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Insulin Resistance and the Cardiometabolic Syndrome: Adipose Tissue and Skeletal Muscle Factors

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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Duffield JA, Vuocolo T, Tellam R, Yuen BS, Muhlhausler 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 ± 0.063; 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 ± 0.063; 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 placentation 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.

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 (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 preadipocytes 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 other adipokine or adipogenic genes in PAT between PR and control fetuses. Thus, restriction of placentation 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.

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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 IGF 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 IGF1 in adipose tissue proliferation (9, 11, 20, 39, 47, 48, 59) and differentiation, the effect of placental restriction on the expression of IGF1 or IGF2 or on the ligand and clearance receptors IGF1R and IGF2R 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 IGF1 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, IGF2, IGF1R, IGF2R, PPAR-γ, 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 caruncleiectomized (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, France). Ewes were fed once daily at 1100 h with 1 kg of lucerne chaff [85% dry matter (DM), metabolizable energy (ME) 8.5 MJ/kg DM] and allowed to recover from surgery for at least 4 days before collection of arterial blood (0800–1100 h). Food, London (52a). Water was provided ad libitum. Animals were housed in individual pens in rooms with a 12:12-h light-dark cycle with lights on at 7:00 AM and a daily temperature range between 19 and 22°C. Ewes were fed once daily at 1100 h with 1 kg lucerne chaff [85% dry matter (DM), metabolizable energy (ME) content = 8.3 MJ/kg DM] and 0.3 kg concentrated pellets containing: straw, cereal, hay, clover, barley, oats, lupins, almond shells, oat husks, and limestone (90% DM, ME content = 8.0 MJ/kg DM; Johnson & Sons, Kapunda, Australia). This diet provided 100% of the energy requirements for the maintenance of a ewe bearing a singleton pregnancy as specified by the Ministry of Agriculture, Fisheries and Food, London (52a). Water was provided ad libitum. Animals were allowed to recover from surgery for at least 4 days before collection of arterial blood samples commenced.

Blood sampling protocol. Fetal arterial (3.5 ml) blood samples were collected between 0800 and 1100 h, before the ewes were fed, three times each week between 116 and 140 days of gestation. Blood samples were centrifuged at 1,500 g for 10 min, and plasma was separated into aliquots and stored at −80°C. At times, fetal blood samples could not be collected due to technical problems (primarily related to blocked vascular catheters). Fetal arterial blood (0.5 ml) samples were also collected for the measurement of arterial blood gas status (ABL 520 blood gas analyzer; Radiometer, Copenhagen, Denmark).

Tissue collection. Ewes were killed between 140 and 145 days of pregnancy with a lethal overdose of pentobarbital sodium (Virbac, Peakhurst, Australia). Fetuses were delivered by hysterectomy, weighed, and killed by decapitation. Fetal perirenal adipose tissue (PAT) was dissected and weighed, and samples from control (n = 6) and PR (n = 8 out of 9) fetuses were frozen in liquid nitrogen and stored at −80°C for subsequent gene analysis.

Plasma nonesterified fatty acids, glucose, and hormone assays. Plasma nonesterified fatty acids were measured by an in vitro enzymatic colorimetric method (Wako, Osaka, Japan). The method relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple-colored adduct that can be measured colorimetrically at 550 nm (Konelab 20, version 6.0 automated analysis system; Thermo Fisher Scientific, Waltham, MA). The sensitivity of the assay was 0.25 μmol/l and the intra- and interassay 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 interassay CVs were both <5%.

Fetal plasma insulin concentrations were measured in control (n = 6) and caruncleectomized (n = 6) fetuses by using a commercial kit (Phasadeph radioimmunoassay kit; Pharmacia & Upjohn, Uppsala, Sweden). The detection range of the assay was 1.5–250 μU/ml. Guinea pig anti-insulin antiserum 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 intraassay CVs were both <10%.

Plasma leptin concentrations were measured in control (n = 6) and caruncleectomized (n = 6) fetuses by using a competitive ELISA (37). Briefly, an ELISA plate was preincubated with recombiant 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 to the wells, followed by the addition of samples (100 μl) in duplicate. Following an overnight incubation at 37°C, a biotinylated phoshatase-streapavidin conjugate (Amrad Biotech, Boronia, Australia) 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 interassay 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, Valencia, California). 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 PPAR-γ, LPL, G3PDH, leptin, and adiponectin mRNA transcripts (53), and RXRα (forward: 5'-ctttgacgaagagtcg-3'; reverse: 5'-ctttgagaactctcg-3') and GAPDH (forward: 5'-ctttgagaacactgcgaat-3' reverse: 5'-
The relative expression of IGF1, IGF2, IGF1R, and IGF2R mRNA transcripts (46) were measured for each fetus by 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 2× 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 IGF1, IGF2, IGF1R, and IGF2R mRNA transcripts during late gestation (116–145 days gestation) was compared with control fetuses during late gestation (116–145 days gestation compared with controls, but there was no difference in the relative mass of perirenal fat between the two groups (Table 1). IGF1, IGF2, IGF1R, and IGF2R mRNA expression in fetal PAT. IGF1 mRNA expression was significantly lower (P < 0.01) in PAT from PR fetuses compared with controls at 140–145 days gestation (Fig. 1). IGF1 mRNA expression was directly related to arterial PO2 (r² = 0.61, P < 0.001), mean plasma glucose (r² = 0.47, P < 0.05), and insulin concentrations (r² = 0.42, P < 0.05) during late gestation. Partial correlation analysis showed that these relationships were interdependent.

