Up-Regulation of Toll-Like Receptor 4/Nuclear Factor-κB Signaling Is Associated with Enhanced Adipogenesis and Insulin Resistance in Fetal Skeletal Muscle of Obese Sheep at Late Gestation

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Maternal obesity is increasing at an alarming rate. We previously showed that maternal obesity induces an inflammatory response and enhances adipogenesis in fetal skeletal muscle at midgestation. The objective of this study was to evaluate effects of maternal obesity on adipogenesis, inflammatory signaling, and insulin pathways at late gestation when ovine fetal skeletal muscle matures. Nonpregnant ewes were assigned to a control diet (Con, fed 100% of National Research Council nutrient recommendations, n = 6) or obesogenic diet (OB, fed 150% of National Research Council recommendations, n = 6) from 60 d before to 135 d after conception (term 148 d) when the fetal semitendenosus skeletal muscle was sampled. Expression of the adipogenic marker, peroxisome proliferator-activated receptor-γ, was increased in OB compared with Con fetal semitendenosus muscle, indicating up-regulation of adipogenesis. More intramuscular adipocytes were observed in OB muscle. Phosphorylation of inhibitor-κB kinase-α and nuclear factor-κB RelA/p65 were both increased in OB fetal muscle, indicating activation of nuclear factor-κB pathway. Phosphorylation of c-Jun N-terminal kinase and c-Jun (at Ser 63 and Ser 73) was also elevated. Toll-like receptor 4 expression was higher in OB than Con fetal muscle. Moreover, despite higher insulin concentrations in OB vs. Con fetal plasma (2.89 ± 0.53 vs. 1.06 ± 0.52 ng/ml; P < 0.05), phosphorylation of protein kinase B at Ser 473 was reduced, indicating insulin resistance. In conclusion, our data show maternal obesity-induced inflammatory signaling in late gestation fetal muscle, which correlates with increased adipogenesis and insulin resistance, which may predispose offspring to later-life obesity and diabetes. (Endocrinology 151: 380–387, 2010)
Obesity induces chronic low-grade inflammation, which can further induce insulin resistance, linking obesity, insulin resistance, and diabetes (10). Toll-like receptors (TLRs), which recognize microbial antigens, play an important role in inducing inflammation (11). Nuclear factor-κB (NF-κB) pathway, which can be activated by TLR4, mediates the inflammatory response (12). NF-κB is an eukaryotic transcription factor, which binds to a specific DNA sequence (GGG ACT TTC C) (13). In its inactive state, NF-κB binds to inhibitors of NF-κB (IκB). When signals such as TLR ligands activate IκB kinases (IKKs), IKKα and IKKβ, they phosphorylate IκB, resulting in degradation of these inhibitors. This process releases NF-κB from IκB and allows the translocation of NF-κB to the nucleus in which it activates transcription of specific genes (14). IKKβ and NF-κB are both involved in the pathogenesis of insulin resistance and type 2 diabetes (12, 15).

The hormone insulin exerts its multiple functions through activation of downstream signaling pathways (16), including phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT) pathway and Ras-MAPK pathway (17). Accumulation of lipids in skeletal muscle increases intracellular fatty acyl-CoA, which activates the protein kinase C (PKC) pathway, phosphorylating insulin receptor substrate (IRS)-1, and resulting insulin resistance (18).

Sheep pregnancy has been extensively studied to evaluate systems active in precocial species in contrast to those in altricial species such as rodents, thereby providing power for translation to human pregnancy (19, 20). We used the pregnant sheep model to investigate the impact of maternal obesity on adipogenesis and inflammatory and insulin signaling pathways in fetal sheep skeletal muscle at late gestation. Our data showed that maternal obesity upregulates adipogenesis, TLR expression, and the IKK/NF-κB, and c-Jun N-terminal kinase (JNK) pathways, whereas insulin-related pathways involving Akt and AMP-activated protein kinase (AMPK) were down-regulated in fetal muscle of obese mothers.

