes, is associated with low body weight at birth (Barker *et al.* 1993). It has been suggested that cardiovascular alterations occur in fetal life and are progressively amplified from the newborn into adult life (Azar *et al.* 1986, Law *et al.* 1993). Intrauterine growth restriction is thought to result, in part, from nutritional deficiencies, including reduced transport of oxygen and nutrients across the placenta and reduced fetal–placental blood flow, both of which PTHrP is known to regulate (Care *et al.* 1990, Mandsager *et al.* 1994, Kovacs *et al.* 1996, Philbrick *et al.* 1996, Macgill *et al.* 1997, Farrugia *et al.* 2002). Furthermore, PTHrP and calcium in milk (which is usually the only source of nutrition for the suckling neonate) are known to play major roles in promoting postnatal growth (Moseley & Gillespie 1995, Philbrick *et al.* 1996). While many perinatal factors are suggested to be involved in the development of growth restriction, calcium is known to play an important role. Thirty to forty percent of low birth weight infants have neonatal hypocalcaemia (Tsang & Oh 1970) which may be a consequence of impaired placental calcium transport, reduced milk calcium content or altered mammary and neonatal PTHrP and calcium homeostasis. PTHrP is involved in the regulation of calcium in the microenvironment and has specific effects on cellular growth (Moseley & Gillespie 1995, Philbrick *et al.* 1996). However, little is known regarding PTHrP and calcium in association with growth restriction during lactation.

The spontaneously hypertensive rat (SHR) of the Okamoto strain is an inbred strain exhibiting spontaneous, genetically determined hypertension whose aetiology has many similarities to human essential hypertension. The SHR rat fetus is underweight late in gestation when compared with the normotensive Wistar Kyoto (WKY) (Lewis *et al.* 1997, Di Nicolantonio *et al.* 2000, Wlodek *et al.* 2000, 2001). We have reported that fetal plasma, placental and amniotic fluid PTHrP concentrations are reduced in the growth-restricted SHR compared with the WKY (Wlodek *et al.* 2000, 2001). Recently, using embryo transfer techniques, we have demonstrated that the reduced fetal weight in the SHR occurs independently of

maternal genetics, hypertension, altered electrolyte or hormonal factors (Di Nicolantonio *et al.* 2000, Wlodek *et al.* 2001). Furthermore, it has been demonstrated that the SHR mother delivers less milk to her pups and the maternal milk contains reduced ionic calcium concentrations compared with WKY (McCarty *et al.* 1992, McCarty & Fields-Okotcha 1994, McCarty & Tong 1995, McCarty & Lee 1996, Gouldsborough *et al.* 1998). We hypothesise that alterations in milk composition and hence nutrient supply to the neonate after birth are due in part to altered activity of the pup, milk or maternal PTHrP and calcium axes which are associated with reduced pup growth. The primary aim of this study was to investigate the role of PTHrP postnatally following normal pregnancies and those complicated by growth restriction in the SHR, by quantifying pup and maternal plasma and milk PTHrP. Total and ionic calcium concentrations in pup and maternal plasma and milk, as well as mammary morphological development and PTHrP tissue content were also examined. A secondary aim was to determine, by cross fostering WKY and SHR pups, the relative roles of pup suckling and maternal lactational environment on these parameters.

Materials and Methods

Experimental protocol

Our primary aim involved studying WKY and SHR mothers and their pups on 1, 3, 6 and 14 days after birth. A secondary aim involved a cross foster study design with mothers and their pups being studied 6 days after birth and cross fostering. SHR pups were cross fostered (on the day of birth) onto WKY mothers (SHR-on-WKY) while WKY pups were cross fostered onto SHR mothers (WKY-on-SHR) and both groups were compared with normally fostered WKY (WKY-on-WKY) and SHR (SHR-on-SHR) pups.

Virgin female SHR and WKY rats, 8–20 weeks old, were obtained from the Biological Research Facility, Departments of Pharmacology and Physiology, University of Melbourne. They were kept in plastic cages of three to four rats each in a temperature-controlled room at 22–26 °C and with lighting from 0600 to 1800 h. They were mated with a breeder of the same strain after vaginal smears in the afternoon indicated that they were in pro-oestrus and presumably in oestrus that night. The presence of sperm in the vaginal smear the following morning was taken as day 1 of pregnancy. Pregnant rats were housed separately from 20 days of gestation and lactation. Systolic blood pressure was measured by an indirect, tail-cuff method using a programmed electro-sphygmomanometer with a pneumatic pulse transducer (PE-300; Narco Bio-System Inc, Houston, TX, USA) on day 1 of gestation. This study had the ethical approval of the University of Melbourne's Animal Experimentation Ethics Sub-committee.

On the mornings of 1, 3, 6 and 14 days after birth, pups up to 6 days old were killed by decapitation and pups over 1 week old and mothers were anaesthetised by intravenous injection with pentobarbitone sodium (Nembutal; Boehringer Ingelheim, Sydney, Australia; 120 mg/kg body weight). For both strains, each age group had a sample size greater than seven. Larger animal numbers were required at the younger ages to obtain sufficient milk and pup plasma volume for the analyses of PTHrP, ionic calcium or total calcium. Maternal and pup (14 day) blood was obtained by cardiac puncture, while blood from pups aged 6 days and younger was obtained from those decapitated, using heparinised capillary tubes. Pup blood was pooled within a litter for analysis. Pup body weight and crown–rump lengths were measured. Ponderal index was calculated as body weight (in g) \times 100/crown–rump length (in cm³) as another indicator of growth incorporating weight and length. Litters were removed from their mothers 6 h prior to milking to allow milk accumulation and milk (approximately 200 μ l) was then collected following gentle massaging of the third and fifth mammary glands and teats on both left and right sides. Milk and plasma samples and maternal tissues were frozen in liquid nitrogen and stored at -80°C until analysed. The fourth left and right mammary glands were immersion fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin for immunohistochemistry.

Protein extraction and DNA and protein assays

Samples of frozen mammary tissue (1.0 g) were homogenised for 20 s at 24 000 r.p.m. (Polytron P3100 homogeniser, Kinematica, Littau, Switzerland) in 5 ml acetic acid (1 M) using previously established techniques (Wlodek *et al.* 2000). Duplicate 500 μ l aliquots of the homogenate were removed for DNA assay. The remaining homogenate was incubated for 2–3 h at 4°C and centrifuged at 20 000 g for 15 min at 4°C . The supernatant was then dialysed against 5 litres deionised water for 22–26 h at 4°C using Spectra-Por 3 dialysis tubing (molecular weight 6000–8000; Cole Palmer, Niles, IL, USA) as previously described (Wlodek *et al.* 2000). The extract was aliquoted and stored at -20°C for PTHrP radioimmunoassay. Tissue DNA concentrations were determined using a modified diphenylamine method as previously described (Wlodek *et al.* 2000).

