Proceedings, Western Section, American Society of Animal Science

Vol. 67, 2016

Insulin-associated and insulin-independent impacts of β adrenergic agonists and pro-inflammatory cytokines on glucose metabolism in primary rat soleus muscle.

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ABSTRACT: Recent studies show that catecholamines and pro-inflammatory cytokines may help regulate skeletal muscle growth and metabolism even at sub-stress levels. The objective of this study was to determine the acute effects of β1 and β2-specific adrenergic agonists as well as TNFα and IL-6 on muscle glucose uptake and oxidation under basal and insulin-stimulated conditions. Primary soleus muscle was collected from adult Sprague-Dawley rats, separated tendon-to-tendon into 25-45 mg strips, and incubated in KHB spiked with or without insulin, and/or ractopamine HCl (β1 agonist), zilpaterol HCl (β2 agonist), TNFα, and IL-6. Glucose uptake was determined from cellular content of [^{3}H]-2-deoxyglucose after 20 min. Glucose oxidation of [^{14}C-U] glucose was determined after 2 h. Phospho-Akt/total Akt (**p-Akt/Akt**) was determined from protein isolated after 1 h. Compared to muscle incubated in un-spiked (basal) media, incubation with insulin increased ($P < 0.05$) glucose uptake by ~47%, glucose oxidation by ~32%, and p-Akt/Akt by ~238%. Muscle incubated with β2 agonist exhibited ~20% less ($P < 0.05$) glucose uptake but ~32% greater ($P < 0.05$) glucose oxidation than basal. Moreover, incubation with β2 agonist+insulin increased ($P < 0.05$) glucose oxidation and p-Akt/Akt over insulin alone. Muscle incubated with β1 agonist did not differ from basal for any output. Likewise, β1 agonist+insulin incubations did not differ from insulin alone. Glucose oxidation was ~23% and ~33% greater ($P < 0.05$), respectively, in muscle incubated with TNFα and IL-6 compared to basal, yet glucose uptake and p-Akt/Akt did not differ. Glucose uptake, glucose oxidation, and p-Akt/Akt were similar among muscle incubated with TNFα+insulin, IL-6+insulin, and insulin alone. In addition, glucose oxidation in muscle incubated with TNFα+insulin and IL-6+insulin did not differ from TNFα alone or IL-6 alone. These results show that acute β2 stimulation had opposite effects on glucose uptake and glucose oxidation in muscle, and that acute β1 stimulation had no evident impact on muscle metabolism. Moreover, β2 stimulation was synergistic with insulin, as glucose oxidation and Akt phosphorylation were greater with the two products together than with either individually. Lastly, acute stimulation with TNFα or IL-6 increased glucose oxidation rates independently of insulin or Akt phosphorylation. Together, our findings demonstrate that adrenergic and inflammatory mediators can have insulin-associated or insulin-independent effects on glucose metabolism and that these effects may differ for glucose uptake and oxidation.

Key words: β-agonist, glucose metabolism, stress hormones

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INTRODUCTION

Skeletal muscle makes up ~40% of total body mass but accounts for >80% of insulin-stimulated glucose utilization (DeFronzo et al., 1981; Brown, 2014). In addition to insulin, muscle metabolism appears to be influenced by adrenergic and cytokine activity (Glund et al., 2007; Pillon et al., 2013; Fernandes et al., 2014). Adrenergic activity in muscle is mediated primarily by β receptors. β2 receptors are the most prevalent, but β1 receptors and to a lesser extent β3 receptors are also present (Kim et al., 1991). Performance studies (Lopez-Carlos et al., 2012) have led to the development of isomer-specific adrenergic growth promoters (Johnson et al., 2014), but far less is known about their effects on metabolism. Likewise, pro-inflammatory cytokines have complex metabolic effects that are only beginning to be understood. Inflammation is known to cause insulin resistance (Marette et al., 2014), but recent studies show that two major pro-inflammatory cytokines, TNFα and IL-6, may stimulate glucose metabolism in muscle (Glund et al., 2007; Gray and Kamolrat, 2011; Remels et al., 2015). Insulin activates a pathway that ultimately translocates the glucose transporter Glut4 to the cell membrane where it is imbedded (James et al., 1988). A major step in this pathway is phosphorylation of the kinase Akt, which is often used to indicate insulin sensitivity. Insulin-stimulated glucose uptake and oxidation are often assumed to be proportional, but we postulate that this relationship may not be maintained when adrenergic factors or cytokines are present. Moreover, adrenergic factors and cytokines may influence glucose metabolism via insulin-independent effects. Our objective was to determine the impact of β1 and β2-specific adrenergic agonists as well as TNFα and IL-6 on muscle glucose uptake and oxidation. Furthermore, we sought to determine whether these effects were insulin-associated or insulin-independent by incubating muscle strips with each factor alone or with insulin.

