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Placental restriction of fetal growth decreases IGF1 and leptin mRNA expression in the perirenal adipose tissue of late gestation fetal sheep

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1Discipline of Physiology, School of Molecular and Biomedical Sciences, The University of Adelaide, South Australia; 2Commonwealth Scientific and Industrial Research Organisation Livestock Industries, Queensland Biosciences Precinct, St. Lucia, Queensland; and, 3Early Origins of Adult Health Research Group, Sansom Research Institute, School of Pharmacy and Medical Sciences, University of South Australia, South Australia, Australia

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Duffield JA, Vuocolo T, Tellam R, Yuen BS, Muhlhauser BS, McMillen IC. Placental restriction of fetal growth decreases IGF1 and leptin mRNA expression in the perirenal adipose tissue of late gestation fetal sheep. Am J Physiol Regul Integr Comp Physiol 294: R1413–R1419, 2008. First published February 13, 2008; doi:10.1152/ajpregu.00787.2007.—Placental restriction (PR) of fetal growth results in a low birth weight and an increased visceral fat mass in postnatal life. We investigated whether PR alters expression of genes that regulate adipogenesis [IGF1, IGF1 receptor (IGF1R), IGF2, IGF2R, proliferator-activated receptor-γ, retinoid-X-receptor-α], adipocyte metabolism (lipoprotein lipase, G3PDH, GAPDH) and adipokine signaling (leptin, adiponectin) in visceral adipose tissue before birth. PR was induced by removal of the majority of endometrial caruncles in nonpregnant ewes before mating. Fetal blood samples were collected from 116 days gestation, and perirenal visceral adipose tissue (PAT) was collected from PR and control fetuses at 145 days. PAT gene expression was measured by quantitative RT-PCR. PR fetuses had a lower weight (PR 2.90 ± 0.32 kg; control, 5.12 ± 0.24 kg; P < 0.0001), mean gestational arterial PO2 (PR 0.332 ± 0.083; control, 0.741 ± 0.083; P < 0.01), and insulin concentrations (P < 0.02), than controls. The expression of IGF1 mRNA in PAT was lower in the PR fetuses (PR, 0.332 ± 0.083; control, 0.741 ± 0.083; P < 0.01). Leptin mRNA expression in PAT was also lower in PR fetuses (PR, 0.077 ± 0.009; control, 0.115 ± 0.013; P < 0.05), although there was no difference in the expression of other adipokine or adipogenic genes in PAT between PR and control fetuses. Thus, restriction of placental and hence, fetal substrate supply results in decreased IGF1 and leptin expression in fetal visceral adipose tissue, which may alter the functional development of the perirenal fat depot and contribute to altered leptin signaling in the growth-restricted newborn and the subsequent emergence of an increased visceral adiposity.

placenta; leptin; obesity

A WORLDWIDE SERIES OF EPIDEMIOLOGICAL and clinical studies has demonstrated that there are associations between the patterns of growth in fetal and early postnatal life and the risk of insulin resistance, type 2 diabetes, and obesity in adult life (6, 7, 33, 41, 44, 50–52). Small-for-gestational-age (SGA; birth weight and/or length < 2 SDs below the mean for gestational age), (2, 72) infants have low circulating IGF1 concentrations and a reduced body fat mass at birth (18) and then undergo a period of accelerated postnatal growth during the first few years of life (1, 21) with a relative increase in body fat mass from as early as 2–12 mo of age (27) and truncal fat mass during childhood (56) and in adult life (40, 58). An accelerated postnatal growth rate is associated with an early increased insulin sensitivity followed by the emergence of insulin resistance (7, 12, 19, 43, 70, 71) and, in particular, insulin resistance of adipose tissue in later life (33, 35, 36, 60).