Materials and Methods

Care and use of animals

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. Twelve multiparous Rambouillet/Columbia ewes were studied. Ewes were all mated with a single ram. Beginning 60 d before conception and continuing to d 135 of gestation (first day of mating = d 0), ewes were individually fed either a highly palatable diet at 100% [control diet (Con)] of National Research Council recommendations for energy (21) (n = 6), or 150% [obesogenic diet (OB)] of recommended energy requirements for early gestation (n = 6) as previously reported (3). Ewes were housed in individual pens within a temperature controlled room (~20 C). All ewes were weighed at weekly intervals and rations were adjusted for weekly changes in metabolic body weight (0.75) (23). Body condition was scored at monthly intervals to evaluate changes in fatness. A body condition score of 1 (emaciated) to 9 (obese) was assigned by two trained observers after palpation of the transverse and vertical processes of the lumbar vertebrae (L2-L5) and the region around the tail head (24).

Immediately before slaughter, on d 135 of gestation, ewes were weighed, sedated with iv ketamine (10 mg/kg), and anesthesia induced and maintained by isoflurane inhalation (1–2%). Fetal blood was obtained from the umbilical vein via a 20-gauge 1.5-in. needle and 3-ml syringe. Serum and plasma were collected and stored for other studies. After fetal blood collection, ewes were exsanguinated via heart puncture while under general anesthesia and fetuses quickly removed. No difference in body weight was observed between twins, so only one fetus was used for analyses. Four ewes with twin fetuses and two ewes with singletons were analyzed for each treatment. Each group had three male and three female fetuses. Fetal semitendinosus (St) muscle samples from the left side were analyzed. Surface tissues were trimmed and a piece of muscle (about 1 g) was sampled at the anatomical center of the muscle and snap frozen in liquid nitrogen for biological analyses.

Antibodies

Antibodies against AMPK, phospho-AMPK at Thr 172, acetyl-CoA carboxylase (ACC), phospho-ACC at Ser 79, Akt, phospho-Akt at Ser 473, IKKα, IKKβ, phospho-IKKα/β, JNK, phospho-JNK, c-Jun, phospho-cJun at 63/73, phospho-PKC, and β-tubulin were purchased from Cell Signaling (Danvers, MA). Peroxisomal proliferator-activated receptor (PPAR)-γ antibodies were purchased from 6 Bioslabs (Gilroy, CA). TLR4 antibody was from Santa Cruz biotechnology (Santa Cruz, CA). IRDye 800CW goat antirabbit secondary antibody and IRDye 680 goat antimouse secondary antibody were purchased from LI-COR Biosciences (Lincoln, NE).

Glucose and insulin analyses

Glucose and insulin in fetal circulation were analyzed as previously described (25). The intra- and interassay coefficient of variation (CV) for glucose were 5 and 7%, respectively, and sensitivity was 10 mg/dl. The intraassay CV was less than 10%, whereas the interassay CV was less than 5% and sensitivity was 0.05 ng/ml for insulin.

Histochemical analyses

Semitendinosus muscle samples were fixed in 4% (wt/vol) paraformaldehyde in phosphate buffer (0.12 m; pH 7.4), embedded in paraffin, and sectioned at 10 μm. Sections were rehydrated by a series of incubations in xylene and ethanol solutions and then used for Masson trichrome staining (26). With this staining, muscle was stained red, nuclei black, and collagen blue. Ten fields were randomly selected for quantification of muscle fiber and adipocyte diameters. The majority of muscle fibers, and adipocytes were circular, and thus, diameter was easily measured. For irregular muscle fibers and adipocytes, a maximum and minimum distance of the two opposite sides of the muscle fiber circle and adipocytes were measured and the average value

(7); myogenesis and adipogenesis should be considered to be competitive processes. For example, inhibition of adipogenesis promotes myogenesis (8, 9).
regarded as the fiber or adipocyte diameter. The diameter of 50 muscle fibers and all adipocytes per field were measured and a total of more than 500 muscle fibers for each muscle sample were quantified using the Image J software (version 1.30; National Institutes of Health, Bethesda, MD). Muscle fiber and adipocyte diameter were measured in a blind fashion. Averaged data were used for calculations (3).

