Impact of embryo number and periconceptional undernutrition on factors regulating adipogenesis, lipogenesis, and metabolism in adipose tissue in the sheep fetus

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1Sansom Institute for Health Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, Australia; 2Discipline of Physiology, University of Adelaide, Adelaide, Australia; 3University of Cambridge, Metabolic Research Laboratories, Institute of Metabolic Science, Addenbrooke’s Hospital, Cambridge, United Kingdom; 4South Australian Research and Development Institute, Turrettfield Research Centre, Rosedale, Australia; and 5Discipline of Obstetrics and Gynaecology, University of Adelaide, Adelaide, Australia

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Lie S, Morrison JL, Williams-Wyss O, Ozanne SE, Zhang S, Walker SK, Kleemann DO, MacLaughlin SM, Roberts CT, McMillen IC. Impact of embryo number and periconceptional undernutrition on factors regulating adipogenesis, lipogenesis, and metabolism in adipose tissue in the sheep fetus. Am J Physiol Endocrinol Metab 305: E931–E941, 2013. First published August 6, 2013; doi:10.1152/ajpendo.00180.2013.—Maternal undernutrition around the time of conception has also been shown to result in glucose intolerance and insulin resistance in the human and in the sheep (36, 41, 47). However, the impact of maternal undernutrition during the periconceptional period on the factors that regulate adipogenesis, lipogenesis, and insulin sensitivity in visceral fat is not known.

In the sheep fetus, fat is deposited at a rate of ~0.8 g·kg body wt−1·day−1 in late gestation, and the proportion of body fat at term is between 0.3 and 2.0%. The perirenal adipose tissue (PAT) is the major fat depot present in the sheep fetus and is comprised predominantly of brown fat cells that express adipogenic and lipogenic factors, including peroxisome proliferator-activated receptor-γ (PPARγ), lipoprotein lipase (LPL), and the adipokines leptin and adiponectin (10, 28, 54). Brown fat is also characterized by the expression of the thermogenic protein uncoupling protein 1 (UCP1), which is highly expressed from midgestation, with a peak in expression occurring soon after birth in the lamb (8, 55). After this peak, there is then a rapid reduction in UCP1 expression, followed by a more gradual decrease until UCP1 is no longer detected from around 2 wk of age in the lamb (14, 55). The decrease in UCP1 is associated with the transformation of PAT from thermogenic brown adipose tissue (BAT) to white adipose tissue (WAT) characterized by the capacity to store lipid (44). Insulin signaling regulates lipogenesis in adipose tissue through the stimulation of glucose uptake (3, 45, 48). Insulin acts through the insulin receptor (IR), which is stabilized by caveolin-1 (Cav-1) and phosphorylates insulin receptor substrate-1 (IRS-1), resulting in the activation of phosphatidylinositol 3-kinase (PI3K), leading to the phosphorylation and thus conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). Conversion of PIP2 to PIP3 is negatively regulated by the phosphatase and tensin homolog (PTEN), and PIP3 phosphorylates 3-phosphoinositide-dependent kinase 1 (PDPK1) and/or protein kinase B/Akt. Phosphorylation of PDPK1 may then result in the phosphorylation of the atypical protein kinase Cζ (PKCζ), and phosphorylation of Akt results in the phosphorylation of the Akt substrate 160 kDa (AS160). Phosphorylated PKCζ and AS160 each play major roles in the translocation of the glucose transporter 4 (GLUT4) to the plasma membrane to facilitate glucose uptake into

A RANGE OF EXPERIMENTAL AND CLINICAL STUDIES has shown that poor maternal nutrition experienced around the time of conception or during pregnancy, placental insufficiency, and being of low birth weight are each associated with an increase in visceral fat mass in postnatal life (5, 9, 11, 17, 21, 27, 30, 31). Interestingly, exposure to a poor nutrient supply in early pregnancy, such as which occurred in the Dutch Winter Fam-

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adipocytes (45). However, in fetal life, glucose uptake into adipocytes is maintained primarily through the activity of the insulin-independent glucose transporter 1 (GLUT1) (18). The transformation of PAT from thermogenic BAT to lipogenic WAT is also characterized by activation of insulin signaling via the Akt and mammalian target of rapamycin (mTOR) pathway, which in turn upregulates the key adipogenic and lipogenic factor PPARγ (56). There is also evidence that exposure to maternal overnutrition and an increase in fetal insulin and glucose concentrations upregulate the expression of PPARγ, LPL, and leptin mRNA in fetal PAT in the sheep (28). Interestingly, there is also a positive relationship between plasma insulin concentrations present at birth and the size of adipocytes in the perirenal fat depot in lambs at 3 wk of age, which suggests that insulin exposure in late fetal life plays an important role in determining the lipogenic capacity of the visceral adipocyte in later life (10).