IGF1 is one of the most important growth factors in the development of adipose tissue. The experimental induction of a global IGF1-receptor (IGF1R) deficiency in mice produces growth restriction, and reduces the growth of adipose tissue relative to that of other tissues (30). At physiological levels, IGF1 stimulates both proliferation and differentiation of pre-adipocytes in cell culture and, along with cAMP and glucocorticoids, has been shown to be required for induction of adipose tissue differentiation in both serum-containing and serum-free media (24, 68). It has been shown that after experimental induction of differentiation growth, arrested preadipocytes go through clonal expansion prior to a significant increase in the expression of proliferator-activated receptor-γ (PPARγ), a prominent adipogenic transcription factor when heterodimerized with retinoid-X-receptor-α (RXRα). This initiates the transcription of the regulatory genes involved in lipid accumulation and glucose metabolism, particularly lipoprotein lipase (LPL), leptin, and adiponectin and marks the entry of the cell into terminal differentiation (24, 61). In species such as the sheep or pig, adipogenesis and lipogenesis occur before birth as in the human, and low birth weight offspring also grow faster in the postnatal period and have a higher proportion of body fat than their higher birth weight counterparts in later life (23, 45, 62). In a cohort of lambs born after experimental restriction of placental growth, there was an increase in relative visceral fat mass and a relationship between visceral adiposity and the action of insulin to suppress circulating free fatty acids

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at 6 wk of age (14). In the fetal sheep, body weight is also related to circulating IGF1 concentrations (10, 38, 57), and to hepatic IGF1 mRNA expression (64). Experimental restriction of placental and hence, fetal growth in the sheep reduces both circulating IGF1 protein and IGFI mRNA expression in fetal tissues, such as the liver, kidney, and skeletal muscle (38). While there is evidence for a significant autocrine/paracrine role of IGFI in adipose tissue proliferation (9, 11, 20, 39, 47, 48, 59) and differentiation, the effect of placental restriction on the expression of IGFI or IGFI2 or on the ligand and clearance receptors IGFI1R and IGFI2R in fetal adipose tissue, is not known. We have therefore used the placentally restricted (PR) fetal sheep as a model to test the hypothesis that poor fetal growth results in a decreased expression of IGFI mRNA in perirenal adipose tissue, the major fetal adipose depot at 140–145 days gestation. We further hypothesize that PR of fetal substrate supply will result in an altered pattern of expression of adipogenic, lipogenic, and adipokine genes in the visceral adipose tissue before birth. We have therefore investigated the effect of PR on the expression of IGF1, IGFI2, IGFI1R, IGFI2R, PPARγ, RXRα, LPL, G3PDH, GAPDH, leptin, and adiponectin in fetal perirenal adipose tissue.

MATERIALS AND METHODS

Animals and surgery. All procedures were approved by The University of Adelaide Animal Ethics Committee. Fifteen Merino ewes were used in the study. Nine nonpregnant ewes underwent surgery to remove the majority of endometrial caruncles from the uterus, leaving three to eight caruncles in each horn to induce experimental restriction of placental and fetal growth (65).

Surgery was performed on control (n = 6) and caruncleomized (n = 9) pregnant ewes under aseptic conditions between 109 and 124 days of gestation (term = 147 ± 3 days) with general anesthesia induced by sodium thiopentone (1.25 g iv; Pentothal; Rhone Merieux, Pinkenba, Australia) and maintained with 2.5–4% (vol/vol) halothane induced by sodium thiopentane (1.25 g iv; Pentothal; Rhone Merieux, Pinkenba, Australia) and maintained with 2.5–4% (vol/vol) halothane in oxygen. Tissue collection.

Fetal plasma insulin concentrations were measured in control (n = 6) and caruncleomized (n = 6) fetuses by using a commercial kit (Phasadeph radioimmunoassay kit; Pharmacia & Upjohn, Uppsala, Sweden). The detection range of the assay was 1.5–240 μU/ml. Guinea pig anti-insulin antisera and 125I-labeled human insulin (100 μl) were added to plasma samples (100 μl), which were then incubated for 2 h at room temperature before the addition of 2 ml of sheep anti-guinea pig immunoglobulin G. Samples were allowed to stand at room temperature for a further 30 min before being centrifuged at 1,500 g for 10 min as described previously (16). The inter- and intra-assay CVs were both <5%.

