Maternal Obesity-Impaired Insulin Signaling in Sheep and Induced Lipid Accumulation and Fibrosis in Skeletal Muscle of Offspring

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ABSTRACT

The prevalence of maternal obesity is increasing rapidly in recent decades. We previously showed that maternal obesity affected skeletal muscle development during the fetal stage. The objective of this study was to evaluate the effects of maternal obesity on the skeletal muscle properties of offspring. Ewes were fed a control diet (100% energy requirement, Con) or an obesogenic diet (150% energy requirement, OB) from 2 mo before pregnancy to weaning. After weaning, the offspring lambs were fed a maintenance diet until 19 mo of age and then ad libitum for 12 wk to measure feed intake. At 22 mo old, the longissimus dorsi (LD) muscle was biopsied. The downstream insulin signaling was lower in OB than Con lambs as shown by reduction in the phosphorylation of protein kinase B, mammalian target of rapamycin, and 4E binding protein 1. On the other hand, the phosphorylation of protein kinase C and insulin receptor substrate 1 was higher in OB compared to Con lambs. More intramuscular adipocytes were observed in OB compared to Con offspring muscle, and the expression of peroxisome proliferator-activated receptor gamma, an adipocyte marker, was also higher, which was consistent with the higher intramuscular triglyceride content. Both fatty acid transport protein 1 and cluster of differentiation 36 (also known as fatty acid translocase) were increased in the OB group. In addition, higher collagen content was also detected in OB compared to Con offspring. In conclusion, our data show that offspring from obese mothers had impaired insulin signaling in muscle and increased intramuscular adipocytes and fibrosis and insulin resistance, supporting the fetal programming of adult metabolic diseases.

INTRODUCTION

Obesity is increasing at an alarming rate. According to a recent National Health and Nutrition Examination Survey (1999–2002), 29% of nonpregnant women between 20 and 39 years of age are obese. Meanwhile, obesity in children and teenagers is also increasing [1]. Maternal obesity predisposes offspring to obesity, hypertension, and insulin resistance [2–4]. However, the underlying mechanisms responsible for such a link remain missing.

Skeletal muscle constitutes about 40%–50% of body mass [5] and is the main peripheral tissue responsive to insulin-stimulated uptake of glucose and fatty acids [6]. Development of insulin resistance in skeletal muscle is an essential step in the development of type 2 diabetes [7–9]. The fetal period is crucial for skeletal muscle development because no net increase in the number of muscle fibers occurs after birth [10, 11]. In addition, its lower priority in nutrient partitioning during fetal development compared to organs such as the brain, heart, and liver renders skeletal muscle particularly vulnerable to nutrient alteration [12]. Furthermore, the late fetal stage is also very important for adipogenesis, which forms intramuscular adipocytes [13]. Increase of adipogenesis in fetal skeletal muscle will induce muscle insulin resistance as a result of the paracrine effect of intramuscular adipocytes [14–16]. Fibrogenesis, which forms endomysium and perimysium, is also actively ongoing during the fetal stage, and an increase in fibrogenesis leads to impaired muscle functions [17]. Therefore, any change in fetal skeletal muscle development has important negative physiological consequences for the offspring, including predisposing offspring to obesity and type 2 diabetes and muscle weakness [18–21].

During aging, a progressive loss of muscle mass occurs accompanied by increased adiposity and fibrosis [17, 22], resulting in a decline in muscle structural integrity and functional capacity [23]. Our previous studies demonstrated that maternal obesity induces changes in fetal skeletal muscle at an early developmental stage, including an increase in intramuscular adipocytes and fibrosis and insulin resistance [24, 25], that typically are not observed until later in life. Here, we report that the effect of maternal obesity on fetal skeletal muscle development is maintained in offspring skeletal muscle, which might provide a reason for the increased incidence of obesity and diabetes in offspring born to obese mothers.