Real-time quantitative PCR
Total mRNA was extracted from the fetal semitendinosus muscle using TRI reagent (Sigma, St. Louis, MO) and reverse transcribed into cDNA by using a kit (QIAGEN, Valencia, CA). Reverse-transcribed cDNAs were used for real-time PCR analyses by using SYBR Green RT-PCR kit from Bio-Rad (Hercules, CA). Primers of adipogenesis markers used were: PPARγ forward, 5'-CCGCAACTCCAGGGGTGTC-3', and reverse, 5'-CAAGGGGACACCATGCTGAAT-3'. Inflammation-related gene primers used were: cluster of differentiation 14 (CD14) forward, 5'-CTAGCTGCTTGATCTCGAG-3', and reverse, 5'-AAGGATTCTGCAAGAGCGAAGT-3'. TLR2 forward, 5'-AAAGGGACCCAGGAGACG-3', and reverse, 5'-TGGAACCAGAGGCTCA-3'; TLR4 forward, 5'-TCGCTGGCTGAAACTATAT-3', and reverse, 5'-CCTGTAAGTGAAGGACAGCAC-3'; tubulin forward, 5'-CGAGAGCTGTGACTGTCTG-3', and reverse, 5'-GGCATGACGCTAAAGCC-3'. Each reaction yielded amplicons between 80 and 200 bp. PCR conditions were as follows: 20 sec at 95 °C, 20 sec at 56 °C, and 20 sec at 72 °C for 35 cycles. After amplification, a melting curve (0.01 °C/sec) was used to confirm product purity. Results are expressed relative to tubulin.

Immunoblotting analysis
Immunoblotting analyses were conducted according to the procedures previously described (3, 27). Membranes were visualized by Odyssey infrared imaging system (LI-COR Biosciences). Density of bands was quantified and then normalized by Odyssey infrared imaging system (LI-COR Biosciences). Results are expressed relative to tubulin.

Statistical analysis
Statistical analyses were conducted according to our previous studies in sheep (3, 6, 27). Briefly, each animal was considered as an experimental unit. Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, 2000; SAS, Cary, NC). Differences in mean values were compared by Tukey’s multiple comparison test, and mean ± SEM are reported. Statistical significance was considered as P < 0.05.

Results

Animal weight
Before dietary treatment, there was no difference in body weight and body condition score among the ewes; at the end of the treatment, both maternal body weight and body condition score were higher in OB than Con groups (P < 0.05), indicating the excessive nutrition resulted in obesity (Table 1). There were no significant differences in fetal body weight or St muscle weight between these two groups.

Histological appearance and adipogenesis in fetal muscle
There were more fat cells in fetal muscle of OB sheep than Con sheep (Fig. 1). Moreover, the total relative area of fat cells increased by 26.3 ± 3.4% (P < 0.05) in fetal muscle of OB mothers (Fig. 1, A, B, and E). In addition, obvious infiltration of adipocytes into muscle fiber bundles was observable (Fig. 1, C and D), and more fat cells were present in OB than Con fetal muscle (Fig. 1C and D). Additionally, fetal muscle of OB sheep contained more large fat cells than Con sheep, whereas fetal muscle of Con sheep contained more small fat cells (Fig. 1F). Along with that, the relative number of muscle cells in OB fetal muscle was decreased by 11.1 ± 3.7% (P < 0.05) (Fig. 1H).

Inflammation
TLR4 mRNA expression was 60.0 ± 11.64% higher (P < 0.05), and TLR4 protein increased by 2.9% in OB vs. Con fetal muscle (Fig. 2A), and PPARγ protein level was also higher (14.2 ± 2.9% increase, P < 0.05) in OB fetal muscle (Fig. 2B).

### TABLE 1. Maternal and fetal body weight and fetal St muscle weight

<table>
<thead>
<tr>
<th>Category</th>
<th>Con</th>
<th>OB</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>At the beginning of treatments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body condition score</td>
<td>5.5 ± 0.3</td>
<td>5.9 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>75.7 ± 7.3</td>
<td>65.1 ± 4.7</td>
<td>NS</td>
</tr>
<tr>
<td>At the end of treatments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body condition score</td>
<td>6.1 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>90.4 ± 7.7</td>
<td>107.6 ± 7.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fetal</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Body weight (g)</td>
<td>5180.3 ± 232.4</td>
<td>4977.1 ± 268.5</td>
<td>NS</td>
</tr>
<tr>
<td>St muscle (g)</td>
<td>8.7 ± 0.3</td>
<td>7.9 ± 0.5</td>
<td>NS</td>
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Means ± SEM (n = 6). NS, Not significant.
Expression of CD14 mRNA, a coreceptor of TLR4, was also increased (P < 0.05) as well as TLR2 mRNA expression (P < 0.05) (Fig. 3C). The content of TNF-α, a proinflammatory cytokine, was also increased (P = 0.07, Fig. 3D).