In postnatal life, lipid accumulation in adipose tissue is also dependent on the rate of fatty acid metabolism (20). AMP-activated protein kinase (AMPK) is a master regulator of cellular energy status. Phosphorylation of AMPK stimulates fatty acid oxidation through phosphorylation of acetyl-CoA carboxylase (ACC), which inhibits malonyl-CoA synthesis (34). Malonyl-CoA inhibits the action of carnitine palmitoyltransferase-I (CPT-I) in facilitating the transport of long-chain fatty acyl-CoA into the mitochondria, which is the first and rate-limiting step in mitochondrial fatty acid β-oxidation (26). PPARγ coactivator-1α (PGC-1α) is also an important metabolic regulator through its role in stimulating mitochondrial biogenesis and increasing the expression of factors regulating mitochondrial fatty acid β-oxidation (37). Additionally, in postnatal life, pyruvate dehydrogenase kinase (PDK)2 and PDK4 play major roles in regulating the rate of fatty acid β-oxidation (43) by inhibiting the pyruvate dehydrogenase complex (PDC), which catalyzes glucose oxidation via the tricarboxylic acid cycle, resulting in an increase in the rate of fatty acid β-oxidation.

In humans, individuals who had a low birth weight have a lower abundance of some of the key insulin signaling factors in subcutaneous adipose tissue in later life, and it has been suggested that this may in part explain the link between low birth weight and type 2 diabetes in adult life (32). In the sheep, exposure to poor maternal nutrition around the time of conception (47) and in early gestation (36) results in an increased risk of insulin resistance in later life, and these effects are different in the offspring of singleton and twin pregnancies. Interestingly, it has also been shown that periconceptional undernutrition in sheep has a differential impact on the development of the fetal cardiovascular system (13), kidney (24), adrenal (23), hypothalamo-pituitary-axis (12), and postnatal insulin sensitivity in singletons and twins (47). These studies suggest that the early hormonal environment of a twin or singleton pregnancy may result in a differential programming of metabolic signaling pathways in singletons and twins. It is not known, however, whether exposure to maternal undernutrition in the periconceptional period can act to program changes in the expression or abundance of insulin signaling molecules, adipogenic and lipogenic factors, adipokines, or fatty acid β-oxidation factors in PAT during early development or whether these effects are different in singleton and twin pregnancies. In the current study, we hypothesized that exposure to periconceptional and/or preimplantation undernutrition would result in a decrease in UCP1 expression, a decrease in the abundance of key signaling molecules that control insulin signaling, and an increase in the abundance of those factors that regulate adipogenesis and lipogenesis in fetal adipose tissue and that these effects would be different in singletons and twins. Therefore, we have investigated the separate effects of maternal undernutrition in the periconceptional period (PCUN; for ≥2 mo before and 1 wk after conception) and in the preimplantation period (PIUN: for 1 wk after conception) on the expression and/or abundance of factors within the insulin signaling and fatty acid β-oxidation pathways and the adipogenic and lipogenic factors in PAT in singleton and twin fetal sheep.

MATERIALS AND METHODS

All procedures were approved by The University of Adelaide Animal Ethics Committee and by the Primary Industries and Resources South Australia Animal Ethics Committee.

Nutritional Management

South Australian Merino ewes were fed a diet that consisted of lucerne chaff and pellets containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime, and molasses (Johnsons & Sons, Kapunda, South Australia, Australia). Eighty percent of the total energy requirements were obtained from the lucerne chaff (8.3 MJ/kg metabolizable energy, 193 g/kg crude protein, containing 90% dry matter), and 20% of the energy requirements were obtained from the pellet mixture (8.0 MJ/kg metabolizable energy, 110 g/kg of crude protein, containing 85% dry matter). All ewes received 100% of nutritional requirements to provide sufficient energy for the maintenance of a nonpregnant ewe as defined by the Agricultural and Food Research Council in 1993.

At the end of an acclimatization period, ewes were randomly assigned to one of three feeding regimes: control (n = 12); the control ewes received 100% of the nutritional requirements from ∼60 days prior to mating until 6 days after mating), periconceptional undernutrition (PCUN; n = 13; the PCUN ewes received 70% of the control allowance from ∼60 days prior to mating until 6 days after mating), and periconceptional undernutrition and preimplantation undernutrition (PIUN; n = 9; the PIUN ewes received 70% of the control diet from mating until 6 days after mating, and all of the dietary components were reduced by an equal amount in the restricted diet), and preimplantation undernutrition (PIUN; n = 9; the PIUN ewes received 70% of the control diet from mating until 6 days after mating, and all of the dietary components were reduced by an equal amount in the restricted diet). From 7 days after conception, all ewes were fed 100% of requirements.