MATERIALS AND METHODS

Care and Use of Animals

All the animal procedures were approved by the University of Wyoming Animal Care and Use Committee. Animal procedures have been previously described in detail [26]. Briefly, multiparous Rambouillet/Columbia ewes were mated to a single ram. From 60 days before conception to weaning (first day of mating = d0), ewes were individually fed either a highly palatable diet at 100% (Con) of National Research Council recommendations for energy [27] or 150% (OB) of recommended energy requirements [19]. Ewes were housed in...
individual pens within a temperature-controlled room (~20°C). All ewes were weighed at weekly intervals, and the rations were adjusted for weekly changes in metabolic body weight (BW0.75) [28, 29]. The 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 through L5) and the region around the tail head [30]. There was no difference in body weight (emaciated) to 9 (obese) was assigned by two trained observers after palpation of the transverse and vertical processes of the lumbar vertebrae (L2 through L5) and the region around the tail head [30]. There was no difference in body weight (emaciated) to 9 (obese) was assigned by two trained observers after palpation of the transverse and vertical processes of the lumbar vertebrae (L2 through L5) and the region around the tail head [30].

Collagen Measurement Using Chemical Method

Ground muscle powders (0.1 g) were dried in a convection oven at 60°C, and samples were weighed and then hydrolyzed in 6 N HCl at 105°C for 16 h. An aliquot was removed for hydroxyproline determination as previously described [33]. Collagen concentration (% dry muscle weight) was calculated assuming collagen weight = 7.25 × hydroxyproline measured weight [34].

Real-Time Quantitative PCR (RT-PCR)

Total mRNA was extracted from the LD muscle using TRI reagent (Sigma) and reverse transcribed into cDNA by using a kit (Qiagen). Reverse-transcribed cDNAs were used for real-time PCR analyses using a SYBR Green RT-PCR kit from Bio-Rad. The primers were used: PPARγ forward, 5′-CCGAGTCTCAGGGTGGTC-3′ and reverse, 5′-CAAAAGGCAGCCAGATCTGAAAT-3′; SLC27A1 forward, 5′-A5CTGTCCTGCCCTTACG-3′ and reverse, 5′-GGCCTGGCAAAACTTCCTG-3′; CD36 forward, 5′-GAAGGTAGTTGGACCAAG-3′ and reverse, 5′-GAAGGTAGTTGGACCAAG-3′; CD36 forward, 5′-AATGTGGTGGAGGAGGACAC-3′; CD14 forward, 5′-CTTCAGGCTCGTTGTCAC-3′ and reverse, 5′-AAGGATTGTCCGTCCAGAT-3′; TLR2 forward, 5′-CAAGAGGAAGCCCGGAAG-3′ and reverse, 5′-TGAGACCTAGGAGTTTCCTCA-3′; TLR4 forward, 5′-TGTCCTGCTCAAAATTGAG-3′ and reverse, 5′-CCCTGTAGTGAAGCAGAC-3′ and tubulin forward, 5′-CGAGAAGCTGAGTACTGTCG-3′ and reverse, 5′-GGCATGACCTGACTGATTT-3′. Each reaction yielded amplifications between 80 and 200 bp. PCR conditions were as follows: 10 sec at 95°C followed by 30 sec at 95°C for 40 cycles. After amplification, a melting curve (0.01°C/sec) was used to confirm product purity. In addition, electrophoresis was conducted to confirm that only one fragment with the right size was amplified. The results are expressed relative to tubulin [25].

Statistical Analysis

Statistical analyses were conducted according to the procedures previously described [12, 19]. Membranes were visualized by the Odyssey Infrared Imaging System (LI-COR Biosciences). The density of the bands of all the animals was quantified and then normalized to the tubulin content. Only representative bands are shown in the figures.

RESULTS

Insulin Resistance Was Detected in Skeletal Muscle of the Progeny of OB Sheep

The muscle content of insulin receptor protein decreased by 22.5% ± 7.1% (P < 0.05) in the progeny of OB lambs compared to Con lambs (Fig. 1A), and the IGF1 receptor tended to decrease (by 20.2% ± 8.8%; P < 0.10) in OB compared to Con muscle (Fig. 1B). Because the sequences surrounding the phosphorylation sites of insulin and IGF1 receptor are identical, the antibody recognizes the phosphorylated receptors of both insulin and IGF1. No difference in
Insulin and IGF1 receptor phosphorylation was observed between the two types of sheep (Fig. 1B).

