The effect of maternal prolactin infusion during pregnancy on fetal adipose tissue development

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Abstract

The present study determines whether maternal administration of prolactin (PRL) to dams promotes the abundance of the brown adipose tissue-specific uncoupling protein-1 (UCP1) in fetal and neonatal rat pups. Recombinant PRL (24 µg/kg per day), or an equivalent volume of saline, were infused into dams (n=19 per group) throughout pregnancy from 12 h after mating. Interscapular brown adipose tissue was sampled either from fetuses at 19·5 days of gestation (term=21·5 days) or from neonatal rat pups at approximately 18 h after birth. The abundance of UCP1 was determined by immunoblotting on adipose tissue samples from individual pups and pooled from groups of pups. This analysis was complemented by immunocytochemistry on representative adipose tissue samples. Maternal PRL infusion resulted in a greater abundance of UCP1 in fetal rats at 19·5 days of gestation (control: 97·2 ± 8·4% reference; PRL: 525·6 ± 74·4% reference; P<0·001) and in neonates 18 h after birth. In contrast, the abundance of the outer mitochondrial membrane protein voltage-dependent anion channel was unaffected by PRL. Neonatal adipose tissue sampled from pups born to PRL-infused dams possessed fewer lipid droplets, but more UCP1, as determined by immunocytochemistry. Fetal, but not maternal, plasma leptin concentrations were also increased by maternal PRL administration. In conclusion, as rats are altricial, and the potential thermogenic activity of brown adipose tissue develops over the first few days of postnatal life, these changes prior to, and at the time of, birth implicate PRL in fetal and neonatal adipose tissue maturation.


Introduction

Lambs and human infants need to achieve independent thermoregulation expeditiously after birth and, as a consequence, the rapid function of brown adipose tissue is crucial for neonatal survival (Clarke et al. 1997a). However, altricial species, such as the rat, are immature at birth and huddling with littersmates ensures maintenance of body temperature in the immediate newborn period. Only several days after birth, with gradual venturing from the nest, does brown adipose tissue function become necessary to maintain the temperature of the newborn rat (Nedergaard et al. 1986). Fetal adipose tissue maturation is characterised by the appearance of the brown adipose tissue-specific uncoupling protein-1 (UCP1), which occurs over the final half of gestation in the ovine fetus (Clarke et al. 1997b). In rat pups, however, the potential thermogenic activity and UCP1 content primarily increase over the first few days of postnatal life (Obregon et al. 1996), reaching a maximum at around day 5 (Sundin & Cannon 1980).

Maturation of fetal adipose tissue is not confined to changes in UCP1 but may include voltage-dependent anion channel (VDAC) which is an outer mitochondrial membrane protein. The role of VDAC in brown adipose tissue has not been elucidated, although it may be important in regulating the supply of mitochondrial ATP and ADP (Gottlieb 2000). It is not known whether fetal UCP1 and VDAC expression are subject to similar endocrine regulation. As the amounts of both UCP1 and VDAC are maximal at birth in lambs (Mostyn et al. 2001) their modulation is difficult to study at that time. Examination of the late fetal and early newborn rat permits study of the effects on UCP1 and VDAC around the time of birth, but before full recruitment of brown adipose tissue function has taken place.

During pregnancy, prolactin (PRL) is produced by the pituitary of the mother and by the placenta but not by the fetus (Tong et al. 1989). In the rat, maternal circulating PRL concentrations peak at the beginning of pregnancy, declining to preconception levels by the ninth day (Forsyth 1991, Ho Yuen et al. 1993). Administration of
PRL to the mother results in an appreciable increase in PRL in the fetal circulation in which pup blood contains 60% of the level of compound administered to the dam (Yang et al. 2002). It is established that the expression of PRL receptors (PRLR) is widespread in fetal rats, increasing in late gestation in multiple fetal tissues including brown adipose tissue (Royster et al. 1995). However, although PRL is postulated to promote cell proliferation and differentiation (Kelly et al. 1991), details of its role in pregnancy and fetal maturation remain to be fully determined.