Animals and Surgery

Ewes were released in a group every evening with rams of proven fertility that were fitted with harnesses and marker crayons. Ewes were individually housed the following morning, and the occurrence of mating was confirmed by the presence of a crayon mark on the ewe’s rump. The first day of mating was defined as day 0. Ewes were weighed weekly after the feeding regime was commenced until postmortem at 136–138 days of gestation. Pregnancy and fetal number were estimated by ultrasound between 40 and 80 days of gestation. Surgery was performed in the pregnant ewes under general anesthesia between 105 and 110 days of gestation, as described previously (12). At surgery, catheters were implanted in a fetal carotid artery, jugular vein, and amniotic cavity. Vascular catheters were filled with heparinized saline, exteriorized through an incision in the ewes’ flank, and flushed with heparinized saline on alternate days. All ewes and fetal sheep received a 2-ml intramuscular injection of antibiotics (250 mg/ml procaine penicillin, 250 mg/ml dihydrostreptomycin, and 20 mg/ml procaine hydrochloride, Penstrept IIIium; Troy Laboratories) at

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of the assay was 0.03 ng/ml. Plasma insulin concentrations in all samples were measured in one assay, and the intra-assay coefficients of variance were <10%.

Quantification of mRNA Expression

RNA was extracted from ~100 mg of PAT using Trizol reagent (Invitrogen, Groningen, The Netherlands) from singleton fetuses and from each of the twin fetuses in each pregnancy where tissue was available (Table 1). RNA was purified using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). The quality and concentration of the RNA was determined by measuring absorbance at 260 and 280 nm and RNA integrity confirmed by agarose gel electrophoresis. cDNA was synthesized using the purified RNA and Superscript 3 reverse transcriptase (Invitrogen) with random hexamers.

The relative expression of mRNA transcripts of UCP1, IRA, IRB, IRS-1, P3K (p85), P3K (p110β), PKCζ, GLUT1, GLUT4, AMPKα1, AMPKα2, PGC-1α, PDK2, PDK4, mTOR, PPARγ, Ileptin, adiponecin, and the housekeeper gene acidic ribosomal protein large subunit-P0 (RPPO) was measured by quantitative real-time reverse transcription-PCR (qRT-PCR), using the SYBR Green system in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA).

Primer sequences were validated for use in the sheep in this (Table 2) or in prior studies (12, 28, 29, 35). Each amplicon was sequenced to ensure the authenticity of the DNA product, and a dissociation melt curve analysis was performed after each run to demonstrate amplicon homogeneity. Each qRT-PCR reaction well contained 50 ng of cDNA (50 ng/µl). Controls for each sample containing no cDNA were also used to confirm absence of DNA contamination. The cycling conditions consisted of 40 cycles of 95°C for 15 min and 60°C for 1 min.

Table 2. Primer sequences for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward (5′)</th>
<th>Sequence</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS-1</td>
<td>TATGGCAAGCTTTGGAGAAACGGG 3′</td>
<td>XM_581382.3</td>
<td></td>
</tr>
<tr>
<td>IRS-2</td>
<td>ACAAGGATTGCGACATCCTGCTT 3′</td>
<td>NM_003749</td>
<td></td>
</tr>
<tr>
<td>P3K (p85)</td>
<td>GTGCTGAAGCTTGCCATGAGG 3′</td>
<td>M61745.1</td>
<td></td>
</tr>
<tr>
<td>P3K (p110β)</td>
<td>TCCGTTCTGGTGGCAGGATCC 3′</td>
<td>NM_001206047.1</td>
<td></td>
</tr>
<tr>
<td>PKCζ</td>
<td>ATCAGACGCGAGCTTTGGAGATGC 3′</td>
<td>NM_00159481.1</td>
<td></td>
</tr>
<tr>
<td>PDK2</td>
<td>TGGAAAGGCTAGGGAAGATGCATA 3′</td>
<td>NM_001077833.1</td>
<td></td>
</tr>
<tr>
<td>PDK4</td>
<td>GCACGGAAACGCTGTGATGAAAT 3′</td>
<td>NM_001101883.1</td>
<td></td>
</tr>
<tr>
<td>mTOR</td>
<td>AGATCTACGCTTTCTGATCTGGCA 3′</td>
<td>FJ617140.1</td>
<td></td>
</tr>
<tr>
<td>UCP1</td>
<td>TCAAGGCTACAGATGCAAGACAGA 3′</td>
<td>AJ689125.1</td>
<td></td>
</tr>
</tbody>
</table>