Plasma leptin concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (Konelab 20, Program Version 6.0 automated analysis system; Thermo Fisher Scientific, Waltham, MA). The sensitivity of the assay was 0.25 mg/l and the intra- and inter-assay coefficients of variation (CVs) were both <10%.

Plasma glucose concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (Konelab 20, Program Version 6.0 automated analysis system, Thermo Fisher Scientific). The sensitivity of the assay was 0.5 mmol/l and the intra- and inter-assay CVs were both <5%.

Fetal plasma insulin concentrations were measured in control (n = 6) and caruncleomized (n = 6) fetuses by using a competitive ELISA (37). Briefly, an ELISA plate was preincubated with recombinant bovine leptin in 50 μl of 0.1 M bicarbonate buffer and blocked with 200 μl of 5% skim milk in ELISA buffer. Chicken antirecombinant bovine leptin antiserum (50 μl) was added, incubated for 1 h, and the plate developed with p-nitrophenylphosphate disodium salt hexahydrate. The sensitivity of the assay was 0.25 ng/ml and the intra- and inter-assay CVs were both <15.0%.

Isolation of RNA, production of cDNA, and quantitative RT-PCR analysis. RNA was extracted from 100 mg PAT (Tri Reagent, prod. no. T9424; Sigma) from eight fetuses in the PR group and six fetuses in the control group. RNA was treated for genomic DNA contamination using Ambion Dnase1, and after enzyme deactivation, the RNA was run through a secondary purification process using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm, and RNA integrity was confirmed by agarose gel electrophoresis. cDNA was then synthesized using the purified RNA (~5 μg) and Superscript 3 RT (Invitrogen, Mount Waverley, Australia) with random hexamers. The relative expression of PPARγ, LPL, G3PDH, leptin, and adiponectin mRNA transcripts (53), and RXRs (forward: 5′-ctttggcaacaggctgttgc-3′; reverse: 5′-cttggcaacacctgg-3′) and GAPDH (forward: 5′-cttggcaacacctggcgaacctg-3′; reverse: 5′-
mRNA transcripts were measured for each fetus by real-time quantitative RT-PCR (qRT-PCR) using the Sybr Green system in an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Each qRT-PCR reaction well (5 µl total volume) contained 2.5 µl 2X Sybr Green Master Mix (PE Applied Biosystems); 0.25 µl of each primer giving a final concentration of 450 nM, 1.0 µl of molecular grade H₂O and 1.0 µl of a 1:10 dilution of the stock template.

The relative expression of IGFI, IGF2, IGFR1, and IGFR2 mRNA transcripts (46) were measured for each fetus by qRT-PCR using the Sybr Green system in an ABI Prism 7300 Sequence Detection System (PE Applied Biosystems). Each qRT-PCR reaction well (10 µl total volume) contained 6 µl 2X Sybr Green Master Mix (PE Applied Biosystems); 1 µl of each primer giving a final concentration of 450 or 900 nM, 1.0 µl of molecular grade H₂O, and 1.0 µl of a 50 ng/µl dilution of the stock cDNA template.

Primers for each transcript were designed with the aid of Primer Express software (PE Applied Biosystems), and, where possible, one primer of each pair was positioned over a splice site to prevent amplification of any residual genomic DNA. For each transcript, RT-PCR was performed using the appropriate primers to ensure the amplification of a single amplicon of the expected size. The product was viewed on an ethidium bromide-stained electrophoresis gel. Each amplicon, which was designed to be ~200 bp in length, was sequenced to ensure the authenticity of the DNA product, and qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. Controls containing no RT were also used, and for the qRT-PCR measurements, the cDNA primer concentrations were equivalent for all genes. Amplification efficiencies were determined from the slope of a plot of the threshold cycle with the lowest significant increase in fluorescence against the log of the cDNA template material (ranging from 1 to 100 ng/µl) and were 0.997–0.999. A constant amount of cDNA equating to 10 ng of total RNA was used for each qRT-PCR measurement and four technical replicates were performed for each gene. For all qRT-PCR runs, the cycling conditions consisted of 40 cycles of 95°C for 15 min and 60°C for 1 min.