Mammary morphological development

The paraffin-embedded blocks were sectioned at 5 μ m and samples for comparative analysis taken from three levels representing the entire tissue sample. Samples from at least six animals in each group and at each age were examined. Morphological features were observed in sections stained by standard haematoxylin and eosin methods.

PTHrP and calcium measurements

Plasma, milk and mammary tissue concentrations of PTHrP were quantified by a sensitive N-terminal radioimmunoassay that does not recognize PTH (Grill *et al.* 1991, Wlodek *et al.* 2000). Blood samples were collected into the protease inhibitor aprotinin (0.4 trypsin inhibitor unit (TIU)/ml; Sigma, St Louis, MO, USA). The PTHrP radioimmunoassay uses a polyclonal goat antiserum against synthetic PTHrP(1–40) and recombinant PTHrP(1–84) as standard. The detection limit is 2 pmol/l and intra- and interassay coefficients of variation are 4.8% and 13.6%, respectively. Total and ionic calcium concentrations were determined using a Beckman Synchron CX-5 Clinical System (colorimetric spectrometry; Beckman Coulter Inc, Fullerton, CA, USA) and using ion selective electrodes correcting for pH (Radiometer ABL615 Blood Gas/Electrolyte Analyser, Copenhagen, Denmark) respectively from milk and pup and maternal plasma. Due to the limited milk and pup plasma volume, PTHrP and calcium measurements were performed on milk and plasma obtained from different mothers.

Statistical analysis

Homogeneity of variance was analysed using Bartlett's test. Postnatal data were analysed by two-way analysis of variance (SPSS-X; SPSS Inc. Encinitas, CA, USA) with age and strain as factors. Differences across the ages were determined by post-hoc Student–Newman–Keuls test following a one-way analysis of variance, and differences between strains at each age were determined with a *t*-test. Cross foster data were analysed by one-way analysis of variance and differences among the cross foster groups were determined by post-hoc Student–Newman–Keuls test. A Pearson's correlation was used to determine the association between certain variables. Data are presented as means \pm S.E.M. and $P < 0.05$ was taken as statistically significant.

Results

Blood pressures and weights

Paternal (data not shown) and maternal systolic blood pressure at day 1 of pregnancy were significantly higher in SHR than WKY for all age groups ($P < 0.0005$; Table 1). Litter size on day 1 postpartum was significantly lower in the SHR ($P < 0.05$; Table 1). However, for all other ages there were no significant differences between the SHR and WKY, nor were there differences across ages (Table 1). There were no significant differences in uterine weight (as a percentage of maternal body weight) between WKY and SHR (Table 1). As lactation progressed, uterine weight progressively decreased ($P < 0.05$; Table 1).

Table 1 Blood pressure, uterine weight, pup parameters and litter size. Maternal systolic blood pressure at mating, maternal uterine weight, pup crown–rump length, pup ponderal index and litter size for WKY and SHR at 1, 3, 6 and 14 days postpartum (means \pm S.E.M.). Sample size was 6–12 and 11–19 mothers per group for maternal and pup parameters respectively

	WKY				SHR			
	1	3	6	14	1	3	6	14
Age (days)								
Maternal blood pressure (mmHg)	117 \pm 3	113 \pm 5	118 \pm 2	126 \pm 7	170 \pm 5*	173 \pm 9*	157 \pm 7*	161 \pm 11*
Dimensions								
Maternal uterine (% body weight)	0.71 \pm 0.04 ^d	0.28 \pm 0.03 ^c	0.19 \pm 0.02 ^b	0.09 \pm 0.01 ^a	0.56 \pm 0.07 ^C	0.28 \pm 0.03 ^B	0.11 \pm 0.01 ^A	0.11 \pm 0.01 ^A
Pup crown–rump (cm)	3.7 \pm 0.05 ^a	4.1 \pm 0.04 ^b	4.6 \pm 0.07 ^c	7.1 \pm 0.14 ^d	3.7 \pm 0.01 ^A	4.2 \pm 0.08 ^B	4.5 \pm 0.06 ^C	5.9 \pm 0.16 ^{D*}
Pup ponderal index (g \times 100/cm ³)	9.2 \pm 0.46 ^b	8.5 \pm 0.2 ^b	9.0 \pm 0.3 ^b	7.0 \pm 0.3 ^a	10.0 \pm 0.6 ^A	8.8 \pm 0.4 ^A	9.1 \pm 0.3 ^A	9.3 \pm 0.5 ^{A*}
Litter size	11.4 \pm 0.4	10.7 \pm 0.6	9.8 \pm 0.6	9.6 \pm 0.5	7.6 \pm 0.8*	9.0 \pm 0.7	9.6 \pm 0.9	9.0 \pm 0.7

Significant differences between strains at any age are indicated by * ($P < 0.05$). Significant differences across the ages for a given strain are indicated by letters, with different letters indicating significant differences such that data with an 'a' are different from data with a 'b', 'c' or 'd' (significant differences for WKY in lower case and those for SHR in upper case letters; $P < 0.05$).

Table 2 Maternal and pup plasma and milk calcium concentrations. Maternal plasma total and ionised calcium ($n = 11$ –18), pup plasma total and ionised calcium ($n = 5$ –13) and milk total calcium ($n = 4$ –8) concentrations at 1, 3, 6 and 14 days postpartum (means \pm S.E.M.)

	WKY				SHR			
	1	3	6	14	1	3	6	14
Age (days)								
Maternal plasma								
Total calcium (mmol/l)	2.58 \pm 0.06 ^b	2.68 \pm 0.10 ^b	2.49 \pm 0.06 ^b	2.25 \pm 0.06 ^a	2.93 \pm 0.17 ^{B*}	2.72 \pm 0.04 ^B	2.63 \pm 0.06 ^B	2.21 \pm 0.12 ^A
Ionic calcium (mmol/l)	0.95 \pm 0.04	0.89 \pm 0.04	0.86 \pm 0.05	0.83 \pm 0.08	0.94 \pm 0.07	0.94 \pm 0.05	0.85 \pm 0.06	1.00 \pm 0.04
Milk								
Total calcium (mmol/l)	45 \pm 8.0 ^a	74 \pm 10.8 ^{ab}	85 \pm 10 ^b	77 \pm 2.9 ^{ab}	45 \pm 5.5 ^A	54 \pm 9.0 ^{AB}	64 \pm 6.6 ^{AB*}	73 \pm 2.9 ^B
Pup plasma								
Total calcium (mmol/l)	0.82 \pm 0.14 ^a	1.27 \pm 0.19 ^a	0.76 \pm 0.10 ^{ab}	1.69 \pm 0.21 ^b	1.14 \pm 0.27 ^A	0.95 \pm 0.33 ^A	0.76 \pm 0.12 ^A	1.68 \pm 0.16 ^B
Ionic calcium (mmol/l)	0.45 \pm 0.04 ^a	0.53 \pm 0.05 ^a	0.53 \pm 0.08 ^a	0.78 \pm 0.06 ^b	0.35 \pm 0.05 ^A	0.42 \pm 0.04 ^A	0.39 \pm 0.04 ^A	0.62 \pm 0.06 ^B

Significant differences between strains at any age are indicated by * ($P < 0.05$). Significant differences across the ages for a given strain are indicated by letters, with different letters indicating significant differences such that data with an 'a' are different from data with a 'b' but the same as data with an 'ab' (significant differences for WKY in lower case and those for SHR in upper case letters; $P < 0.05$).