The long and short forms of PRLR have been implicated in fetal adipose tissue development, as their expression appears at the gestational age at which UCP1 is initially detected in adipose tissue of the ovine fetus (Symonds et al. 1998). During late gestation there is also a developmental increase in leptin mRNA abundance in adipose tissue (Yuen et al. 1999). Then, after birth in lambs, a rapid loss of leptin mRNA occurs in conjunction with a decrease in plasma concentration (Bispham et al. 2002). This contrasts with the newborn rat in which, although starvation results in a substantial decrease in both leptin mRNA abundance and plasma concentrations (Dessolin et al. 1997), there is little change in plasma leptin after birth. Studies in adult rats have shown that PRL stimulates leptin secretion (Guallillo et al. 1999) by a mechanism that may be dependent on insulin (Ling & Billig 2001). It is not known whether similar effects are observed during pregnancy or at birth. The aim of the present study was, therefore, to determine whether the UCP1 abundance of brown adipose tissue and fetal plasma leptin can be enhanced by maternal PRL administration throughout gestation.

**Materials and Methods**

**Experimental design**

Female Sprague–Dawley rat dams of similar body weight (240–260 g) were provided with feed (laboratory chow) and water *ad libitum* and maintained in a constant photoperiod of 12 h light:12 h darkness. Recombinant PRL was produced from a human PRL cDNA clone (American Type Culture Collection, Rockville, MD, USA), as detailed by Chen et al. (1998) and expressed in *E. coli* so that it contained no posttranslational modifications. Before mating, five randomly selected rats were killed by an overdose of metafane (Mallinkrodt, Mundelein, IL, USA), decapitated and trunk blood was collected. Plasma was separated by centrifugation (800 g for 15 min, 4 °C) and stored at −20 °C for later analysis. The remaining dams were mated and randomly allocated to control (i.e. no PRL, *n*=19) or PRL treatment groups (*n*=19). These were randomised for tissue sampling during pregnancy (*n*=12 per group) or 18 h after birth (i.e. neonatal sampling, *n*=7 per group).

Initial, samples of interscapular brown adipose tissue, dissected from individual fetuses, were used to prepare mitochondria as described by Symonds et al. (1992). As a result of the very small amount of tissue available from both fetuses and neonates, the mitochondrial yields and UCP1 abundance of preparations of mitochondria from individual pups and tissue pooled within age and treatment groups were compared. This demonstrated that very similar amounts of mitochondrial protein were obtained. Twelve hours after mating, and on observation of a vaginal plug, minipumps (model 2004; Alza, Palo Alto, CA, USA) were implanted subcutaneously under aseptic conditions with local anaesthesia (0·7 ml 2% lidocaine subcutaneously). Pumps continuously infused recombinant PRL diluted with 0·9% NaCl to administer 24 µg/kg per day throughout pregnancy to the PRL group or an equivalent volume of saline to controls. This regime achieves stable administered plasma PRL concentrations of 50 ng/ml from day 4·5 of pregnancy (i.e. 4 days after pump implantation) (Coss et al. 2000, Yang et al. 2001).

At 6·5 and 11·5 days, dams were bled from the tail and plasma was stored at −20 °C until analysis. In preparation for delivery, dams were individually housed from day 17·5 of gestation. At 19·5 days of gestation, where term is 21·5 days, dams randomised for fetal sampling were killed by decapitation, and trunk blood and the fetuses were removed from the uterus. Two randomly selected fetal pups from each litter were snap-frozen in liquid nitrogen and stored at −80 °C, whilst five litters were decapitated and blood was collected into heparinised tubes for later analysis. Dams randomised for neonatal sampling delivered spontaneously at term into a constant ambient temperature. Then at approximately 18 ± 1 h of postnatal age, the newborn rats were weighed. Five randomly selected newborn rats from each litter were decapitated and blood was collected into heparinised tubes for later analysis. Newborn rats randomised for brown adipose tissue sampling and analysis were killed by an overdose of metafane. From each dam, two littersmates were placed on ice and interscapular brown adipose tissue was dissected and then either snap-frozen in liquid nitrogen and stored at −80 °C or immersed in fixative and embedded in paraffin for later immunocytochemical analysis. The presence or absence of milk in the stomach of neonatal pups at dissection was recorded. Dams randomised for neonatal sampling were killed, trunk blood was collected and the uterus of each dissected and inspected for visible implantation sites. The animal study protocol was designed and performed in accordance with the US National Institutes of Health guidelines and was prospectively approved by the local animal ethics committee (University of California, Riverside, Committee on Laboratory Animal Care).