There was no difference in the expression of IGF2, IGF1R, or IGF2R mRNA in PAT from PR and control fetal sheep (Table 2). When data from both treatment groups were combined, the expression of IGF2 mRNA (r² = 0.32, P < 0.05) and IGF2R mRNA (r² = 0.34, P < 0.05) were each directly related to the expression of PPARγ mRNA.

Leptin, adiponectin, PPARγ, RXRα, LPL, G3PDH, and GAPDH mRNA expression in fetal visceral adipose tissue. Leptin mRNA expression in PAT was lower (P < 0.05) in PR fetuses when compared with controls (Fig. 2) and was directly related to the absolute mass of perirenal fat (r² = 0.35, P < 0.01). Leptin and PPARγ mRNA expression were also directly related (r² = 0.55, P < 0.01).

There was no difference in the expression of adiponectin, PPARγ, RXRα, LPL, G3PDH, and GAPDH mRNA in PAT between the PR and control groups at 140–145 days gestation (Table 2). Adiponectin mRNA expression was strongly related to G3PDH mRNA expression (r² = 0.64, P < 0.001), and PPARγ mRNA expression was related to the expression of both RXRα (r² = 0.31, P < 0.05) and adiponectin mRNA (r² = 0.35, P < 0.05).

**DISCUSSION**

In this study we have investigated the extent to which the expression of the IGF1 and IGF2, their receptors (IGF1R, IGF2R), 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 IGF1 and leptin mRNA in adipose tissue...
were each decreased in response to placental restriction of fetal substrate supply.

Consistent with previous studies of the placently 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 whole body insulin sensitivity at 45 days of postnatal life (13).

We have shown that PR results in a decreased expression of IGF1, but not IGF2, IGF1R, nor IGF2R mRNA expression in fetal PAT at 140–145 days gestation. It has previously been reported that IGF1 mRNA expression is decreased in the fetal liver, kidney, and skeletal muscle, and that circulating IGF1 concentrations are decreased in PR, compared with control fetuses at 121 days gestation (38, 57). It is currently unknown whether IGF1 mRNA expression in adipocytes is directly related to protein expression; however, in hepatocytes, IGF1 has been shown to be readily synthesized and secreted, with little preformed or stored (49, 67).

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).

**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**

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<tr>
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<th>PR Group</th>
<th>Control Group</th>
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<tbody>
<tr>
<td>No. of fetuses</td>
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<td>6</td>
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<tr>
<td>IGF2</td>
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<td>0.81±0.10</td>
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<td>0.005±0.000</td>
</tr>
<tr>
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<td>0.004±0.000</td>
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<td>PPARγ</td>
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<td>1.16±0.14</td>
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<tr>
<td>RXRα</td>
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<td>0.07±0.01</td>
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<tr>
<td>LPL</td>
<td>0.31±0.05</td>
<td>0.28±0.03</td>
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<tr>
<td>G3PDH</td>
<td>0.66±0.08</td>
<td>0.53±0.07</td>
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<tr>
<td>GAPDH</td>
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<tr>
<td>Adiponectin</td>
<td>2.79±0.29</td>
<td>2.49±0.36</td>
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R, receptor; PPARγ, proliferator-activated receptor-γ; RXRα, retinoid-X-receptor-α; LPL, lipoprotein lipase; ARP-P0, acidic ribosomal protein P0.

Fig. 1. Expression of IGF1 mRNA [expression relative to acidic ribosomal protein P0 (ARP-P0) mRNA expression] in fetal perirenal visceral adipose tissue (PAT) in placental restriction (PR) and control fetuses. There was a significantly lower expression of IGF1 mRNA in the PAT of PR fetuses compared with control fetuses at 140–145 days gestation.

Fig. 2. Expression of leptin mRNA (expression relative to ARP-P0 mRNA expression) in fetal PAT in PR and control fetuses. Adipose expression of leptin mRNA was significantly lower in PR than control fetuses at 140–145 days gestation.
hancer binding protein (C/EBP)β, an adipocyte differentiation factor that induces PPARγ mRNA expression (5). While IGFI 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 IGF2 related to the expression of PPARγ mRNA in PAT at 140–145 days gestation, which may indicate that during fetal life IGF2 binding at the IGFIIR, 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 RXXα, 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 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 placentally 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 IGFI and leptin mRNA in fetal adipose tissue is decreased by placentation restriction of fetal substrate supply. It is possible that a reduced expression of IGFI1 mRNA in adipose tissue is associated with a direct reduction in the concentration of IGFI1 available for paracrine activation of differentiation in developing adipose tissue. One intriguing possibility is that the low expression of IGFI1 in adipose tissue of IUGR fetuses results in a lower 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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