Metabolic regulation by stress systems in bovine myoblasts and ovine fetal skeletal muscle.

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ABSTRACT: Stress factors including catecholamines and inflammatory cytokines are known to regulate glucose metabolism. However, the underlying mechanisms and the impact on developing muscle are not fully understood. Therefore, the objective of this study was to determine how β adrenergic agonists and inflammatory cytokines alter glucose metabolic homeostasis in myoblasts and fetal skeletal muscle fibers. In Experiment 1, glucose uptake and glucose oxidation rates were determined in primary bovine myoblasts (muscle stem cells) incubated for 4 days in differentiation media with or without insulin, and/or β_1 adrenergic agonist (Ractopamine HCl), or β_2 adrenergic agonists (Zilpaterol HCl). In Experiment 2, pregnant ewes were treated with saline (controls) or bacterial endotoxin (**LPS**) from day 100-115 of gestation (term = 150 days). On gestational day 122, ewes were euthanized and fetal soleus muscle was isolated tendon-to-tendon and split longitudinally. To determine glucose uptake and glucose oxidation rates, soleus strips were incubated in KHB that was un-spiked (basal) or spiked with either insulin or TNFα. In differentiated bovine myoblast cultures, insulin increased ($P < 0.05$) glucose uptake and oxidation when compared to basal. β_2 adrenergic agonist likewise increased ($P < 0.05$) glucose uptake and oxidation from basal to levels that did not differ from insulin alone. β_1 adrenergic agonist did not change glucose uptake for basal levels and only slightly increased ($P < 0.05$) glucose oxidation. Moreover, when β_1 adrenergic agonist was added in combination with insulin, glucose uptake and oxidation were both less ($P < 0.05$) than with insulin alone. Interestingly, when β_2 adrenergic agonist was added in combination with insulin, glucose uptake was not different from insulin alone but glucose oxidation was decreased ($P < 0.05$). In primary ovine fetal soleus muscle, glucose uptake was not different between control and LPS fetuses under basal, insulin-stimulated, or TNFα-stimulated conditions. However, insulin-stimulated glucose oxidation and TNFα-stimulated glucose oxidation were both greatly decreased ($P < 0.05$) in LPS fetal muscle compared to controls. Our findings show that adrenergic and inflammatory mediators have insulin dependent and insulin independent effects on glucose metabolism and that regulation by these stress systems is present in developmental stages, as demonstrated by responses in both stem cells and fetal muscle.

Key words: β agonist, metabolic syndrome, pro-inflammatory cytokines

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INTRODUCTION

Factors that activate stress systems, including inflammatory cytokines and β adrenergic agonists, are known to impact metabolism in both myoblasts and skeletal muscle fibers. Skeletal muscle accounts for more than 80% of insulin-stimulated glucose metabolism (DeFronzo et al., 1981) and therefore plays a critical role in glucose homeostasis. Inflammatory cytokines such as TNFα can acutely enhance glucose metabolism despite antagonizing insulin signaling (Cadaret et al., 2016). However, we speculate that chronic exposure of developing muscle to inflammatory cytokines may reduce sensitivity to them and thus dampen their direct capacity to stimulate glucose oxidation. Inflammation is known to cause insulin resistance (Marette et al., 2014), and thus fetuses that are chronically exposed to inflammatory cytokines during late gestation are likely at a greater risk for developing metabolic dysfunction from the combination of insulin resistance and reduced sensitivity to cytokines (Pickup and Crook, 1998; Spranger et al., 2003).

The β adrenergic agonists, ractopamine HCl (β_1) and zilpaterol HCl (β_2), enhance muscle growth efficiency and decrease fat accumulation in finishing cattle when used as feed additives (Mersmann, 1998), although the mechanisms underlying this increased efficiency are not fully understood. Due to our growing population, increased beef consumption, and greater competition for available land, producers benefit from using these products. The use of β adrenergic agonists yields more meat from the same number or even fewer animals (Strydom, 2016), thus benefiting the US economy (Centner et al., 2014). Our objectives for this study were to determine the effects of sustained inflammation on the ability of inflammatory cytokines to regulate insulin-stimulated glucose metabolism in fetal skeletal muscle and to determine whether β adrenergic agonists have similar effects on glucose metabolism in bovine myoblasts (muscle stem cells) as previously found in mature muscle.