Figure 4A shows that phosphorylation of key components IKK and NF-κB p65 of the IKK/NF-κB signaling pathway was increased (23.1 ± 6.9 and 15.9 ± 4.4%, respectively, P < 0.05) in OB fetal muscle. Phosphorylation of JNK was increased (41.3 ± 11.2%, P < 0.05) in OB fetal muscle (Fig. 5A). Phosphorylation of c-Jun was increased both at serine 63 (37.2 ± 11.9% increase, P < 0.05) and 73 (38.3 ± 12.4% increase, P < 0.05) in OB fetal muscle (Fig. 5B).

**Insulin resistance-related pathways**

**PKC and insulin receptors**

A 27.1 ± 9.1% increase (P < 0.05) in phosphorylation of PKC was observed in OB fetal muscle (Fig. 6A), whereas the content of insulin receptor was lower (by 27.2 ± 12.0%, P < 0.05) in OB than Con fetuses (Fig. 6B). Because PKC phosphorylates IRS-1 at serine to inhibit the insulin pathway, we further attempted to measure the phosphorylation of IRS-1 at Ser 1101, but the bands were too weak.

**PI3K/Akt signaling pathway activity in fetal St muscle**

PI3K/Akt is the main downstream signaling pathway of insulin signaling. There was no difference in the total content of Akt between the control and OB group; however, the phosphorylation of Akt at Ser 473, which is the active form, was reduced by 27.4 ± 8.3% (P < 0.05) (Fig. 6C).

**AMPK and ACC phosphorylation**

Phosphorylation at Thr 172 of AMPK-α-subunit and at Ser 79 of its downstream target ACC is commonly used to assess AMPK activity. AMPK phosphorylation was decreased by 15.2 ± 3.7% (P < 0.05) (Fig. 6D). No difference in the total content of AMPK-α-subunits was observed. A decrease (23.5 ± 0.9%, P < 0.05) in the level of ACC phosphorylation was observed in the fetal muscle of OB sheep without any change in total ACC concentration (data not shown).

**Insulin and glucose concentration**

Insulin concentrations in OB vs. Con fetal plasma were 2.89 ± 0.53 vs. 1.06 ± 0.52 ng/ml (P < 0.05), and the
glucose concentrations were 29.49 ± 2.28 and 33.00 ± 2.18 mg/dl for Con and OB fetuses, respectively.

Discussion

The value of the pregnant sheep maternal obesity studies as a model of altered fetal development in a species that delivers precocial young

Obesity is currently a very serious problem in the United States as well as many areas around the world. It is known that obesity is associated with a high risk of several chronic diseases, such as hypertension, type 2 diabetes, dyslipidemia, and coronary heart diseases (28). Accompanying the increase of obesity in general, maternal obesity is becoming more and more prevalent. Due to the similarity between sheep and human pregnancy, the sheep has been widely used to investigate fetal development (3). Whereas studies on altricial rodent species are of great value, there is also a need to determine changes that occur prenatally in precocial species at a time when key cellular processes are occurring in a very different environment (e.g., $PO_2$, hormonal concentrations, and nutrients such as glucose) from those to which the same tissues are exposed postnatally.
Inflammation was observed in fetal skeletal muscle

Obesity is associated with low-grade inflammation (10) in the presence of elevated serum free fatty acid concentrations, and free fatty acids activate TLR4 (29, 30). In the TLR4/NF-κB pathway, the kinase complex IKK is phosphorylated and activated on the binding to TLR4, thereby activating NF-κB signaling, which induces expression of inflammatory cytokines such as TNFα (29). Activation of TLRs also activates JNKs (32). In the current study, we detected up-regulation of TLR2 and TLR4 expression in OB compared with Con fetal muscle. In addition, the NF-κB signaling pathway and JNK pathway were up-regulated, indicating an inflammatory response. In a recent study, enhanced expression of TLRs was linked to insulin resistance and diabetes (29). This finding prompted us to analyze insulin-related signaling pathways in fetal muscle.

Decreased insulin signaling in OB fetal skeletal muscle

Muscle is the primary site of glucose disposal and fatty acid oxidation (5). Intracellular lipids induce insulin resistance through activation of PKC, and previous studies showed a strong correlation between muscle insulin resistance and intramyocellular lipid pools (33, 34). In this study, we observed an increase in the phosphorylation of PKC in OB compared with Con fetal muscle, which should be partially responsible for the observed insulin resistance.