IRS-1 and -2, insulin receptor substrate-1 and -2, respectively; P3K, phosphatidylinositol 3-kinase; PDK, pyruvate dehydrogenase kinase; mTOR, mammalian target of rapamycin; UCP1, uncoupling protein 1.
fetuses in late gestation

lipogenesis in perirenal adipose tissue in singleton and twin

expression of factors regulating adipogenesis and

Impact of PCUN and PIUN on the mRNA

difluoride hybridization transfer membrane (Perkin-Elmer, Waltham, MA) using a semidry blotter (Hoefer, Holliston, CA). The membranes were blocked with 5% BSA in Tris-buffered saline with 1% Tween-20 at room temperature for 1 h and then incubated overnight with primary antibody against IRB, PKCζ, GLUT1 and, CPT-1 (Santa Cruz Biotechnology, Santa Cruz, CA); Cav1, PTEN, Akt1, Akt2, pAkt (Ser473), PDK1, p-PDK1 (Ser241), pPKCζ (Thr410), AS160, p-AS160 (Thr642), total AMPK, p-AMPK (Thr172), PGC-1α, ACC, p-ACC (Ser79), mTOR, p-mTOR (Ser2448), p-mTOR (Ser2481), and PPARγ (Cell Signaling Technology, Danvers, MA); IRSp51 and p85 (Merck Millipore); p110β and PDK2 (Epitomics, Burlingame, CA); and GLUT4 and PDK4 (Abcam, Cambridge, UK). Membranes were washed, and bound antibody was detected using anti-rabbit or antimouse (Cell Signaling Technology) horseradish peroxidase-conjugated secondary IgG antibodies at room temperature for 1 h. Enhanced chemiluminescence reagents SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and ImageQuant LAS 4000 (GE Healthcare, Rydalmere, NSW, Australia) were used to detect the protein-antibody complexes. AlphaEaseFC (Alpha Innotech, Santa Clara, CA) was utilized to quantify specific bands of the target proteins.

**Statistical Analyses**

All data are presented as means ± SE. All data were analyzed using the Statistical Package for the Social Sciences Software (SPSS, Chicago, IL). Two-way analysis of variance (ANOVA) was used to determine the effects of maternal nutritional treatment (PCUN and PIUN) and fetal number (singleton or twin) on mRNA expression and protein abundance in fetal PAT. When there was an interaction between the effects of nutritional treatment and fetal number, data from singletons and twins were split and the effects of nutritional treatment determined using a one-way ANOVA. The Duncan post hoc test was used to determine the level of significant difference in mean values between nutritional treatment groups. A probability level of 5% (P < 0.05) was taken as significant.

**RESULTS**

**PAT Weight**

There was no effect of either PCUN, PIUN, or fetal number on total PAT mass (singleton: controls = 17.8 ± 2.5 g, PCUN = 18.5 ± 1.0 g, PIUN = 18.1 ± 2.0 g; twins: controls = 21.5 ± 1.7

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Table 3. Impact of PCUN and PIUN on the mRNA expression of factors regulating adipogenesis and lipogenesis in perirenal adipose tissue in singleton and twin fetuses in late gestation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>PCUN</th>
<th>PIUN</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>1.106 ± 0.047</td>
<td>1.164 ± 0.050</td>
<td>1.124 ± 0.057</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.080 ± 0.008</td>
<td>0.083 ± 0.006</td>
<td>0.071 ± 0.007</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>2.277 ± 0.138</td>
<td>2.339 ± 0.091</td>
<td>2.337 ± 0.111</td>
</tr>
<tr>
<td>LPL</td>
<td>1.462 ± 0.106</td>
<td>1.808 ± 0.103</td>
<td>1.499 ± 0.115</td>
</tr>
</tbody>
</table>

Data presented as means ± SE. RPPO, acidic ribosomal protein large subunit-P0; PPARγ, peroxisome proliferator-activated receptor-γ; LPL, lipoprotein lipase.

The abundance of each mRNA transcript was measured, and expression relative to RPPO was calculated using the comparative threshold cycle (CT) method (Q-gene qRT-PCR analysis software), which provides a quantitative measurement of the relative abundance of a specific transcript in different samples that takes into account any differences in the amplification efficiencies of the target and reference genes. The CT value was taken as the lowest statistically significant (>10 SD) increase in fluorescence above the background signal in an amplification reaction.