MATERIALS AND METHODS

Animals and Treatments

The following experiments were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. Studies were conducted at the University of Nebraska-Lincoln Animal Science Complex, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

Experiment 1. Primary myoblasts were isolated as previously described (Yates et al., 2014) from four Angus-cross steers slaughtered at one year of age. Isolated

myoblasts were seeded onto 12-well tissue culture plates to measure glucose uptake or 6-well tissue culture plates to measure glucose oxidation at an initial seeding density of 20,000 cells per well for both plates. All plates were coated with poly-L-lysine and bovine fibronectin. Cells were incubated in complete growth media (DMEM, 20% FBS, 1% Ab/Am, 0.4% Fungizone, and 0.5% Gentamicin) for 24 hours then incubated in treatment-spiked differentiation media (DMEM, 2% FBS, 1% Ab/Am, 0.4% Fungizone, and 0.5% Gentamicin) for four days. Differentiation media was spiked with one of the following treatments: no additive (basal), insulin (5 mU/ml Humulin-R), β1 agonist (1 μM ractopamine HCl), β2 agonist (0.05 μM zilpaterol HCl), β1 agonist + insulin, or β2 agonist + insulin. All additives were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of Humulin-R (Eli Lilly, Indianapolis, IN)

To determine glucose uptake, cells were pre-incubated at 37°C for 1 hour in treatment-spiked Krebs-Henseleit Buffer (KHB) media containing 0.1% bovine serum albumin (BSA), 5mM glucose, and 35 mM mannitol. Cells were then washed at 37°C for 20 minutes in treatment-spiked glucose-free KHB media with 0.1% BSA and 40 mM mannitol. Cells were then incubated at 37° C for 20 minutes in treatment-spiked KHB media with 1 mM [^{3}H] 2-deoxyglucose (300 μCi/mmol) and 39 mM [U-^{14}C] mannitol (12.5 μCi/mmol), and 0.1% BSA. Media was then removed and cells were thrice washed with ice-cold PBS and nuclear stained with 5 μg/ml Hoehst 3342 (Thermo-Fischer). Cells were imaged and cell number was estimated by counting with Olympus CellSense Software. Cells were then lysed with 2 mM NaOH and lysates were transferred to scintillation vials and mixed with 20 ml UltimaGold scintillation fluid. Specific activities of [^{3}H] to determine glucose uptake and [^{14}C] to estimate extracellular fluid volume were measured using a Beckman-Coulter 1900 TA LC counter (Brea, CA). Specific activity of media was estimated from 10-μl aliquots mixed with 500-μl double-distilled water. All radioactive materials and scintillation fluids were purchased from Perkin-Elmer (Waltham, MA). Four technical replications per condition were performed and averaged for each animal.

Before measuring glucose oxidation rates, cell populations in each well were imaged using Olympus CellSense Software. Three images per well were taken using bright field and used to estimate the number of cells per well. Cells were then pre-incubated at 37°C for 1 hour in treatment-spiked KHB with 0.1% BSA and 5 mM glucose and washed at 37°C for 20 minutes in glucose-free treatment-spiked KHB with 0.1% BSA. To determine glucose oxidation, cells were sealed in dual-well chambers using a rubber gasket and incubated at 37° C for 120-min. in treatment-spiked gassed (95% O_2: 5% CO_2) KHB media and containing 5 mM [^{14}C-U] D-glucose (2.5 μCi/mmol) and 0.1% BSA. In the adjacent well, 2 mM NaOH was injected to capture CO_2 produced. After the incubation period, plates were cooled for 2 minutes at -20° C and 2M HCl was injected into the media through the rubber seal. Plates were then incubated at 4°C for 1 hour to release bicarbonate-bound CO_2 from media. Chambers were

opened and the NaOH was collected and mixed with 20 ml UltimaGold scintillation fluid in a scintilation vial. Specific activity of $^{14}CO_2$ and media were determined as described above. Background was determined from no-cell control wells with tracer media. Three technical replications per condition were averaged for each animal.