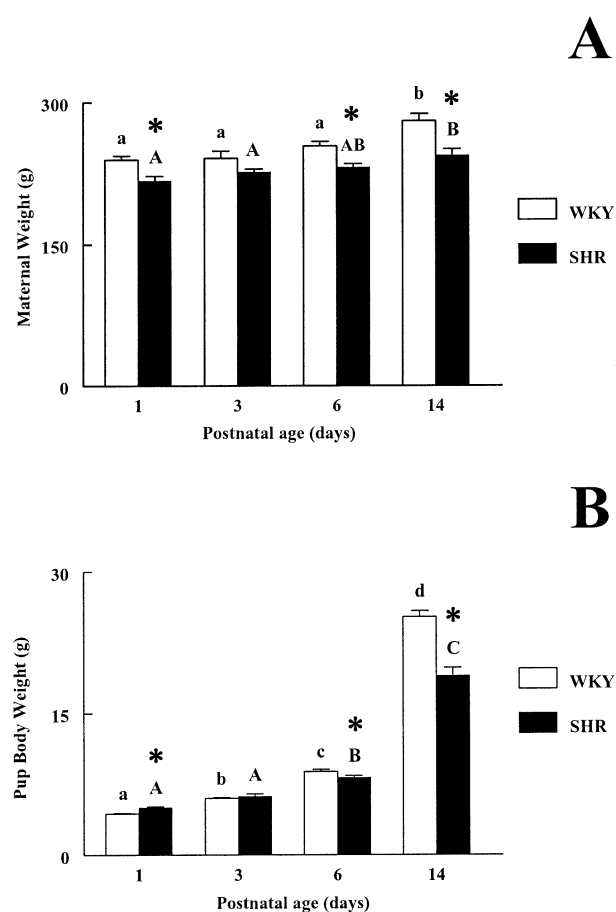


Figure 1 Maternal (A) and pup (B) body weight in the first 14 postnatal days in the WKY (open bars) and SHR (closed bars) (means \pm S.E.M.; $n=11-19$). The maternal body weight of WKY was significantly greater than SHR ($P<0.01$) at all ages except day 3. Pup body weight increased significantly and progressively over the early postnatal period for both strains ($P<0.01$). Like maternal body weight, SHR pups were significantly growth restricted compared with WKY pups from day 6 ($P<0.05$). Significant differences between strains at any age are indicated by * ($P<0.05$). Significant differences across the ages for both strains are indicated by letters, with different letters indicating significant differences such that data with an 'a (A)' are different from data with a 'b (B)', 'c (C)' or 'd', but the same as data with an 'AB' (significant differences for WKY in lower case and those for SHR in upper case letters; $P<0.05$).

Maternal body weight was significantly greater in WKY than SHR at all ages ($P<0.01$; Fig. 1) except day 3 where there was no difference.

Pup weight ($P<0.01$; Fig. 1) and crown-rump length ($P<0.05$; Table 1) increased over the postpartum period for both SHR and WKY pups such that WKY weight increased significantly and incrementally from day 1 to 14 and SHR weight increased from day 3 to 14. SHR pups weighed significantly more on day 1 and significantly less on days 6 and 14 than WKY ($P<0.05$; Fig. 1) and were

significantly shorter than WKY on day 14 ($P<0.001$; Table 1). Ponderal index of WKY was lower on day 14 compared with the other days ($P<0.05$) and was significantly different between WKY and SHR on day 14 ($P<0.02$; Table 1).

PTHrP and calcium concentrations

Postpartum maternal plasma PTHrP concentrations were not altered by age in the WKY; however, in the SHR, maternal plasma PTHrP concentrations varied between ages and were significantly greater on day 14 than on day 3 postpartum ($P<0.05$; Fig. 2). Furthermore, maternal plasma PTHrP concentrations in the SHR were greater than WKY on 1, 6 and 14 days ($P<0.02$; Fig. 2). In the WKY and SHR, maternal plasma total calcium concentrations were lower on day 14 compared with the other postpartum days ($P<0.05$; Table 2). Although pup plasma PTHrP concentrations were not different between the strains on days 1, 3 and 14 they were significantly lower in SHR on day 6 ($P<0.05$; Fig. 2). WKY pup plasma PTHrP concentrations were the same on days 1, 3 and 6 and then decreased on day 14 significantly ($P<0.05$; Fig. 2). In contrast, in the SHR, values decreased significantly from day 1 to 3 with further decreases between days 6 and 14 ($P<0.05$; Fig. 2).

Pup plasma ionic calcium concentrations were not significantly different between SHR and WKY at any age (Table 2). For both strains they were the same on days 1, 3 and 6 and then increased on day 14 ($P<0.05$; Table 2). Total calcium concentrations in pup plasma also increased on day 14 compared with days 1, 3 and 6 in both WKY and SHR. A significant negative correlation was observed between pup weight and pup plasma PTHrP concentrations ($r^2=0.51$; $P<0.0001$). In the first 6 days postpartum, pup plasma PTHrP concentrations were 2.5- to 5-fold higher than maternal plasma values.