The abundance of each mRNA transcript was measured and expression relative to that of acidic ribosomal protein P0 (ARP-P0) was calculated using the comparative threshold cycle method (Q-gene qRT-PCR analysis software) (54).

Statistical analysis. The effects of placental restriction on fetal size, plasma hormone, and nutrient concentrations, the absolute and relative weights of perirenal fat, and the expression of adipose genes were determined using a Student’s unpaired t-test. Relationships between fetal size, plasma hormone, and nutrient concentrations or fat mass and PAT gene expression were determined using linear regression and partial correlation analyses. There was no effect of gestational age on any of the outcomes reported in this study. All data are presented as the means ± SE. A probability of < 5% (P < 0.05) was taken as the level of significance in all analyses.

RESULTS

Effect of placental restriction on arterial PO₂, plasma glucose, insulin concentrations, fetal growth, and fat mass. Mean arterial PO₂ (P < 0.001), plasma glucose (P < 0.01), and plasma insulin concentrations (P < 0.02) were lower in PR compared with control fetuses during late gestation (116–145 days) (Table 1). There was no effect, however, of placental restriction on either the mean plasma nonesterified fatty acids or leptin concentrations during late gestation (Table 1). PR fetuses had a lower weight (P < 0.0001), crown rump length (P < 0.0001), and absolute mass of perirenal fat (P < 0.01) at 140–145 days gestation compared with controls, but there was no difference in the relative mass of perirenal fat between the two groups (Table 1).

Table 1. Fetal weight, crown rump length, perirenal fat mass, and mean fetal arterial PO₂, and plasma NEFA, glucose, insulin, and leptin concentrations (116–144 days), in PR and control fetuses

<table>
<thead>
<tr>
<th></th>
<th>PR Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of fetuses</td>
<td>6–9</td>
<td>6</td>
</tr>
<tr>
<td>Gestational age, days</td>
<td>143.6±0.7</td>
<td>144±0.2</td>
</tr>
<tr>
<td>Fetal weight, kg</td>
<td>2.90±0.32*</td>
<td>5.13±0.24</td>
</tr>
<tr>
<td>Fetal crown rump length, cm</td>
<td>48.6±1.6*</td>
<td>60.6±1.5</td>
</tr>
<tr>
<td>Absolute mass of perirenal fat, g</td>
<td>13.4±1.3*</td>
<td>21.6±1.8</td>
</tr>
<tr>
<td>Relative mass of perirenal fat, g/kg</td>
<td>4.53±0.57</td>
<td>4.20±0.29</td>
</tr>
<tr>
<td>Mean arterial Po₂ 116–145 days, mmHg</td>
<td>13.8±0.5*</td>
<td>21.8±1.3</td>
</tr>
<tr>
<td>Mean plasma glucose 116–145 days, mmol/l</td>
<td>0.68±0.07*</td>
<td>1.01±0.03</td>
</tr>
<tr>
<td>Mean plasma insulin 116–145 days, µU/ml</td>
<td>5.16±0.83*</td>
<td>8.65±0.76</td>
</tr>
<tr>
<td>Mean plasma NEFA 116–145 days, µg/ml</td>
<td>0.053±0.004</td>
<td>0.054±0.004</td>
</tr>
<tr>
<td>Mean plasma leptin 116–145 days, ng/ml</td>
<td>1.96±0.23</td>
<td>2.28±0.31</td>
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PR. placental restriction. NEFA, nonesterified fatty acids. *P < 0.05 compared with control.

DISCUSSION

In this study we have investigated the extent to which the expression of the IGFI and IGF2, their receptors (IGFR1, IGFR2), and other genes involved in adipogenesis (PPARγ, RXRα), adipocyte metabolism (G3PDH, GAPDH), and adipokine signaling (leptin, adiponectin) is regulated by placental substrate supply during fetal life. We have demonstrated that the expression of IGFI and leptin mRNA in adipose tissue...
were each decreased in response to placental restriction of fetal substrate supply.