Experiment 2. Timed-mated Poly Pay ewes carrying twins received an IV injection of sterile saline (Controls; n = 8) or 0.1 μg/kg BW of bacterial lipopolysaccharide (LPS; n = 6) from *E. coli O55:B5 (*Sigma-Aldrich, St. Louis, MO*)* every third day from day 100 to 115 of gestation (term = 150 days).

Animals were euthanized at 140 ± 1 days of gestational age and fetal soleus muscles were isolated tendon-to-tendon, washed in ice-cold PBS, and split longitudinally into intact strips (150-400 mg). Strips were pre-incubated and washed in treatment-spiked KHB as described above. For fetal muscle strips, however, medias were spiked with the following treatments: no additive (basal), insulin (5 mU/ml Humulin-R), or TNFα (20 ng/ml hTNFα). All additives were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of Humulin-R (Eli Lilly, Indianapolis, IN). To determine glucose uptake, muscle strips were incubated at 37° C for 20 minutes in treatment-spiked KHB with 1 mM [^{3}H]2-deoxyglucose and 39 mM [^{14}C] mannitol as described above. Afterward, muscle strips were removed, washed thrice in ice-cold PBS, weighed, and lysed in 2 M NaOH at 37° C for 1 hour. Specific activity of ^{3}H and ^{14}C was determine for lysates and media as above.

To determine glucose oxidation, fetal muscle strips were placed in sealed dual-well chambers, pre-incubated for 1h, and washed for 20 minutes in treatment-spiked KHB as described above. Muscle strips were then incubated at 37° C for 120-min. in treatment-spiked gassed KHB with 5 mM [^{14}C-U] D-glucose (.25 μCi/mmol), and 2M NaOH was put into the adjacent wells to capture CO_2 produced. After the 120-min. incubation, the chambers were cooled at -20° C for 2 minutes, and 2M HCl was injected into the media through the rubber seal to release media-bound CO_2. Chambers were incubated at 4° C for 1 hour. Muscle strips were then thrice washed and weighed, and specific activity of ^{14}C in NaOH and media aliquots were determined as described above.

Statistical Analysis

All data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, Cary, NC). In Experiment 1, steer was the experimental unit and in Experiment 2, fetus was the experimental unit. For both experiments, glucose uptake and oxidation were determined from the same muscle or cell isolates, but from different wells. Values for glucose uptake and oxidation were normalized to the mean of the basal group and are expressed as means ± standard error.

RESULTS

Experiment 1. Incubation of myoblasts with insulin increased ($P < 0.05$) glucose uptake compared to basal conditions (Figure 1). Incubation with β2 agonist alone

also increased ($P < 0.05$) glucose uptake from basal conditions to a degree similar to insulin. However, glucose uptake in myoblasts incubated with insulin and β2 agonist together did not differ from myoblasts incubated with insulin alone. Glucose uptake in myoblasts incubated with β1 agonist alone did not differ from glucose uptake in myoblasts incubated under basal conditions. Moreover, glucose uptake in myoblasts incubated with insulin and β1 agonist together was less ($P < 0.05$) than in myoblasts incubated with insulin alone.

Incubation of myoblasts with insulin increased ($P < 0.05$) glucose oxidation compared to basal conditions (Figure 1). Incubation with β2 agonist alone similarly increased ($P < 0.05$) glucose oxidation compared to basal conditions. Conversely, glucose oxidation in myoblasts incubated with β1 agonist alone did not differ from myoblasts incubated under basal conditions. Furthermore, incubation of myoblasts with insulin and β1 agonist or with insulin and β2 agonist decreased ($P < 0.05$) glucose oxidation compared to myoblasts incubated with insulin alone.