Mammary PTHrP content and milk PTHrP and calcium concentrations

SHR mammary tissue PTHrP content was 35-70% lower than WKY in the first 2 weeks of postnatal life ($P<0.01$; Fig. 3). Both mammary tissue PTHrP content and milk PTHrP concentrations increased between days 6 and 14 in the WKY and SHR ($P<0.05$; Fig. 3). Milk PTHrP concentrations were $\sim 80\%$ lower in the SHR at days 3 and 6 respectively, compared with WKY ($P<0.0001$; Fig. 3). Furthermore, there was a significant correlation between milk PTHrP concentrations and mammary PTHrP content ($r^2=0.75$; $P<0.0001$). A correlation also existed between pup weight and milk PTHrP concentrations ($r^2=0.53$; $P<0.0001$) and between pup weight and mammary PTHrP content ($r^2=0.65$; $P<0.0001$). In both strains, milk ionic calcium concentrations were significantly increased at days 6 and 14 compared with day 1

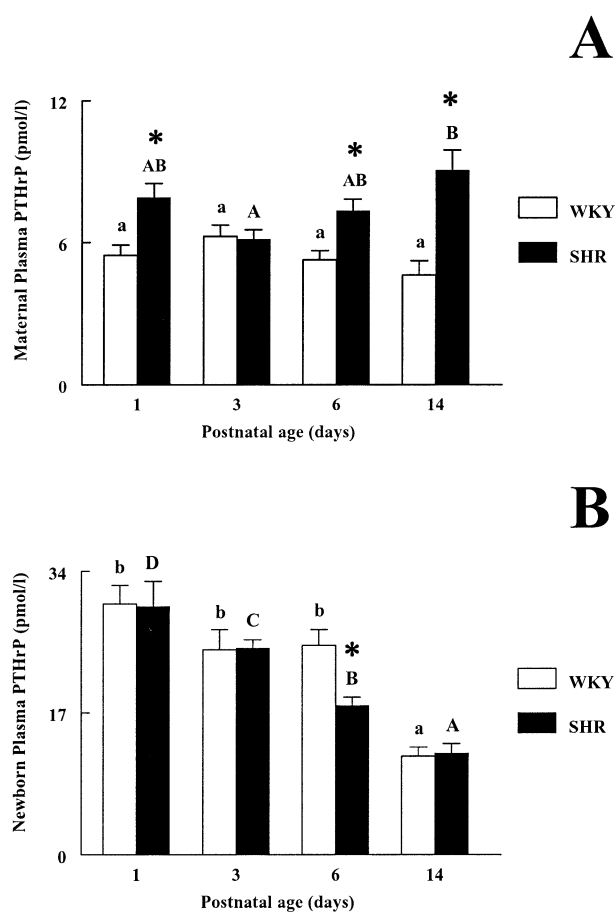


Figure 2 Maternal (A; $n=10-16$) and pup (B; $n=6-15$) plasma PTHrP concentrations in the first 14 postnatal days in the WKY (open bars) and SHR (closed bars) (means \pm S.E.M.). Changes in maternal plasma PTHrP concentrations were not observed in the WKY with age; however, in SHR, PTHrP concentrations increased significantly from day 3 to day 14 ($P<0.01$). Furthermore, SHR PTHrP concentrations were significantly greater on days 1, 6 and 14 compared with WKY. Pup plasma PTHrP concentrations were significantly lower on day 6 in the SHR compared with WKY ($P<0.01$). In the SHR, pup plasma PTHrP concentrations decreased progressively from day 1 whereas the decline was delayed until after day 6 in the WKY ($P<0.05$). Significant differences between strains at any age are indicated by * ($P<0.05$). Significant differences across the ages for a given strain are indicated by letters, with different letters indicating significant differences such that data with an 'a' (A) are different from data with a 'b' (B) or 'C' but the same as data with an 'AB' (significant differences for WKY in lower case and those for SHR in upper case letters; $P<0.05$).

postpartum ($P<0.05$; Fig. 3). At day 3, milk calcium ionic concentrations were reduced in the SHR compared with the WKY ($P<0.05$). WKY milk total calcium concentrations increased between days 1 and 6 ($P<0.05$; Table 2), whereas the increase in SHR was delayed until day 14 ($P<0.05$; Table 2). Milk total calcium concentrations were significantly lower in SHR compared with

WKY on day 6 ($P<0.05$; Table 2). No significant correlations existed between mammary PTHrP and milk calcium (ionic and total) concentrations. Furthermore, a correlation between milk PTHrP and milk calcium concentrations was not observed.

The development of lactating mammary glands

Figure 4 illustrates a comparison of WKY and SHR morphology during lactation. At 3 days postpartum in the WKY, the alveoli were small but still exhibited evidence of epithelial proliferation. Functional alveoli did not occupy the entire gland at this stage. In contrast, the SHR mammary tissue appeared to be more differentiated with little evidence of epithelial proliferation. We also observed that the SHR alveoli occupied less of the mammary gland margins than the WKY. At 6 days, the WKY mammary glands were fully occupied with wide open, active, milk-containing alveoli and very little stromal tissue present. The SHR rat mammary gland looked similar to the WKY at this time although there was less mammary tissue. At 2 weeks, there was little change in the appearance of the SHR lactating tissue but, in the WKY, alveolar shrinkage was evident. By 4 weeks when weaning was achieved, involution and cell death, as indicated by the presence of condensing nuclei, was apparent in both strains, although it was far more advanced in the SHR.

Six day cross foster study

There were no significant differences in pup crown-rump length, ponderal index or litter size across the cross foster groups (Table 3). WKY mothers (WKY-on-WKY and SHR-on-WKY) delivered their pups on day 22 of gestation which was 1 day less than SHR mothers (SHR-on-SHR and WKY-on-SHR; day 23 of gestation) ($P<0.05$; Table 3). On postnatal day 6, WKY pup body weight decreased when suckling on a SHR mother compared with WKY-on-WKY to weights comparable with SHR-on-SHR ($P<0.05$; Fig. 5). When an SHR suckled on a WKY, pup body weight increased above all other groups ($P<0.05$; Fig. 5). Milk total calcium, but not ionic calcium, concentrations were significantly greater in WKY mothers (WKY-on-WKY and SHR-on-WKY) compared with SHR mothers (WKY-on-SHR and SHR-on-SHR), irrespective of the pup it was suckling ($P<0.05$; Fig. 5 and Table 3). A significant positive correlation was found between pup weight and milk total calcium concentrations in the cross foster groups ($r^2=0.18$; $P<0.05$).

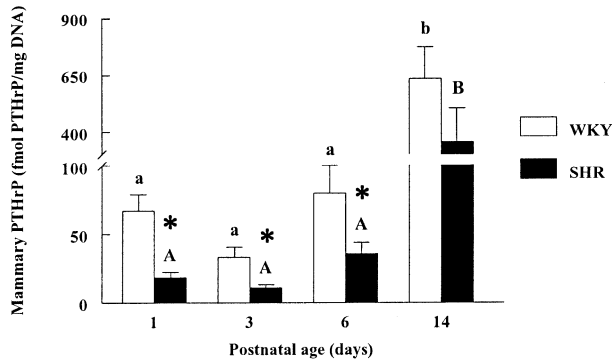
Pup plasma PTHrP concentrations in the cross foster groups (WKY-on-SHR and SHR-on-WKY) were intermediate and not different from the cross foster controls which were significantly different (WKY-on-WKY higher than SHR-on-SHR) ($P<0.05$; Table 3). There was some variation in maternal plasma total calcium

concentrations between the cross foster groups ($P < 0.05$; Table 3) but WKY-on-WKY was not different from SHR-on-SHR. There were no differences between the cross foster groups for maternal plasma ionic calcium concentrations and pup plasma total and ionic calcium concentrations (Table 3).