Consistent with previous studies of the placentally restricted (PR) sheep model, PR fetuses in the present study were chronically hypoxic, hypoglycemic, and hypoinsulinemic. The changes in fetal blood gas status and nutrient supply in PR sheep fetuses are similar to those measured in cordocentesis studies of SGA human infants (15). In the present study, the absolute mass of perirenal fat in PR fetuses at 140 days gestation was significantly lower than that of control fetuses, but when fat mass was expressed relative to body weight, there was no difference in fat mass between PR and control fetuses at 140 days gestation. In a previous study, it was shown that PR lambs have an increased perirenal and visceral fat mass relative to body weight at 45 days of postnatal life (14). Furthermore, there was an inverse relationship between the insulin sensitivity of glucose, circulating amino acids, and free fatty acid metabolism and birth weight in both PR and control lambs, such that a lower birth weight corresponded with a higher metabolism and birth weight in both PR and control lambs (16).

IGF1R, IGF2R, IGF2, PPARγ, and RXRα have been shown to be readily synthesized and secreted, with little preformed or stored (49, 67). It is therefore possible that the decreased expression of IGF1 mRNA in the PAI of PR fetuses may have consequences for the stimulation of the proliferation and differentiation of PAT. At physiologic levels, IGF1 stimulates both proliferation and differentiation of preadipocytes in cell culture and along with cAMP and glucocorticoids is required for induction of differentiation in both serum-containing and serum-free media (24, 68). Furthermore, preadipocyte proliferation can be prevented by stripping IGF1 from adipose tissue-conditioned culture medium (47). Given the role of IGF1 in adipocyte proliferation and differentiation, it may initially appear paradoxical that there is a decrease in adipose IGF1 mRNA expression in IUGR fetuses given the association between being born SGA and the emergence of increased adiposity and insulin resistance in later life (56, 58). Recently it has been suggested, however, that low proliferation and differentiation capacity of adipocytes may be a key factor in a propensity to develop larger fat cells that in turn are associated with insulin resistance in adult life (28). It has been proposed that when proliferation of adipocytes is reduced, mesenchymal stem cells are able to differentiate along several mutually exclusive pathways to form other cell types and that the existing adipocytes are susceptible to hypertrophy under conditions of an increased nutrient supply or fat excess (28).

It has been shown previously that the induction of terminal differentiation of preadipocyte cultures by IGF1 or supraphysiological concentrations of insulin acting at the IGF1R (55) results in a marked increase in the expression of PPARγ mRNA (24, 61, 63). PPARγ, in a heterodimer with RXRα, acts as a transcription factor to initiate transcription of the regulatory genes involved in lipid accumulation and metabolism, particularly LPL, leptin, and adiponectin, which all have functional peroxisome proliferator response elements (PPREs) in their promoter regions (29, 32, 66). There is evidence that the effect of IGF1R signaling on PPARγ mRNA expression may be via the p38MAPK downstream signaling pathway (42), which modulates the transcriptional activity of CCAAT/en-

| Table 2. IGF2, IGF1R, IGF2R, PPARγ, RXRα, adiponectin, LPL, G3PDH, and GAPDH mRNA expression (expression relative to ARP-P0 mRNA expression) in perirenal adipose tissue in PR and control fetal sheep |
|------------------|-------------------|
| PR Group | Control Group |
| No. of fetuses | 8 | 6 |
| IGF2 | 0.66±0.05 | 0.81±0.10 |
| IGF1R | 0.004±0.000 | 0.005±0.000 |
| IGF2R | 0.003±0.000 | 0.004±0.000 |
| PPARγ | 0.94±0.065 | 1.16±0.14 |
| RXRα | 0.05±0.00 | 0.07±0.01 |
| LPL | 0.31±0.05 | 0.28±0.03 |
| G3PDH | 0.66±0.08 | 0.53±0.07 |
| GAPDH | 1.84±0.10 | 1.91±0.20 |
| Adiponectin | 2.79±0.29 | 2.49±0.36 |