Experiment 2. No interaction was observed between maternal treatment and incubation media. However, incubation with insulin increased ($P < 0.05$) glucose uptake in fetal soleus muscle from control and LPS-treated ewes similarly (Figure 2). Moreover, glucose uptake rates in muscle incubated with TNFα was not different from muscle incubated with insulin in either treatment group.

Interacting effects between maternal treatment and incubation media were observed ($P < 0.05$) for glucose oxidation. Incubation in media spiked with insulin increased ($P < 0.05$) glucose oxidation in fetal muscle from both treatment groups, but the increase was greater ($P < 0.05$) in fetal muscle from control ewes than from LPS-treated ewes. Moreover, when muscle from control fetuses was incubated with TNFα, glucose oxidation was greater ($P < 0.05$) than when incubated under basal conditions but less ($P < 0.05$) than when incubated with insulin. Conversely, when fetal muscle from LPS-treated ewes was incubated with TNFα, glucose oxidation rates did not increase above basal conditions.

DISCUSSION

The findings from this study show that adrenergic and inflammatory mediators have a role in the metabolic regulation of developing muscle, as demonstrated by the effects on muscle stem cells (myoblasts) and fetal skeletal muscle fibers. Moreover, these effects can be insulin-dependent or insulin-independent. In primary bovine myoblasts, β2 adrenergic agonists increased glucose uptake and oxidation in the absence of insulin, but β1 adrenergic agonists had no effect on glucose uptake or oxidation, which was similar to findings in mature skeletal muscle (Cadaret et al., 2016). Unlike in mature muscle, however, insulin and β2 adrenergic agonists together resulted in less glucose oxidation than either factor individually. In fetal soleus muscle, insulin-stimulated and TNFα-stimulated glucose oxidation was diminished by maternal inflammation earlier in gestation, although the capacity for insulin-stimulated or TNFα-stimulated glucose uptake did not appear to be affected. This shows that metabolic regulation by cytokines not only occurs in fetal muscle, but it can be diminished by fetal adaptations to chronic inflammation long after the inflammation has subsided.

β2 adrenergic agonists appeared to stimulate more efficient glucose metabolism than β1 adrenergic agonists, as the former increased both glucose uptake and oxidation rates in the primary bovine myoblasts. Since previous evidence shows that both stimulants increase growth efficiency (Centner et al., 2014), it is likely that β1 and β2 adrenergic agonists may be utilizing differing metabolic pathways. We speculate that β1 adrenergic agonists are more efficient mediators of metabolism in adipocytes than myoblasts.

The combination of insulin and β2 adrenergic agonists decreased glucose oxidation in primary bovine myoblasts, which suggests that β2 adrenergic agonists work independently of insulin in myoblasts. Our previous study conducted in adult rat muscle showed that β2 adrenergic agonists worked synergistically with insulin to enhance glucose metabolism (Cadaret et al., 2016), but this was not the case in our muscle stem cells. This indicates that prior to forming myofibers, myoblasts have a different mechanistic balance between metabolic regulators like insulin and the adrenergic system.