**Laboratory analyses**

Initially, samples of interscapular brown adipose tissue, dissected from individual fetuses, were used to prepare mitochondria as described by Symonds et al. (1992). As a result of the very small amount of tissue available from both fetuses and neonates, the mitochondrial yields and UCP1 abundance of preparations of mitochondria from individual pups and tissue pooled within age and treatment groups were compared. This demonstrated that very similar amounts of mitochondrial protein were obtained.

with each procedure (e.g. individual control fetuses, 2·12 mg/fetus; pooled control fetuses, 2·04 mg/fetus). As a consequence, only pooled samples were used for determination of UCP1 in neonatal pups. UCP1 abundance was determined on 10 µg mitochondrial protein as described by Schermer et al. (1996) using an antibody dilution of 1/1000. VDAC abundance was determined using an antibody raised in rabbits to ovine VDAC, purified from the kidney of a newborn lamb based on the method of Schermer et al. (1996) as described for UCP1 and was used at a dilution of 1/2000. Densitometric analysis was performed on each membrane following image detection using a fujifilm LAS-1000 cooled charge-coupled device (CC-D) camera (Fuji Photo Film Co. Ltd, Tokyo Japan) and values were expressed as a percentage of reference run on all gels. All immunoblotting was performed in duplicate and each membrane included a reference sample for determination of interassay variance reference and molecular weight markers. The interassay and intra-assay coefficients of variance were 7·4 and 2·3% (n=5) respectively.

Serial sections were prepared from paraffin-embedded interscapular brown adipose tissue samples and immunocytochemistry for UCP1 was performed using a 1/1000 dilution of antibody using the same techniques as described by Dandrea et al. (2001). This was undertaken using at least four sections per treatment group. Following incubation with enzyme-conjugated second antibody and chromogen substrate, sections were examined by light microscopy. The specificity of the procedure was confirmed by the absence of binding when adjacent sections were incubated with rabbit serum from an unimmunised rabbit in place of rabbit antiovine UCP1 primary antibody. Mean lipid droplet size was determined using the Scion image analysis package (Scion Corporation, MD, USA).

Rat plasma leptin was determined by radioimmunoassay using an American Laboratory Products (ALPCO, Windham, NH, USA) kit, specific for rodent leptin. The interassay and intra-assay coefficients of variance were 7·7 and 4·3% (n=5) respectively.

Statistical analyses

Pups from any one litter are exposed to a single maternal environment and inclusion of multiple littersmates in analyses may amplify or confound the apparent effects of the intervention. Therefore, to eliminate the effects of multiple sampling from littersmates, one pup was randomly selected from each litter for tissue sampling. The abundance of UCP1, determined by immunoblotting, was expressed relative to a reference sample from a single neonatal rat included on all gels in order to ensure that comparable results were obtained from duplicate analyses.

Statistical analysis of UCP1 abundance in individual pups was performed for fetal pups at 19·5 days of gestation. In view of the small numbers of pups in each group and the difficulty in accurately determining whether a non-Gaussian distribution of the data was present, statistically significant differences with respect to maternal intervention were assessed by Mann–Whitney U test. However, for consistency of representation and comparison with other studies, data are presented as mean ± s.e.m. values. For neonatal pups, and for the pooled brown adipose tissue from fetal pups, the abundance of UCP1 for all animals combined was determined giving a single value for UCP1 abundance for each group and only a descriptive analysis is included. In view of the different numbers of pups sampled in fetal compared with neonatal life, the abundance of protein from tissue samples pooled before analysis is described as arithmetic mean abundances per animal (i.e. total protein abundance in pooled tissues/no. of animals from which tissue samples were derived) to allow simple comparison between groups.

For measurement of plasma leptin, blood from five fetuses from each litter was pooled in order to obtain sufficient plasma for analysis. In view of the likely similar maternal influence on individual pups from the same litter, statistical analysis was performed assuming that the number of individuals included in the study is equal to the number of dams from which the pups were derived. As sufficient blood was obtained from dams for individual analysis, maternal plasma leptin concentrations between groups were compared using the Mann–Whitney U test as the Kolmogorov–Smirnov test implied that a normal distribution of values was not present.