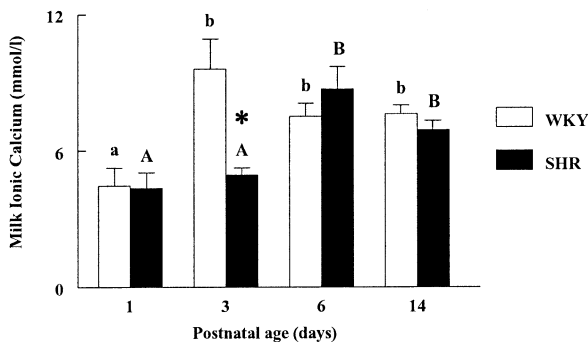
On postnatal day 6, mammary PTHrP content and milk PTHrP concentrations in the SHR-on-SHR group were lower than the WKY-on-WKY ($P < 0.05$; Fig. 6). Milk

PTHrP concentrations in the cross foster groups (WKY-on-SHR and SHR-on-WKY) were higher than cross foster controls (WKY-on-WKY and SHR-on-SHR) ($P < 0.05$; Fig. 6). Mammary PTHrP content was the same for SHR-on-SHR, WKY-on-SHR and SHR-on-WKY groups and all were lower compared with WKY-on-WKY ($P < 0.05$; Fig. 6). Maternal plasma PTHrP concentrations in the SHR-on-SHR and the cross foster groups were greater than the WKY-on-WKY group with WKY-on-SHR values being highest ($P < 0.05$; Fig. 6). Milk PTHrP concentrations were correlated to pup total calcium concentrations ($r^2 = 0.58$; $P < 0.05$).

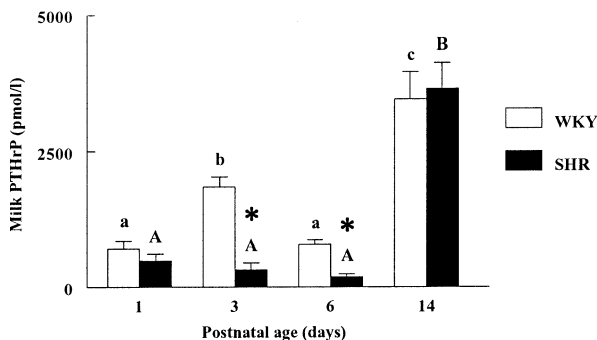
A



B



C



Discussion

The present study shows that SHR mothers have impaired development of the lactating mammary gland associated with reduced mammary PTHrP content. Furthermore, the growth-restricted SHR pups are exposed to milk with reduced PTHrP and calcium concentrations early in life. SHR pups had fallen behind WKY weight by 6 days and remained growth restricted during the lactational period. SHR maternal weight was lower than that of WKY, although there were no significant differences in litter sizes or uterine weights in the WKY and SHR mothers. SHR fetuses are growth restricted *in utero* (Wlodek *et al.* 2000) and our finding that SHR pups are not growth restricted early in neonatal life may be due to the longer gestation in the SHR (WKY, 22 days; SHR, 23 days). Previous work has reported that SHR mothers deliver less milk despite longer suckling times (Gouldsbrough *et al.* 1998). This may be due to impaired development of the SHR mammary gland rendering the SHR mother unable to deliver sufficient milk of an appropriate composition to ensure normal pup growth. The present cross foster study has demonstrated that when an SHR pup suckles on a

Figure 3 Mammary PTHrP content (A; $n = 6-10$), milk ionised calcium concentrations (B; $n = 5-16$) and milk PTHrP concentrations (C; $n = 5-13$) in the first 14 postnatal days in the WKY (open bars) and SHR (closed bars) (means \pm S.E.M.). Mammary PTHrP tissue content was suppressed in the SHR compared with the WKY on days 1, 3 and 6 ($P < 0.01$). It increased at day 14 for both WKY and SHR ($P < 0.05$). In the SHR, milk ionic calcium concentrations were significantly lower than the WKY on day 3 ($P < 0.05$). Milk ionic calcium concentrations were significantly greater on day 14 compared with day 1 for both strains ($P < 0.05$). Milk PTHrP concentrations were significantly lower in SHR on days 3 and 6 postpartum compared with WKY ($P < 0.0001$). In the WKY and SHR, milk PTHrP concentrations increased significantly from day 6 to 14 postpartum ($P < 0.05$). Significant differences between strains at any age are indicated by * ($P < 0.05$). Significant differences across the ages for a given strain are indicated by letters, with different letters indicating significant differences such that data with an 'a (A)' are different from data with a 'b (B)' or 'c' (significant differences for WKY in lower case and those for SHR in upper case letters; $P < 0.05$).