AKT is the main downstream mediator of the insulin-signaling pathway. Despite a lack of difference in the phosphorylation of insulin and IGF1 receptors, there was a decrease in the phosphorylation of AKT at Ser-473 (21.0% ± 5.3%; *P < 0.05) as well as a lower ratio of phospho-AKT to total AKT (26.6% ± 6.0%; *P < 0.05) in the muscle of the progeny of OB compared to Con lambs (Fig. 2A). In addition, both MTOR and EIF4EBP1 are downstream effectors of AKT; the phosphorylation of both MTOR (by 19.9% ± 5.5%; *P < 0.05) and EIF4EBP1 (by 55.4% ± 10.7%, *P < 0.05) decreased in the muscle of the progeny of OB compared to Con lambs (Fig. 2, B and C). These data show that the downstream signaling of insulin/IGF1 was attenuated despite a lack of difference in receptor phosphorylation, indicating insulin/IGF1 resistance.

PRKCθ (PRKCQ) phosphorylates IRS1 at Ser-1101, which attenuates downstream insulin signaling, leading to insulin resistance. The phosphorylation of PRKCθ appeared to increase.

FIG. 1. Insulin receptor content and receptor phosphorylation in LD muscle of progeny of obese (OB) versus control group (Con). A) Western blot showed less insulin receptor in OB than in Con muscle. B) The total protein content of IGF1 displayed a decreasing trend in OB compared to Con muscle. No significant differences were observed in the phosphorylation of IGF1 receptor (IGF1R) and the ratio of phospho-IGF1R to total IGF1R. (*P < 0.05, #P < 0.10; mean ± SEM; n = 8 in Con and n = 9 in OB group).

FIG. 2. Down-regulation of downstream insulin signaling in LD muscle of progeny of obese (OB) versus control group (Con). A) Decreased phosphorylation in AKT at Ser-473 in OB compared to Con muscle. B) Phosphorylation of MTOR as well as the ratio of phospho-MTOR to total MTOR were decreased in OB compared to Con muscle. C) Phosphorylation of EIF4EBP1 was decreased in OB muscle without a change in the total protein content of EIF4EBP1. (*P < 0.05; mean ± SEM; n = 8 in Con and n = 9 in OB group).
FIG. 3. Protein kinase C (PRKC) and insulin substrate 1 (IRS1) phosphorylation in LD muscle of progeny of obese (OB) versus control group (Con). A) Phosphorylation of PRKC tended to increase in OB compared to Con muscle. B) Phosphorylation of IRS1 at Ser-1101 was increased in OB muscle. (*P < 0.05, #P < 0.10; mean ± SEM; n = 8 in Con and n = 9 in OB group).

FIG. 4. Masson trichrome staining showed more intramuscular adipocytes and collagen in LD muscle of progeny of obese (OB) versus control group (Con). Using Masson trichrome staining, muscle cells stained red, collagen stained blue, while adipocytes were colorless. A) LD muscle of Con sheep (top: 100× magnification; bottom: 400× magnification). B) LD muscle of OB sheep (top: 100× magnification; bottom: 400× magnification). C) Larger fat area in muscle tissue of OB sheep. D) Total triglycerides content in Con and OB LD muscle. The results showed a higher content of total triglycerides in OB muscle than in Con muscle, displayed in mg of total triglycerides per gram of muscle powder. E) Increased expression of PPARG mRNA in OB muscle determined by quantitative PCR. F) PPARG protein was enhanced in OB muscle as shown by Western blot analysis (*P < 0.05; mean ± SEM; n = 8 in Con and n = 9 in OB group).
in the muscle of the progeny of OB sheep ($P < 0.10$) (Fig. 3A). In addition, the phosphorylation of IRS1 at Ser-1101 was higher in the OB group (by 23.8% ± 8.4%; $P < 0.05$) (Fig. 3B), which further confirmed that there was insulin resistance in the muscle of the progeny of the OB sheep.