**Quantification of Protein Abundance**

Protein abundance was determined using Western blotting (32). Briefly, PAT samples (~200 mg) from singletons and one twin fetus from each pregnancy (Table 1) were homogenized in 800 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM Na3VO4, 30 mM NaF, 10 mM Na4P2O7, 10 mM EDTA, and 1 protease inhibitor tablet) and centrifuged at 12,000 g at 4°C for 15 min to remove insoluble material. Protein content of the clarified extracts was quantified using bicinchoninic acid protein assay. Prior to Western blot analysis, samples (10 μg protein) were subjected to SDS-PAGE and stained with Coomassie blue reagent (Thermo Fisher Scientific, Rockford, IL) to ensure equal loading of the proteins. Equal volumes and concentrations of protein were subjected to SDS-PAGE. The proteins were transferred onto a PolyScreen polyvinylidene difluoride hybridization transfer membrane (Perkin-Elmer, Waltham, MA) using a semidry blotter (Hoefer, Holliston, CA). The membranes were blocked with 5% BSA in Tris-buffered saline with 1% Tween-20 at room temperature for 1 h and then incubated overnight with primary antibody against IRB, PKCζ, GLUT1 and, CPT-1 (Santa Cruz Biotechnology, Santa Cruz, CA); Cav1, PTEN, Akt1, Akt2, pAkt (Ser473), PDK1, p-PDK1 (Ser241), pPKCζ (Thr410), AS160, p-AS160 (Thr642), total AMPK, p-AMPK (Thr172), PGC-1α, ACC, p-ACC (Ser79), mTOR, p-mTOR (Ser2448), p-mTOR (Ser2481), and PPARγ (Cell Signaling Technology, Danvers, MA); IRSp51 and p85 (Merck Millipore); p110β and PDK2 (Epitomics, Burlingame, CA); and GLUT4 and PDK4 (Abcam, Cambridge, UK). Membranes were washed, and bound antibody was detected using anti-rabbit or antimouse (Cell Signaling Technology) horseradish peroxidase-conjugated secondary IgG antibodies at room temperature for 1 h. Enhanced chemiluminescence reagents SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and ImageQuant LAS 4000 (GE Healthcare, Rydalmere, NSW, Australia) were used to detect the protein-antibody complexes. AlphaEaseFC (Alpha Innotech, Santa Clara, CA) was utilized to quantify specific bands of the target proteins.

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Fig. 1. Protein abundance of peroxisome proliferator-activated receptor-γ (PPARγ) and uncoupling protein 1 (UCP1) mRNA in singleton and twin fetuses in late gestation. A and B: protein abundance of PPARγ (A) and UCP1 mRNA expression (B) in the periconceptional (PCUN) and preimplantation undernutrition (PIUN) groups compared with controls in singletons and twins. C: immunoblots of PPARγ in the control, PCUN, and PIUN groups in singletons and twins. Different letters denote significant differences between treatment groups compared with controls in singletons and twins. PAT, perirenal adipose tissue; RPPO, acidic ribosomal protein large subunit-P0.

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Fig. 2. Fetal plasma insulin concentration and protein abundance of p110β, Akt1, and phosphorylated PKCζ (Thr410) in singleton and twin fetuses in late gestation. Fetal plasma insulin concentration in singletons and twins (A), protein abundance of p110β in singletons (B) and twins (C), abundance of Akt1 in singletons and twins (D), and abundance of phosphorylated PKCζ (Thr410) in singletons and twins (E) in the PCUN and PIUN groups compared with controls. Immunoblots of p110β (F), Akt1 (G), and phosphorylated PKCζ (Thr410; H) in the control, PCUN, and PIUN groups in singletons and twins. Different letters denote significant differences between treatment groups compared with controls in singletons and twins. AU, arbitrary units.
Impact of PCUN and PIUN on Expression and/or Abundance of Adipogenic and Lipogenic Factors, Adipokines, and UCP1 in Fetal PAT

There was no effect of either PCUN or PIUN on PPARγ, leptin, adiponectin, or LPL mRNA expression in PAT in either singleton or twin fetuses (Table 3). There was, however, a lower abundance of PPARγ (P < 0.05) in PAT in the PIUN group compared with controls in both singletons and twins (Fig. 1).

UCP1 mRNA expression was also lower (P < 0.05) in fetal PAT in both PCUN and PIUN groups compared with controls in singletons and twins (Fig. 1).

Impact of PCUN and PIUN on Circulating Insulin Concentrations and the Expression and Abundance of Insulin-Signaling Molecules in Fetal PAT

Fetal plasma insulin concentrations were higher in the PIUN but not the PCUN group compared with controls in both singletons and twins (Fig. 2).

There was no effect of PCUN or PIUN on IRα, IRβ, or GLUT4 mRNA expression in the fetal PAT in either singletons or twins (Table 4). The mRNA expression of GLUT1 in fetal PAT was lower (P < 0.05) in the PIUN group compared with the PCUN but not in control fetuses in singleton and twin pregnancies (Table 4). There was also no difference in the protein abundance of IRβ, Cav-1, IRS-1, IRS-2, PI3K (p85), PTEN, PDK1, phosphorylated PDK1 (Ser410), PKCζ, Akt2, phosphorylated Akt (Ser473), phosphorylated mTOR (Ser2448), AS160, phosphorylated AS160 (Thr642), GLUT4, or GLUT1 in fetal PAT in either the PCUN or PIUN groups compared with controls in either singletons or twins (Table 4).

Singletons. The abundance of PI3K (p110β) in fetal PAT was lower (P < 0.01) in the PCUN and PIUN groups; the abundance of Akt1 was lower (P < 0.05) and the abundance of phosphorylated PKCζ (Thr410) higher (P < 0.01) in fetal PAT in the PIUN group compared with controls (Fig. 2). The abundance of mTOR in fetal PAT was lower in the PCUN group; however, the abundance of phosphorylated mTOR (Ser2481) was only lower (P < 0.05) in the PIUN group compared with controls (Fig. 3).