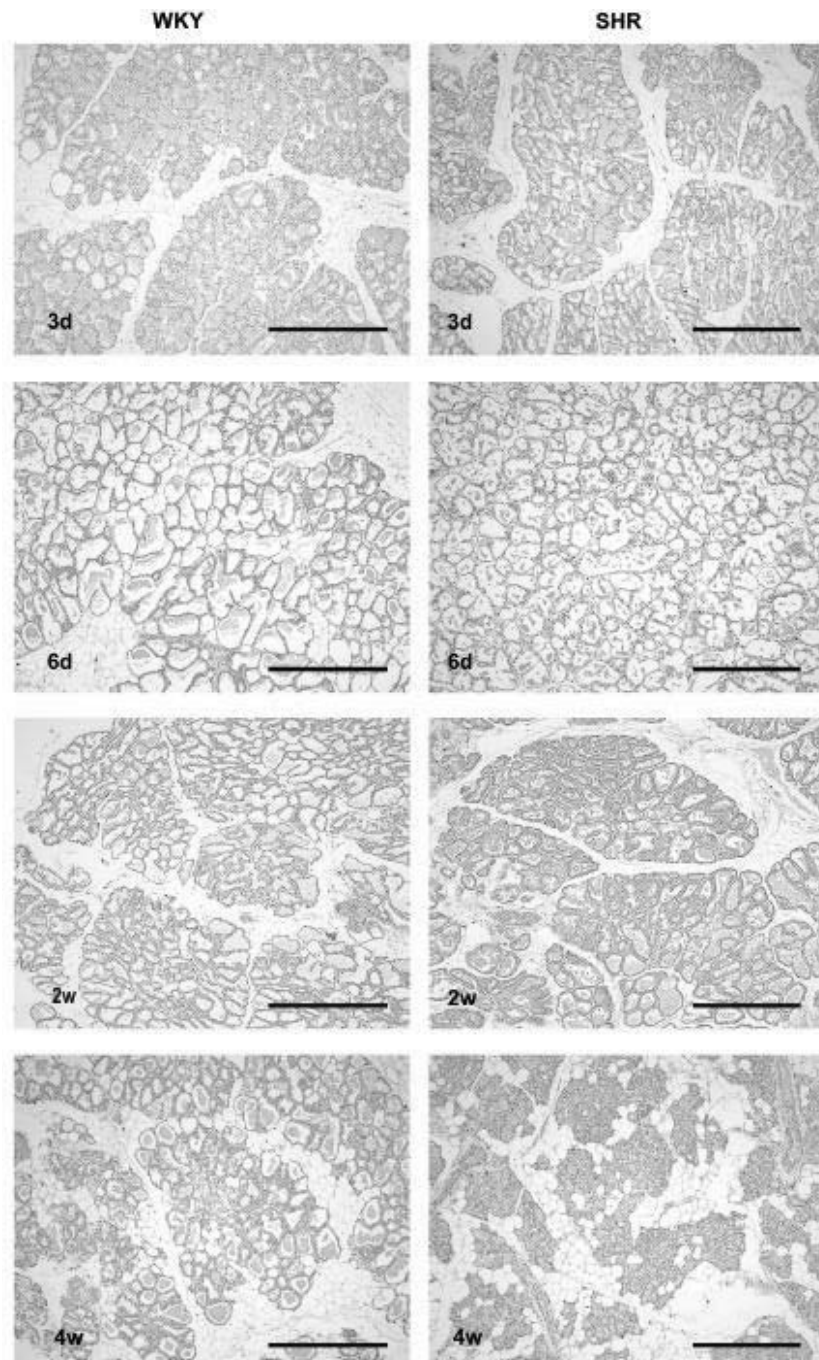


Figure 4 Haematoxylin and eosin staining of SHR and WKY mammary tissue at 3 days (d), 6 days, 2 weeks (w) and 4 weeks postpartum. Between 3 and 6 days in the WKY there was continued proliferation of alveoli, which also expanded to contain more milk over that time. In contrast, the alveoli in the SHR remained small and occupied less of the entire area of the mammary gland. By 2 weeks, there was some shrinkage of alveoli in the WKY while there was little change in the SHR. By 4 weeks, involution was underway in both strains, but was more advanced in the SHR. Bar=0.5 mm.

Table 3 Pup parameters, litter size and maternal and pup plasma PTHrP and calcium concentrations. Pup parameters, litter size and maternal and pup plasma PTHrP and calcium concentrations at 6 days postpartum in the cross foster groups (means \pm S.E.M.; $n=4-11$)

	Cross foster group			
	WKY-on-WKY	WKY-on-SHR	SHR-on-WKY	SHR-on-SHR
Dimensions				
Pup crown-rump (cm)	4.6 \pm 0.07	4.6 \pm 0.05	4.7 \pm 0.14	4.6 \pm 0.05
Pup ponderal index (g \times 100/cm ³)	6.9 \pm 1.2	8.2 \pm 0.36	10.3 \pm 1.4	7.9 \pm 0.76
Litter size	9.9 \pm 0.7	10.3 \pm 1.1	9.0 \pm 0.7	9.8 \pm 1.0
Gestational age at delivery	22.1 \pm 0.1 ^a	23.0 \pm 0.0 ^b	22.0 \pm 0.0 ^a	23.2 \pm 0.2 ^b
Maternal plasma				
Total calcium (mmol/l)	2.47 \pm 0.07 ^{ab}	2.43 \pm 0.14 ^{ab}	2.32 \pm 0.06 ^a	2.66 \pm 0.06 ^b
Ionic calcium (mmol/l)	0.85 \pm 0.05	0.66 \pm 0.09	0.62 \pm 0.06	0.82 \pm 0.08
Milk				
Ionic calcium (mmol/l)	7.66 \pm 0.64	7.53 \pm 1.02	7.13 \pm 0.50	8.87 \pm 1.12
Pup plasma				
PTHrP (pmol/l)	23.6 \pm 0.86 ^b	21.4 \pm 1.86 ^{ab}	19.2 \pm 1.30 ^{ab}	18.3 \pm 1.23 ^a
Total calcium (mmol/l)	0.78 \pm 0.11	1.18 \pm 0.25	1.12 \pm 0.16	0.73 \pm 0.14
Ionic calcium (mmol/l)	0.50 \pm 0.08	0.34 \pm 0.08	0.48 \pm 0.07	0.38 \pm 0.03

Significant differences across the groups are indicated by letters, with different letters indicating significant differences such that data with an 'a' are different from data with a 'b' but the same as data with an 'ab' ($P<0.05$).

WKY mother its weight increases above the SHR-on-SHR and WKY-on-WKY as a result of the normal WKY mammary function. This demonstrates that catch-up growth is possible. In contrast, when a WKY pup suckles on an SHR mother with impaired mammary function its growth is reduced to the level of the SHR-on-SHR. These results are supported by previous studies demonstrating that when SHR pups suckle on WKY mothers the pups have reduced blood pressure and increased weight compared with SHR-on-SHR (McCarty & Fields-Okotcha 1994, Gouldsbrough *et al.* 1998). The first 2 weeks of lactation have been identified as a critical period for influencing pup growth and blood pressure (McCarty & Fields-Okotcha 1994, Gouldsbrough *et al.* 1998) and are consistent with our identification of reduced SHR pup growth and mammary and milk PTHrP during this time. Mammary PTHrP content and milk PTHrP concentrations were lower in the SHR relative to the WKY in the first 14 postnatal days when morphological examination showed open milk-filled alveoli although the amount of lactating tissue in the SHR was less. The SHR mammary gland showed more extensive involution late in lactation, a further indication of compromised lactational function. Our results, in addition to previous studies, highlight and confirm the important role of mammary function and milk for normal pup growth and development and that impaired mammary function is a major contributor to SHR postnatal growth restriction. However, in this model, growth restriction may also be the consequence of maternal genetic factors influencing lactation.