R, receptor; PPARγ, proliferator-activated receptor-γ; RXRα, retinoid-X-receptor-α; LPL, lipoprotein lipase; ARP-P0, acidic ribosomal protein P0.
hancer binding protein (C/EBP)β, an adipocyte differentiation factor that induces PPARγ mRNA expression (5). While IGF1 expression in PAT was decreased in PR fetal sheep, there was no effect of PR on PPARγ mRNA expression in this tissue. Interestingly, however, the expression of IGFB2 related to the expression of PPARγ mRNA in PAT at 140–145 days gestation, which may indicate that during fetal life IGFB2 binding at the IGFB1R, significantly contributes to the regulation of PPARγ mRNA expression in adipose tissue. Alternatively PR may act to alter the expression of the IGF binding proteins and hence influence IGF bioavailability within the PAT. There was no effect of PR on the expression of RXRα, adiponectin LPL, G3PDH, or GAPDH in fetal PAT, although there was a positive relationship between PPARγ and adiponectin mRNA, indicating that there may be functional activation of the PPRE in the promoter region of the adiponectin gene in PAT of PR and normally grown fetal sheep in late gestation.

There was a significant decrease in the expression of leptin mRNA in the PAT of PR fetal sheep at 140–145 days gestation. It has previously been shown that the expression of leptin mRNA and the size of lipid locules in the perirenal adipocytes were increased in the hyperglycemic sheep fetuses of over-nourished ewes (53). Therefore, the reduction in leptin mRNA expression in the PR fetus could reflect a decrease in lipid content of the fetal PAT.

The leptin gene promoter region contains C/EBP (26, 31, 69), Sp1 transcription factor, cAMP, glucocorticoid (22), and hypoxia (4, 25) response elements in addition to the PPRE. In the present study, there was a relationship between the expression of leptin and PPARγ mRNA in perirenal fat, which could reflect either direct activation of the PPRE in the promoter of the leptin gene, or indirect activation of both the leptin and PPARγ promoters by the same transcription factor, such as C/EBP.

In contrast to adult life, there was no relationship between the expression of leptin mRNA in adipose tissue and circulating plasma leptin concentrations in the fetal sheep at 140–145 days gestation. It has been suggested that leptin may be derived from other fetal tissues, such as the liver and lung (17), or from the placenta or maternal circulation (8). It is possible that after the transition to postnatal life and the induction of adipose tissue as the main source of circulating leptin that the reduction in leptin mRNA expression in the adipose tissue of lambs, which were placently restricted, might result in a functional immaturity of the leptin-fat mass axis with consequences for appetite regulation, lipid metabolism, and insulin sensitivity. Interestingly, evidence for a reduction in basal circulating leptin and higher relative increases in body mass index have been described in adults who were born IUGR (birth weight < 3rd percentile) (34, 41).

**Perspectives and Significance**

We have demonstrated that the expression of both IGF1 and leptin mRNA in fetal adipose tissue is decreased by placental restriction of fetal substrate supply. It is possible that a reduced expression of IGF1 mRNA in adipose tissue is associated with a direct reduction in the concentration of IGF1 available for paracrine activation of differentiation in developing adipose tissue. One intriguing possibility is that the low expression of IGF1 in adipose tissue of IUGR fetuses results in a reduced proliferation and differentiation capacity of adipose tissue. However, after the transition of the SGA newborn to a nutrient-rich environment at birth, the adipocytes undergo hypertrophy, which in turn has been associated with the emergence of insulin resistance (28). This mechanism could contribute, in part, to explaining how a mismatch between a nutrient-poor prenatal environment and a nutrient-rich postnatal environment results in obesity and insulin resistance. The reduction in leptin mRNA expression in PAT of fetal sheep also raises the possibility that events that occurred in utero, which result in a growth-restricted fetus, may result in a functional immaturity of the leptin-fat mass axis with consequences for the regulation of lipid metabolism and insulin sensitivity during postnatal life.

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