PKC induces serine phosphorylation of IRS-1, a well-studied substrate of insulin receptor, and reduces its ability to serve as a docking center for the recruitment and activation of downstream pathways. Thus, we analyzed the phosphorylation of IRS-1 at Ser 1101, a site phosphorylated by PKC; however, the bands were too weak. Therefore, we further measured the PI3K/Akt pathway, one of the main downstream pathways of IRS-1, which was decreased in OB fetal muscle, showing the attenuation of insulin signaling in OB fetal muscle. Of note, the protein content of insulin receptor was also decreased in OB group, which may also contribute to the down-regulation of insulin signaling. Combining these results with the increased insulin and glucose contents in OB fetal plasma, we concluded that there was insulin resistance in OB fetal muscle.

AMPK is a key regulator of energy metabolism. AMPK activation sensitizes insulin signaling (3, 35), whereas inhibition of AMPK promotes lipogenesis and adipogenesis, which results in intracellular lipid accumulation and insulin resistance (36, 37). We analyzed AMPK phosphorylation at Thr 172, which correlates with its activity (38). Our data showed a decrease of AMPK activity in OB fetal muscle, which could partially explain the insulin resistance in OB fetal muscle. Recently TNFα has been shown to reduce AMPK activity in skeletal muscle, linking inflammation to AMPK activation and insulin resistance (39). We detected a trend of increase in TNFα protein content, which might explain the observed inhibition of AMPK activity in OB fetal muscle.

Adipogenesis

Myocytes, adipocytes, and fibroblasts are all derived from the same pool of mesenchymal stem cells. Fetal muscle development is largely separated into two stages. Primary myofibers are first formed in the embryonic stage, followed by the formation of secondary myofibers in mid and late gestation in human and sheep (40). The formation
of secondary myofibers overlaps with adipogenesis, which is initiated at midgestation in precocial species such as humans and sheep, as well as fibrogenesis, which forms perimysium and epimysium in fetal skeletal muscle. Because the fetal stage is crucial for skeletal muscle development, enhanced adipogenesis in fetal muscle is expected to impair muscle functions in offspring (41). Such changes will impair metabolism of glucose and fatty acids in response to insulin stimulation and thus predispose offspring to diabetes and obesity in later life (42, 43). Consistent with our hypothesis, enhanced adipogenesis was observed in fetal muscle of OB sheep. In addition, the average size of adipocytes in OB fetal muscle was larger than that of Con fetal muscle. Large, lipid-laden adipocytes rather than small adipocytes are responsible for the abnormalities of adipose tissue including inflammation, oxidative stress, and insulin resistance (44).

Mechanisms leading to enhanced adipogenesis in OB fetal muscle are not clear but may be due to the inflammatory response and oxidative stress. High glucose induces adipogenic differentiation of muscle derived stem cells by enhancing oxidative stress (45). Muscle regeneration involves the proliferation and differentiation of muscle derived stem cells. Inflammation may alter the normal progression of downstream regenerative events in injured skeletal muscle and directs myogenic precursor cells in the regenerating milieu toward an adipogenic phenotype (46). Because muscle regeneration involves processes similar to fetal muscle development, it is plausible to suggest that inflammation promotes adipogenesis in fetal muscle.

In late gestation, sheep skeletal muscle matures, and changes in skeletal muscle structure and properties are considered to have long-lasting effects (47). Increase in numbers of im adipocytes induces insulin resistance in skeletal muscle through paracrine signaling mediated by adipokines (45). To our knowledge this is the first demonstration of the development of greater numbers of adipocytes exist in OB fetal skeletal muscle compared with Con muscle in late gestation, a potentially very significant alteration in muscle composition. These adipocytes are sites for im lipid accumulation, an event known to induce insulin resistance (3, 45). In addition, adipocytes do not contribute to generation of contractile force and have a much lower metabolic rate compared with muscle cells. As a result, replacing muscle fibers with adipocytes will tend to impair muscle functions such as force generation, oxidative capacity, and insulin sensitivity, all of which could predispose offspring to obesity and type 2 diabetes. Because skeletal muscle is the principal site for glucose and fatty acid use, our data showing the impairment of fetal skeletal muscle development provide a plausible explanation for the increase in maternal obesity and the surge in childhood obesity in recent years (48, 49).

In conclusion, our findings demonstrate that maternal obesity induces inflammation and insulin resistance in fetal muscle at late gestation. In addition, adipogenesis is enhanced in fetal muscle, which is associated with inflammation.

Acknowledgments

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