Twins. There was no difference in the abundance of PI3K (p110β) in fetal PAT in the PCUN or PIUN group compared with controls; however, the abundance of PI3K (p110β) was lower (P < 0.01) in control twins compared with control singletons (Fig. 2). As in singleton fetuses, the abundance of Akt1 was lower (P < 0.05) in PAT in PIUN twins, and the abundance of phosphorylated PKCζ (Thr410) was also higher in PIUN twins compared with controls (Fig. 2). The abundance of mTOR in fetal PAT was higher (P < 0.05) in the PCUN group, but the abundance of phosphorylated mTOR (Ser2481) was lower (P < 0.05) in the PIUN twins compared with controls (Fig. 3).

Impact of PCUN and PIUN on the mRNA expression and protein abundance of factors regulating insulin signaling and fatty acid β-oxidation in perirenal adipose tissue in singleton and twin fetuses in late gestation

Table 4. Impact of PCUN and PIUN on the mRNA expression and protein abundance of factors regulating insulin signaling and fatty acid β-oxidation in perirenal adipose tissue in singleton and twin fetuses in late gestation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>PCUN</th>
<th>PIUN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target gene mRNA expression relative to RPPO mRNA expression</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IRA</td>
<td>0.066±0.004</td>
<td>0.071±0.003</td>
<td>0.067±0.004</td>
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<td>IRB</td>
<td>0.008±0.001</td>
<td>0.009±0.001</td>
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<tr>
<td>GLUT1</td>
<td>0.0027±0.002</td>
<td>0.0029±0.001</td>
<td>0.0023±0.001*</td>
</tr>
<tr>
<td>GLUT4</td>
<td>0.018±0.008</td>
<td>0.018±0.009</td>
<td>0.018±0.011</td>
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<td>AMPKα2</td>
<td>0.144±0.011</td>
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<td>PGC-1α</td>
<td>0.145±0.010</td>
<td>0.138±0.008</td>
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<td>Protein abundance (AU × 10^4)</td>
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<tr>
<td>Protein</td>
<td>Control</td>
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<td>PIUN</td>
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<tr>
<td>IRβ</td>
<td>613±69</td>
<td>748±53</td>
<td>705±32</td>
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<tr>
<td>Cav-1</td>
<td>86±7</td>
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<td>IRS-1</td>
<td>17±1</td>
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<td>13±3</td>
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<td>593±41</td>
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<td>53±3</td>
<td>62±3</td>
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<td>PDKP-1</td>
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<td>Akt2</td>
<td>407±32</td>
<td>455±40</td>
<td>376±31</td>
</tr>
<tr>
<td>p-Akt (Ser473)</td>
<td>489±131</td>
<td>365±88</td>
<td>411±91</td>
</tr>
<tr>
<td>p-mTOR (Ser2448)</td>
<td>21±3</td>
<td>16±2</td>
<td>15±3</td>
</tr>
<tr>
<td>AS160</td>
<td>30±5</td>
<td>39±8</td>
<td>28±4</td>
</tr>
<tr>
<td>pAS160 (Thr642)</td>
<td>32±7</td>
<td>22±5</td>
<td>28±5</td>
</tr>
<tr>
<td>GLUT1</td>
<td>6±1</td>
<td>7±1</td>
<td>8±1</td>
</tr>
<tr>
<td>GLUT4</td>
<td>1,502±147</td>
<td>1,294±158</td>
<td>1,147±185</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>8±1</td>
<td>6±1</td>
<td>8±1</td>
</tr>
<tr>
<td>ACC</td>
<td>47±9</td>
<td>53±9</td>
<td>57±5</td>
</tr>
<tr>
<td>p-ACC (Ser79)</td>
<td>37±4</td>
<td>38±5</td>
<td>44±6</td>
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</table>

Data are presented as means ± SE. IR, insulin receptor; Cav-1, caveolin-1; PTEN, phosphatase and tensin homolog; PDKP1, phosphoinositide-dependent kinase 1; AS160, Akt substrate 160 KDa; PGC-1α, PPARγ coactivator-1α; ACC, acetyl-CoA carboxylase. *Significant difference between treatment groups, P < 0.05.

Impact of PCUN and PIUN on Expression and Abundance of Factors Regulating Cellular Energy Homeostasis and Fatty Acid β-Oxidation in Fetal PAT

AMPKα1 mRNA expression was higher (P < 0.05) in fetal PAT in the PCUN and PIUN groups in both singletons and twins (Fig. 4). However, there was no effect of nutritional treatment or fetal number on either PAT AMPKα2 or PGC-1α mRNA expression (Table 4). There was also no difference in the abundance of PGC-1α, ACC, or phosphorylated ACC (Ser79) in fetal PAT between the nutritional treatment groups or between singletons and twins (Table 4).