**Skeletal Muscle of the Progeny of Obese Sheep Had Enhanced Intramuscular Lipid Accumulation and Fibrosis**

Intramuscular lipid accumulation activates PRKC, which induces insulin resistance. More intramuscular adipocytes were observed in the muscle of the offspring of OB than Con lambs (Fig. 4, A and B). We quantified the fat area in muscle sections and found it to be higher in muscle of the progeny of the OB sheep (by 35.6% ± 11.8%; $P < 0.05$) (Fig. 4C). Furthermore, chemical analysis showed that there were more triglycerides in the OB muscle (by 20.1% ± 2.8%; $P < 0.05$) (Fig. 4D). In addition, both the mRNA and protein levels of PPARG, a marker of adipocytes, were higher in the OB group compared to the Con group (by 33.6% ± 13.6% and 31.1% ± 5.1%, respectively; $P < 0.05$) (Fig. 4, E and F).

Compared to muscle of the progeny of Con sheep, the mRNA and protein levels of SLC27A1 (FATP1) were enhanced in the muscle of the OB group (by 61.8% ± 24.8% and 40.8% ± 9.3%, respectively; $P < 0.05$) (Fig. 5, A and B). In addition, the level of CD36 (cluster of differentiation 36 or fatty acid translocase, FAT) mRNA was increased by 39.4% ± 8.8% (P < 0.05) (Fig. 5C). Taken together, our results indicated that there were more adipocytes and enhanced lipid accumulation in offspring skeletal muscle of OB compared to Con sheep.

As shown in Figure 4, A and B, the muscle of the offspring of OB sheep also appeared to have more connective tissue. Indeed, the collagen area was larger in the muscle tissue of the progeny of OB sheep (by 32.8% ± 8.8%; $P < 0.05$) (Fig. 6A); chemical analysis also showed that the collagen content was higher in the progeny of OB lambs compared to Con lambs (by 50.6% ± 15.3%; $P < 0.05$) (Fig. 6B). Transforming growth factor β (TGFβ) signaling stimulates fibrogenesis. The level of TGFβ mRNA was 27.0% ± 9.1% higher in OB compared to Con muscle ($P < 0.05$) (Fig. 6C). Combining these results, we concluded that fibrogenesis was increased in LD muscle of the progeny of OB sheep.

**Inflammatory Signaling Was Enhanced in LD Muscle of the Progeny of Obese Sheep**

Inflammatory responses are linked with obesity, enhanced lipid accumulation, fibrosis, and insulin resistance. Our previous results showed that toll-like receptor 4 (TLR4) signaling was induced in the fetal muscle of OB mothers. In the current study, we detected an increase of TLR2 and TLR4 mRNA expression (by 60.6% ± 29.7% and 54.5% ± 24.1%, respectively; $P < 0.05$) (Fig. 7A) in LD muscle of the progeny of OB sheep compared to Con sheep. The mRNA level of CD14 (cluster of differentiation 14, mainly expressed in macrophages), which functions as a coreceptor of TLR4, was 50.6% ± 18.6% ($P < 0.05$) higher in the progeny of the OB group (Fig. 7A). TLR2 and TLR4 initiate the inflammatory signaling pathway involving NFKB. The phosphorylation of p65, a key component of the NFKB-signaling pathway, was increased by 37.4% ± 11.5% ($P < 0.05$) in the muscle of the progeny of OB sheep compared to Con sheep (Fig. 7B).