Stimulation of glucose metabolism by insulin was diminished in fetal muscle that had been exposed to chronic inflammation earlier in gestation. This indicates that chronic inflammation during development disrupts insulin signaling in skeletal muscle, and that the deficit remains even after the inflammation has been alleviated. This may help to explain previous findings that chronic inflammation during fetal development increases the risk of developing insulin resistance later in life (Hotamisligil et al., 1993).
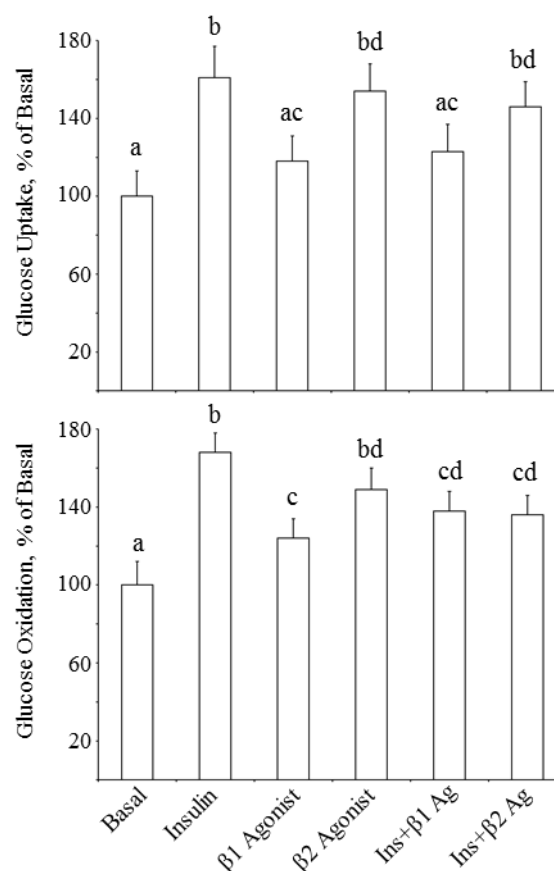
This study also shows that incubation with TNFα after chronic inflammation decreases glucose oxidation when compared to controls. Our previous studies show that acute exposure to inflammatory cytokines increases glucose oxidation in muscle even in the absence of insulin (Cadaret et al., 2016). However, the present study shows that chronic exposure to inflammation decreases this metabolic response and that subsequent exposure to inflammatory cytokines does not produce greater glucose oxidation. Thus, the benefit of this metabolic regulator is lost.
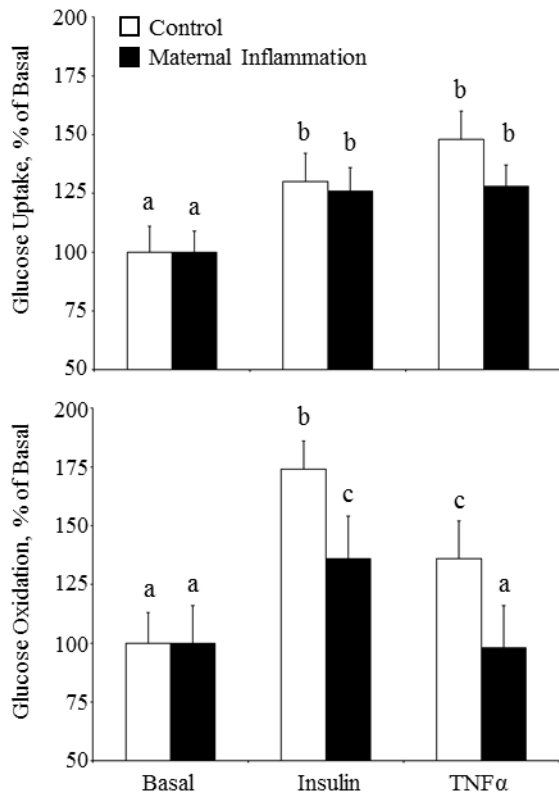
IMPLICATIONS

Our findings indicate that stress factors help regulate glucose metabolism in developing skeletal muscle and that their effects can be diminished by chronic over-exposure, even after exposure is alleviated. Furthermore, although ractopamine HCl and zilpaterol HCl are FDA approved for use as feed additives in animals, this study demonstrates that zilpaterol HCl is more effective in increasing metabolic efficiency.

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Figure 1. Glucose uptake and oxidation in primary bovine myoblasts during 20-min and 120-min incubations, respectively, spiked with one of the following treatments: No additive (basal), insulin (5 mU/ml Humulin-R), β1 agonist (1 μM Ractopamine HCl), β2 agonist (0.05 μM Zilpaterol HCl), β1 agonist + insulin, or β2 agonist + insulin. [a,b,c,d] means with differing superscripts differ ($P < 0.05$).

Figure 2. Glucose uptake and oxidation in primary fetal soleus muscle after maternal inflammation during 20-min and 120-min incubations, respectively, spiked with one the following treatments: No additive (basal), insulin (5 mU/ml Humulin-R), or TNFα (20 ng/ml hTNFα). [a,b,c] means with differing superscripts differ ($P < 0.05$).