Singletons. The abundance of total AMPKα was higher (P < 0.05) in the PIUN group, whereas the abundance of phosphorylated AMPKα (Thr172) was higher (P < 0.01) in the PCUN group compared with controls (Fig. 4). There was no difference in the protein abundance of PDK2 in PAT in the PCUN or PIUN group; however, the abundance of PDK4 was higher (P < 0.01) in the PIUN group compared with controls (Fig. 5).

Twins. In contrast to singletons, the abundance of total AMPKα was lower (P < 0.05) in the PIUN group, although the abundance of phosphorylated AMPKα (Thr172) was higher (P < 0.01) in the PCUN group compared with controls (Fig. 4). The abundance of PDK2 (P < 0.01) and PDK4 (P < 0.01) were each higher in the PIUN group compared with controls (Fig. 5).
DISCUSSION

In this study, we have shown for the first time that exposure to maternal undernutrition during the preimplantation period results in higher circulating insulin concentrations in the late-gestation fetus and that maternal undernutrition during either the periconceptional or the preimplantation period results in changes in the expression and abundance of key factors regulating thermogenesis, insulin signaling, and fatty acid β-oxidation in the major visceral fat depot present in late gestation.

Preimplantation Undernutrition and UCP1 Expression in Fetal Perirenal Fat

UCP1 expression was lower in fetal PAT in both singletons and twins after exposure to maternal undernutrition in the periconceptional and preimplantation periods. This suggests that the first week after conception is a sensitive period during which exposure to maternal undernutrition may program a decreased thermogenic capacity in brown fat in late gestation. Interestingly, it has been shown recently in the mouse that exposure to a low-protein diet for the first 3.5 days after conception also results in a decreased UCP1 expression in interscapular brown fat in male offspring at 1 yr of age (53). The mechanisms by which exposure of the embryo may result in a change in the thermogenic capacity of brown fat in the perinatal period that persists into adult life are unclear. UCP1 expression in brown fat is also regulated by β-adrenergic activation (46), but we found no evidence that downstream factors in the β-adrenergic signaling pathway, including either phosphorylated ACC or PGC-1α, were downregulated in fetal PAT in the PIUN group. Indeed, the abundance of total phosphorylated AMPK in the PCUN group and PDK4 abun-

Fig. 3. Protein abundance of mammalian target of rapamycin (mTOR) and phosphorylated mTOR (Ser2481) in singleton and twin fetuses in late gestation. Protein abundance of mTOR in singletons (A) and twins (B) and abundance of phosphorylated mTOR (Ser2481) in singletons and twins (C) in the PCUN and PIUN groups compared with controls. Immunoblots of mTOR (D) and phosphorylated mTOR (Ser2481; E) in the control, PCUN, and PIUN groups in singletons and twins. Different letters denote significant differences between treatment groups compared with controls in singletons and twins.
dance in the PIUN group was upregulated rather than downregulated (40, 49, 52). One possibility is that exposure to poor maternal nutrition in early embryonic life results in an epigenetic downregulation of UCP1 within the progenitors of brown adipocytes as an adaptation to decreased energy utilization within brown adipocytes in later life. These findings in fetal BAT may have significance in both perinatal and later life, as it has been demonstrated that brown adipocytes are present in adult life (2, 50) and that BAT thermogenic activity is reduced in obese individuals (51).

**Insulin, Insulin Signaling, and Perirenal Fat**

In the present study, we demonstrated that PIUN, but not PCUN, resulted in higher plasma insulin concentrations in singletons and twins. This suggests that there is a differential impact of PCUN and PIUN on either insulin secretion or the sensitivity of fetal tissues to insulin. It has been shown in the sheep that plasma insulin concentrations at birth are correlated directly with the size of adipocytes in the perirenal fat depot in male and female lambs and with perirenal fat mass in male lambs at 3 wk of age (10).

In the present study, whereas plasma insulin concentrations were higher in the PIUN group, there was no difference in the protein abundance of total AMPK (Thr172), total phosphorylated AMPK (Thr172) in singletons and twins (C), and mRNA expression of AMPKα1 in singletons and twins (D) in the PCUN and PIUN groups compared with controls. Immunoblots of total AMPK (E) and phosphorylated AMPK (Thr172; F) in the control, PCUN, and PIUN groups in singletons and twins. Different letters denote significant differences between treatment groups compared with controls in singletons and twins.

**Fig. 4.** Protein abundance of total AMP-activated protein kinase (AMPK), total phosphorylated AMPK (Thr172), and AMPKα1 mRNA expression in singleton and twin fetuses in late gestation. Protein abundance of total AMPK in singletons (A) and twins (B), protein abundance of phosphorylated AMPK (Thr172) in singletons and twins (C), and mRNA expression of AMPKα1 in singletons and twins (D) in the PCUN and PIUN groups compared with controls. Immunoblots of total AMPK (E) and phosphorylated AMPK (Thr172; F) in the control, PCUN, and PIUN groups in singletons and twins. Different letters denote significant differences between treatment groups compared with controls in singletons and twins.