Results

Brown adipose tissue development

Fetal rats exposed to maternal PRL infusion during development had five times more UCP1 per µg mitochondrial protein (Fig. 1), a difference that persisted after birth although the magnitude of response declined. There was no difference in the amount of mitochondrial protein extracted between groups (control fetuses: 2·12 ± 0·17 (n=4); PRL fetuses: 1·85 ± 0·39 mg (n=4)) which decreased tenfold after birth to 0·24–0·26 mg/pup. As a result of the greater increase in UCP1 abundance with PRL in fetal compared with neonatal pups, when the transition from 19·5 days of gestation to 18 h of postnatal age is considered, there was a greater increase in UCP1 abundance per µg mitochondrial protein after birth in controls, i.e. 9·8-fold increase compared with PRL-treated, i.e. 4·2-fold increase. However, in view of the falling mitochondrial protein content of brown adipose tissue over this period, the mean UCP1 content of the interscapular tissue depot was maintained in control offspring whilst it was lower in PRL-exposed offspring at 18 h of postnatal age than at 19·5 days of gestation (Fig. 1). There was no difference between groups with respect to VDAC abundance (data not shown).
Representative samples of interscapular adipose tissue were taken for histological and immunohistochemical analyses. This confirmed the above UCP1 results for neonatal pups as obtained by immunoblotting. Pups born to PRL-treated dams possessed adipose tissue with a much darker visual appearance than that of controls (Fig. 2). Adipose tissue of these pups also had markedly reduced smaller lipid droplets (controls: 0.05 ± 0.01; PRL: 0.17 ± 0.02 arbitrary units ($P < 0.001$)) and there was consistently more immunocytochemical staining for UCP1 in these samples (Fig. 3).

**Plasma leptin concentrations**

At 19.5 days of gestation, fetal pups born to PRL-treated dams had 2.6-fold greater plasma leptin concentrations than controls (control: 0.32 ± 0.04; PRL: 0.82 ± 0.103 ng/ml; $P < 0.001$). Maternal plasma leptin increased to 11.5 days of gestation, when values were significantly greater than after delivery (11.5 days: 2.90 ± 0.23; post delivery: 1.73 ± 0.30 ng/ml; $P < 0.05$), and there were no differences in maternal plasma leptin concentrations between the PRL- and saline-infused groups.

**Fetal weight and viability**

There was no effect of maternal PRL administration on the duration of pregnancy as all dams gave birth at 21.5 days of gestation. The numbers of pups per litter (control: 13 ± 1 ($n=7$); PRL: 13 ± 1 ($n=7$)), visible implantation sites at term (control: 14 ± 1; PRL: 13 ± 1) and percentages of neonatal rats surviving to 18 h of age (control: 97.78; PRL: 97.75%) were all similar between groups. No effect of PRL administration was observed on average pup weight (e.g. control: 6.6 ± 0.1 g; PRL: 6.5 ± 0.1 g) or litter weight at 18 h of postnatal age or on maternal weight gain throughout gestation (control: 66.4 ± 14.0 g; PRL: 69.0 ± 9.6 g). There was no difference between the groups in the numbers of animals with milk present in their stomachs at 18 h, which has no influence on lipid droplet size in adipose tissue.

**Discussion**

This study demonstrates, for the first time, that administration of PRL to the pregnant rat throughout gestation results in substantially increased UCP1 abundance in both the late gestation fetus and the newborn rat pup. Maternal PRL administration resulted in a fivefold greater abundance of UCP1 in late gestation fetal offspring and this effect persisted after birth, although the magnitude of response declined. The increase in UCP1 was associated with a concomitant decline in lipid content of adipose tissue, thereby providing further evidence of enhanced UCP1 function (Bird et al. 2001). As a consequence of the greater fall in the mitochondrial content of brown adipose tissue after birth in PRL-treated pups, the total UCP1 content of interscapular brown adipose tissue decreased between 19.5 days of gestation and 18 h after birth. This adaptation was not found in controls. Thus, augmented maternal PRL concentrations during fetal development led to accelerated brown adipose tissue maturation. The effect of PRL appeared to be specific to UCP1, as maternal PRL had no influence on mitochondrial VDAC abundance. The present study, therefore, suggests that PRL, acting through its receptor, is critical in regulating both the initial appearance of UCP1 in fetal adipose tissue (Symonds et al. 1998) and the gradual increase in its abundance around the time of birth. To this extent, PRL and/or its receptor have been shown to have a significant effect on adipose tissue function and development. For example, administration of PRL at birth can improve both thermoregulation and thermogenic potential in the newborn lamb (Pearce et al. 2000). Conversely, mice lacking PRL receptors become fat depleted (Freemark et al. 2001).