Both WKY and SHR pup plasma PTHrP concentrations are at least 2.5-fold higher than maternal plasma

values, suggesting a neonatal source of PTHrP which is consistent with suggestions of fetal production of PTHrP (Thiebaud *et al.* 1993, Papantoniou *et al.* 1996, Wlodek *et al.* 2000). Alternatively, it is plausible that the high levels of milk PTHrP reach the neonatal circulation, since pup plasma PTHrP concentrations, although raised during lactation, decline to maternal values by 4 weeks of age (data not shown) coinciding with the time of weaning. The higher concentrations of PTHrP in pups may be required for growth and development of bone and gut as shown in PTHrP knockout mice (Karaplis *et al.* 1994). The decline in SHR pup plasma PTHrP concentrations occurs earlier than in the WKY pup and may be due to impaired PTHrP production by the growth-restricted neonate and/or reduced milk supply of PTHrP. Pup plasma PTHrP concentrations in the cross fostering groups are intermediate between WKY-on-WKY and SHR-on-SHR, strongly supporting the suggestion that both endogenous pup PTHrP production and milk PTHrP from its mother contribute to circulating pup PTHrP concentrations.

It has been suggested that PTHrP from mammary tissue and the high levels in milk 'spill' into the maternal circulation but no mechanisms have been established (De Papp & Stewart 1993). However, mammary PTHrP content in the SHR, in comparison with the WKY, was lower at a time when maternal plasma PTHrP concentrations were higher. Since the SHR mammary gland contains less PTHrP than one would expect less 'spill' into maternal plasma if this is indeed the mechanism. Alternatively, in the SHR, less mammary PTHrP may be secreted into milk with more 'spilling' into the maternal circulation accounting for the higher SHR maternal

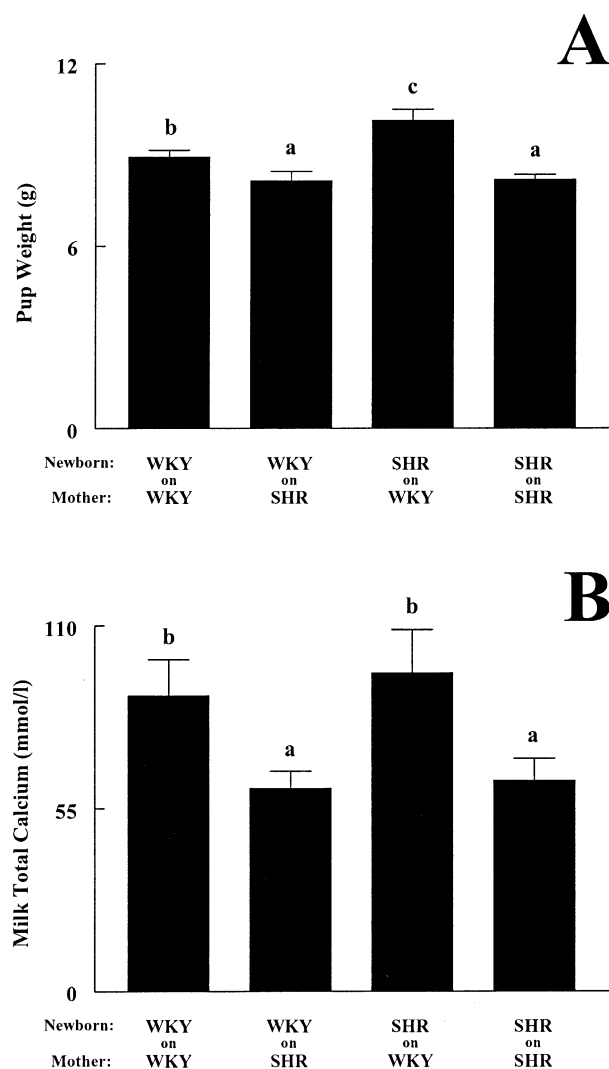


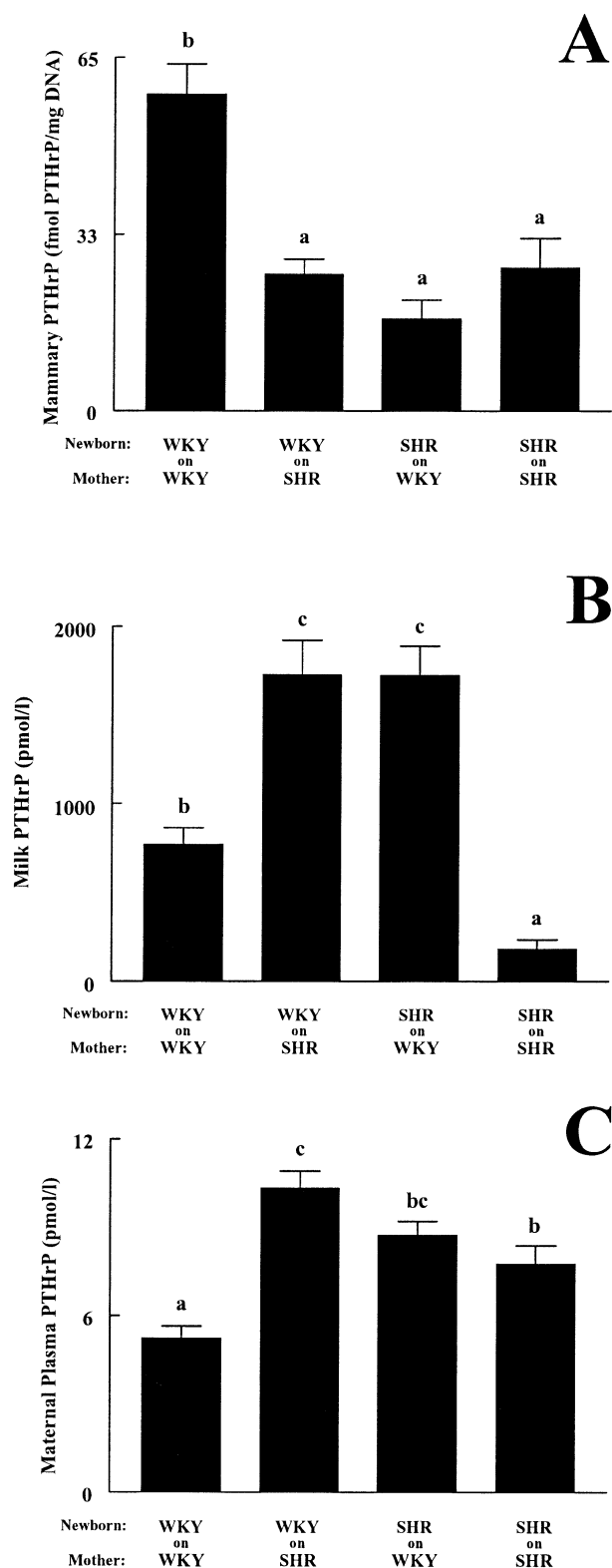
Figure 5 Pup body weight (A; $n=4-11$) and milk total calcium concentrations (B; $n=4-11$) on postnatal day 6 in the cross foster groups (means \pm S.E.M.). WKY pup body weight decreased when suckling on an SHR compared with WKY-on-WKY ($P<0.05$). SHR pup body weight increased when suckling on a WKY compared with all other groups ($P<0.05$). Milk total calcium concentrations were significantly greater in WKY mothers compared with SHR mothers irrespective of pup ($P<0.05$). Significant differences across groups are indicated by letters, with different letters indicating significant differences such that data with an 'a' are different from data with a 'b' or 'c' ($P<0.05$).

plasma PTHrP. Our cross foster studies imply that suckling is the primary determinant of maternal plasma PTHrP concentrations but that they can be influenced by the maternal environment even when mammary function and PTHrP content are impaired.