![Graphs showing protein abundance of total AMPK and phosphorylated AMPK](image-url)
the PI3K (p110β) results in mild glucose intolerance and hyperinsulinemia in adult life (4). Interestingly, in the present study, the abundance of the PI3K (P110β) was also lower in PAT in control twins compared with control singletons, and therefore, this may explain why there was no further reduction after exposure to maternal undernutrition. One possibility is that exposure to either low maternal nutrition in early embryonic life or to the hormonal environment of a twin pregnancy results in a downregulation in the abundance of the PI3K (p110β) catalytic subunit in visceral fat, perhaps because the early environment in each context signals the expectation that there will be less fuel to store as fat in postnatal life. The data from the present study highlight that the programming of a decrease in the abundance of PI3K (p110β) in visceral fat after exposure to poor nutrition in early life can occur as early as the periconceptional period.

We also found that the abundance of Akt1 and phosphorylated mTOR (Ser2481) was lower and the abundance of phosphorylated PKCζ (Thr410) higher in fetal fat in singletons and twins in the PIUN group. Although Akt has been implicated in mediating insulin action in glucose uptake and thus glucose homeostasis (4, 19), Akt1 is essential for cell growth, rather than for the maintenance of insulin sensitivity (6), in contrast to the actions of Akt2 (3, 7). Additionally, insulin-stimulated mTOR phosphorylation at Ser2481 regulates cell growth through the role of the mTOR complex 1 (1). The findings of the current study may be consistent with an impact of undernutrition in early embryonic life acting to limit the actions of high circulating insulin concentrations on adipocyte growth in the PIUN group.

Although there was no change in PPARγ mRNA expression, there was a decrease in PPARγ abundance in fetal PAT in singletons and twins. It would be interesting to determine whether the low PPARγ abundance contributes to a decreased insulin sensitivity in the postnatal visceral fat depot after the transition from the fetal environment, where the circulating free fatty acid concentrations are low, to the postnatal environment, where there are higher free fatty acid concentrations. The
lack of change in the expression of leptin and adiponectin in the PCUN and PIUN groups is consistent with our earlier findings in PCUN fetuses (11) and suggests that leptin expression in fetal fat is not programmed specifically by exposure to poor maternal nutrition around the time of conception.

AMPK has been shown to play an important role in stimulating glucose uptake and fatty acid $\beta$-oxidation in liver and skeletal muscle (22, 42). In contrast, activation of AMPK in rat adipocytes resulted in a decrease in basal and insulin-stimulated glucose uptake, lipid synthesis, and fatty acid $\beta$-oxidation (15). Therefore, an increase in phosphorylation of AMPK (Thr$^{172}$) in the singletons and twins in the PCUN groups may further compound the effects of the lower PI3K (p110$\alpha$/H9251) abundance in PAT present in singleton fetuses after PCUN.

Disruption of AMPK$\alpha_1$ activation also inhibits glucose uptake via AS160 (16). In this study, there was higher AMPK$\alpha_1$ expression in singletons and twins exposed to PCUN and PIUN in the absence of changes in either AS160 or phosphorylated AS160 (Thr$^{422}$) abundance. The increase in AMPK$\alpha_1$ expression occurs in parallel with the increase in the abundance of phosphorylated PKC$\xi$ (Thr$^{140}$) in the PIUN group, which suggests that the rate of glucose uptake into fetal adipocytes may be maintained in late gestation.

Lipid accumulation in adipocytes is also regulated by the rate of fatty acid $\beta$-oxidation. In this study, we found that the abundance of PDK2 was higher in fetal PAT in PIUN twins, and the abundance of PDK4 was also higher in fetal PAT in PIUN singletons and twins. PDK2 and PDK4 maintain the balance between glucose and fatty acid $\beta$-oxidation through their ability to inhibit PDC and thus inhibit glucose oxidation and promote fatty acid $\beta$-oxidation (43). It has been proposed that elevated PDK2 and PDK4 are a result of the failure of insulin to inhibit their expression (25). The cause and consequences of the higher abundance of PDK2 and PDK4 in fetal PAT require further investigation.

In summary, the findings of the present study highlight that exposure of the early embryo to maternal undernutrition programs changes that may limit the thermogenic capacity as well as the insulin sensitivity of visceral fat in postnatal life. Our findings suggest that exposure to undernutrition during the first week of embryonic life in the sheep may recruit epigenetic mechanisms that have specific consequences for the insulin sensitivity of the visceral fat depot in postnatal life. The thermogenic capacity of BAT and insulin sensitivity of visceral fat are each important determinants of the risk of developing obesity and an insulin resistance phenotype, and the current study highlights the importance of maintaining optimal maternal nutrition during the periconceptional period for the later programming of metabolic health in the offspring.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

EARLY NUTRITION PROGRAMS FAT DEVELOPMENT