**DISCUSSION**

Sheep are one of the most commonly used models for pregnancy studies. Using the same sheep model, we previously showed that maternal obesity increases intramuscular adipocytes and connective tissue in fetal muscle at late gestation and that insulin resistance was detected in the fetal muscle of OB sheep [24, 25]. In this study, we further analyzed whether such changes in OB fetal muscle persist in the musculature of the adult offspring. Consistent with our observations in fetal muscle, we detected similar changes in offspring muscle. For instance, the phosphorylation of AKT was lower in muscle of the progeny of OB sheep to an extent that was very similar to the data in fetal muscle. The expression of PPARG and selected inflammatory cytokines in the skeletal muscle of OB offspring was also comparable to those seen in OB fetal muscle. Even though we cannot rule out the possible effects of lactation and postnatal feed intake on the changes of muscle properties, it seems that most of the changes in fetal muscle of OB sheep were maintained postnatally, indicating that maternal obesity generates persistent effects on the properties of offspring muscle.
IRS1 is a key mediator of insulin signaling. When IRS1 is activated by tyrosine phosphorylation, it serves as a docking center for the recruitment and activation of downstream pathways, of which the most important is the phosphoinositide-3-kinases/AKT (PI3K/AKT) pathway [36]. PRKC-induced IRS1 serine phosphorylation reduces the ability of IRS1 to serve as a docking center for the recruitment and activation of downstream pathways, leading to insulin resistance [36]. In the current study, although the circulatory insulin concentration was higher in OB [26], the PI3K/AKT pathway, a major insulin downstream signaling pathway, was down-regulated in the skeletal muscle of the progeny of OB sheep. The phosphorylation of key mediators of insulin signaling, including AKT, MTOR, and EIF4EBP1, was attenuated in the LD muscle of the offspring of the OB group, showing insulin resistance. This observation was in agreement with the lower insulin sensitivity observed in the same pool of OB lambs [26].

To analyze possible reasons for the observed insulin resistance in OB offspring muscle, we analyzed the phosphorylation of PRKC and the content of intramuscular lipids, both of which were higher in OB offspring muscle, consistent with our previous observation in fetal skeletal muscle [25]. Higher intramuscular lipid content might be responsible for the activation of PRKC. Increasing intracellular fatty acyl-CoA is known to activate the PRKC pathway [37, 38].

Fatty acid transporters mediate the cellular uptake of fatty acids. SLC27A1 is a plasma membrane protein that facilitates the uptake of fatty acids into cells [39, 40]. SLC27A1 up-regulation in skeletal muscle of the progeny of OB sheep might be associated with the higher intramuscular fat content and insulin resistance in skeletal muscle of the progeny of OB sheep. In addition, the expression of FAT/CD36 was also higher in OB offspring muscle, consistent with a previous report showing FAT/CD36 up-regulation under obese and type 2 diabetic conditions [41].

Obesity induces chronic low-grade inflammation, which alone can induce insulin resistance [42]. TLRs recognize fatty acids and play an important role in inducing inflammation associated with obesity [43]. TLRs activate the NFkB pathway to mediate the inflammatory response [44, 45]. Consistent with our observation of insulin resistance and enhanced lipid accumulation in OB fetal muscle [25], enhanced TLR4 level and inflammation signaling were also observed. In a recent study, enhanced expression of TLR4 was linked to insulin resistance and diabetes [46].

Inflammation induces the expression of anti-inflammatory factors, one of which is TGFβ [47]. By analyzing the mRNA level, we observed a higher expression of TGFβ in muscle of the offspring of OB sheep. TGFβ signaling induces fibrogenesis [48]. Using Masson trichrome staining, we observed a higher collagen content in OB muscle, which was further confirmed by chemical analyses. During aging, a progressive loss of muscle mass occurs accompanied by increased adiposity and fibrosis [17, 22], resulting in a decline in muscle structural integrity and functional capacity [23]. Our observation that maternal obesity induces changes in offspring skeletal muscle characterized by increased intramuscular adiposity and fibrosis clearly shows that the properties of skeletal muscle in OB offspring were negatively affected by maternal obesity.

In conclusion, we observed intramuscular adiposity and fibrosis in the offspring of overnourished mothers, which was associated with attenuated insulin signaling and enhanced inflammatory signaling when compared with Con muscle. We propose that maternal obesity enhances adipogenesis and fibrogenesis in fetal and offspring muscle, which is expected to impair the physiological function of skeletal muscle. The

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