The mechanisms by which PTHrP is transported into milk in such high amounts are not known. In general, during the first 2 postnatal weeks, milk PTHrP levels

reflected the mammary content of PTHrP; however, this correlation was not present in our cross foster study. That mammary PTHrP content is reduced and milk PTHrP concentrations are increased in the two cross foster groups suggest that the mechanisms regulating mammary PTHrP production and milk PTHrP transport are different. Mammary PTHrP is thought to be stimulated by a suckling-induced rise in prolactin (Thiede 1989). The adult SHR has higher plasma prolactin concentrations (Sowers 1981) but it is not known whether this occurs during lactation. The low mammary PTHrP content in SHR mothers (WKY-on-SHR and SHR-on-SHR) suggest that, regardless of the type of suckling, the impaired SHR mammary gland produces less PTHrP. When an SHR pup suckles on a WKY mother the pup suckling results in reduced mammary PTHrP compared with the WKY-on-WKY, suggesting that both normal mammary function and suckling contribute to normal mammary PTHrP content. In contrast, milk PTHrP concentrations reflected maternal circulating PTHrP in both the WKY-on-SHR and SHR-on-WKY groups, indicating that suckling could lead to an increase in PTHrP in milk and maternal circulation irrespective of the extent of lactational tissue. When both suckling and mammary function are altered, as in the SHR-on-SHR, milk PTHrP concentrations are compromised. It is possible that there is an increased mammary PTHrP synthesis induced by WKY-on-SHR which cannot be stored and thus 'spills' in the maternal circulation and is transported into milk by unknown mechanisms. The role of the pituitary PTHrP in normal physiology has not been fully explored but recent reports suggest that both pituitary prolactin and mammary prolactin contribute to the final milk prolactin concentrations (Ben-Jonathan *et al.* 1996), providing a precedent to suggest that PTHrP could reach milk by similar mechanisms.

Maternal milk is the only source of neonatal calcium and impaired lactation could impact significantly on neonatal calcium homeostasis and bone growth. There is controversy in the literature about the proposed roles for mammary PTHrP in the delivery of calcium to the neonate either by stimulating calcium transport into milk by mechanisms similar to those in the placenta (Barlet *et al.* 1991, Law *et al.* 1991, Yamamoto *et al.* 1992, Uemura *et al.* 1997) and/or by mobilising calcium from the skeleton of the mother (Grill *et al.* 1992). Milk ionic and total calcium concentrations were lower in the SHR compared with the WKY but the difference was only significant at days 3 and 6 respectively, when milk and mammary PTHrP were also significantly lower. Milk calcium concentrations were not correlated with mammary or milk PTHrP in the postnatal or cross fostering studies, suggesting that PTHrP is not the primary regulator of milk calcium transport. Reduced milk total calcium concentrations in the cross foster groups clearly demonstrated that impaired SHR mammary function is associated with reduced milk calcium and the development of postnatal



growth restriction. It is likely that the neonate would be able to compensate for reduced milk calcium intake and hypocalcaemia may not develop until neonatal calcium stores are significantly compromised or if delivery of calcium to the neonate is severely impaired. It is possible that the transient low milk calcium concentrations were detected by the calcium-sensing receptor to mobilise maternal or mammary calcium so it is available for transfer into milk. Others have reported reduced total calcium concentrations in the milk of the SHR compared with the WKY and also that reduced dietary levels of calcium in the SHR are implicated in effects on cardiovascular homeostasis (McCarty & Tong 1995, McCarty & Lee 1996). We cannot eliminate the possibility that WKY and SHR mothers, and hence milk composition, may have responded differently to the time of maternal separation from pups prior to milk collection. Defects in calcium homeostasis and cellular calcium levels are associated with the development of growth restriction as well as hypertension (Pitkin 1975).

Milk PTHrP may have roles in the neonate to promote growth, gut maturation and transport of calcium across the gut wall (Kovacs & Kronenberg 1997). These postulated roles of milk PTHrP in the neonatal pup require substantiation as milk PTHrP concentrations did not correlate with pup weight or pup plasma calcium. The results of this study indicate that the SHR mammary gland does not reach its full potential to produce and/or deliver milk PTHrP which, in turn, may affect the growth of the neonate. Studies have implicated a role for PTHrP in the development and maintenance of hypertension in the SHR (Garcia *et al.* 1998, Wysolmerski & Stewart 1998). Indeed, the present and previous cross fostering studies have demonstrated that only during the first 2 weeks of postnatal life can the blood pressure of SHR pups be reduced and weight increased if suckling from WKY foster mothers (McCarty & Tong 1995). This strongly supports the presence of essential regulatory factors in milk, one of which may be PTHrP (McCarty *et al.* 1992, McCarty & Fields-Okotcha 1994). Given that the post-natal environment is believed to be important to the development of hypertension later in life (Barker *et al.* 1993), we suggest that mammary and milk PTHrP may be

Figure 6 Mammary PTHrP content (A; $n=4-6$), milk PTHrP concentrations (B; $n=4-11$) and maternal plasma PTHrP concentrations (C; $n=4-11$) on postnatal day 6 in the cross foster groups (means \pm S.E.M.). Mammary PTHrP tissue content was highest in the WKY-on-WKY group compared with all other groups ($P<0.05$). Milk PTHrP concentrations in the SHR-on-SHR group were lower than all other groups ($P<0.05$). Maternal plasma PTHrP concentrations in the WKY-on-WKY group was lower than all other groups ($P<0.05$). Significant differences across the groups are indicated by letters, with different letters indicating significant differences such that data with a 'b' are different from data with an 'a' or 'c' but the same as data with a 'bc' ($P<0.05$).

significant modulators of postnatal growth and development and may be a beneficial therapeutic agent in growth restriction. These studies have identified that mammary and milk PTHrP are regulated by independent mechanisms but are dependent on the maternal lactational environment and the suckling pup.

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