There was no effect of maternal PRL administration on maternal leptin concentrations. However, the observed increase in fetal plasma leptin is indicative of additional effects on fetal adipose tissue development as adipose tissue is an important source of plasma leptin in the fetus.
Figure 2 Macroscopic appearance of interscapular brown adipose tissue from neonatal rat pups at 18 h of postnatal age. (A) Control neonate and (B) PRL-exposed neonate.

Figure 3 Light microscopy of interscapular brown adipose tissue from neonatal rat pups at 18 h of postnatal age. (A and B) Control neonate and (C and D) PRL-exposed neonate. (A and C) Haemotoxylin staining and (B and D) chromogen immunocytochemical staining for UCP1.
(Bispham et al. 2002). These findings provide further evidence that PRL regulation of leptin secretion from adipose tissue is not confined to white adipose tissue (Gualillo et al. 1999). This effect may be mediated by concomitant effects on insulin secretion (Ling & Billig 2001). Pregnancy, however, results in raised plasma insulin contributing to insulin resistance (Butte 2000) and increased plasma leptin (Reitman et al. 2001); further enhancement of leptin secretion from maternal adipose tissue by PRL administration may not be detectable.

The extent to which an increase in fetal leptin may directly contribute to enhanced UCP1 abundance remains to be established. Chronic leptin administration to the late gestation ovine fetus promotes UCP1 expression (Evens et al. 2001), an effect that requires not only intact sympathetic innervation (Scarpone & Matheny 1998) but, in particular, norepinephrine action (Commins et al. 1999a). Thus, the effect of leptin following maternal PRL infusion may be mediated indirectly via stimulation of the sympathetic nervous system (Commins et al. 1999b). A stimulatory effect of leptin on brown adipose tissue function acting via increased norepinephrine secretion would act to increase UCP1 abundance (Ricquier & Mory 1984) at the same time as lipid content declines as a consequence of enhanced lipolysis.

The effects of maternal PRL on the fetus are not exclusive to brown adipose tissue, as a range of responses have been reported, including specific effects on bone, lungs, thymus and the heart (Coss et al. 2000, Yang et al. 2001). The mechanisms by which maternal PRL promotes fetal development have yet to be directly explored. A direct effect of maternally administered PRL on fetal brown adipose tissue maturation would require placental transfer which has previously been considered unlikely due to its large molecular weight. Active transport mechanisms have, however, been illustrated for some high molecular weight proteins including immunoglobulins (Huxham & Beck 1981). Importantly, administration of 50 ng/ml PRL through gestation has no effect on the dam’s own release of PRL. It does, however, result in appreciable transfer of PRL to the fetus to the extent that at 19.5 days of gestation 60% of fetal PRL is derived from maternal blood (Yang et al. 2002). The concentration of placental lactogen II is 28 ng/ml compared with maternal levels that are more than 10-fold higher i.e. 400 ng/ml (Freemark et al. 1993). When PRL is administered to dam through pregnancy ~30 ng/ml of PRL is transferred to the fetal circulation despite there being a total of only ~85 ng/ml in maternal blood (Yang et al. 2002). It therefore appears that there is a transfer system that preferentially selects PRL over placental lactogen II, but results in a very similar total lactogen concentration in the fetal circulation. It should also be noted that maternal PRL administration has no effect on the dam’s mothering abilities, any difference in adipose tissue composition between groups is likely to be primarily due to increased fetal exposure to PRL throughout gestation.

In conclusion, maternal PRL administration results in enhanced brown adipose tissue function in the neonate as well as raised plasma leptin. As a consequence, these individuals may be better adapted to thermoregulate following exposure to cold in the extruterine environment.

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