MATERIALS AND METHODS

Animals and tissue isolation

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

Soleus muscles were collected tendon-to-tendon from mature Sprague-Dawley rats (female 252.86 ± 14.93

g, male 378 ± 15.72 g) after decapitation under heavy anesthesia (isoflurane) and used to measure glucose uptake, glucose oxidation, and Akt phosphorylation (n = 10, 9, and 8 rats, respectively). Males and females were spread evenly across all groups. Isolated muscles were washed in ice-cold phosphate buffered saline (PBS), and each muscle was dissected longitudinally into 25-45 mg strips (2 technical replicates per condition for each rat). Strips were then pre-incubated for 1 h at 37°C in gassed (95% O_2, 5% CO_2) Krebs-Henseleit bicarbonate buffer (KHB, pH 7.4; 0.1% bovine serum albumin; Gibco Life, Grand Island, NY) spiked with treatment (see below) and 5mM glucose and then washed for 20 min in treatment-spiked KHB with no glucose. Pre-incubation and wash media for glucose uptake experiments also contained 35 mM and 40 mM mannitol, respectively. To determine glucose uptake, glucose oxidation, and Akt phosphorylation, muscle strips were then incubated in treatment-spiked KHB under the below conditions.

Pre-incubation, wash, and incubation media were spiked with the following treatments: none (basal), insulin (5 mU/ml Humulin-R), β1 agonist (10 μM ractopamine HCl), β2 agonist (0.5 μM zilpaterol HCl), β1+insulin, β2+insulin, TNFα (20 ng/ml hTNFα), IL-6 (1 ng/ml rIL-6), TNFα+insulin, and IL-6+insulin. All additives were purchased from Sigma-Aldrich (St. Louis, MO).

Glucose uptake

Glucose uptake rates were determined by incorporation of [^{3}H]2-deoxyglucose as previously described (Jacob et al., 1996), with some modifications. After being washed, muscle strips were incubated at 37°C for 20 min in treatment-spiked KHB with 1 mM [^{3}H]2-deoxyglucose (300 μCi/mmol) and 39 mM [1-^{14}C] mannitol (1.25 μCi/mmol). Muscle strips were then removed, thrice washed in ice-cold PBS, weighed, and lysed in 2 M NaOH at 37°C for 1 h. Lysate was mixed with UltimaGold scintillation fluid and specific activity of ^{3}H and ^{14}C was measured by liquid scintillation with a Beckman-Coulter 1900 TA LC counter (Brea, CA). Specific activity of the media was likewise determined in triplicate from 10-μl aliquots mixed with 500 μl distilled water. All radioactive compounds and scintillation fluids were purchased from Perkin-Elmer (Waltham, MA).

Glucose oxidation

Glucose oxidation rates were determined by oxidation of [^{14}C-U]D-glucose as previously described (Henriksen and Tischler, 1988), with some modifications. After being washed, muscle strips were placed in sealed dual-well chambers and incubated at 37°C for 2 h in treatment-spiked KHB with 5 mM [^{14}C-U] D-glucose (0.25 μCi/mmol). In the adjacent well, 2M NaOH was placed to capture CO_2. After 2 h, chambers were cooled at -20°C for 2 min, 2M HCl was added to the media through the rubber seal, and chambers were placed at 4°C for 1 h to release bicarbonate-bound CO_2 from the media. Chambers were then opened and muscle strips were washed and weighed. NaOH was collected and mixed with UltimaGold scintillation fluid to determine specific activity of $^{14}CO_2$ via liquid scintillation. Specific activity of media was also determined.

Phosphorylated Akt

Akt phosphorylation was determined by the proportion of phosphorylated Akt to total Akt (p-Akt/Akt) as previously described (Morley et al., 2015). Muscle strips were incubated in treatment-spiked KHB with 5 mM glucose at 37°C for 1 h and then snap-frozen in liquid nitrogen and stored at -80ºC.

Snap-frozen muscle was thoroughly homogenized in 200 μl of RIPA Buffer containing recommended concentrations of Protease and Phosphatase Inhibitor (Thermo Fisher, Carlsbad, CA). Homogenates were then sonicated and centrifuged (14,000 x *g*; 5 min at 4ºC), and supernatant was collected. Total protein concentrations were determined by Pearce BCA Assay (Thermo Fisher). Protein samples were combined with BioRad 4x Laemmli Sample Buffer (BioRad, Hercules, CA) and incubated at 95ºC for 5 min. Protein was loaded into a 15-well gel at 35 μg/well, separated by SDS-page and then transferred to polyvinylidene fluoride low florescence membranes(BioRad). Membranes were incubated in odyssey block (Li-Cor Biosciences, Lincoln, NE) solution for 1 h at room temperature and then washed with 1X TBS-T (20 mM Tris-HCL+ 150 mM NaCl + 0.1% Tween 20). Membranes were subsequently incubated overnight at 4ºC in rabbit anti-pAkt (1:2,000) or rabbit anti-Akt (1: 1,000) antibodies for one hour (Cell Signaling, Danvers, MA) diluted in odyssey block + 10μL Tween 20. An IR800 goat anti-rabbit IgG secondary antibody (1:10,000; Li-Cor) was applied for 1 h at room temperature, imaged on an Odyssey scanner and analyzed with Image Studio Lite Software (Li-Cor).

Statistical analysis

All data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, Cary, NC). Data were analyzed as five separate experiments, each with its own basal and insulin-only conditions: glucose uptake/ adrenergic factors (n = 10), glucose uptake/ cytokines (n = 10), glucose oxidation/ adrenergic (n = 9), glucose oxidation/ cytokine (n = 9), and Akt phosphorylation (n = 8). A separate muscle strip from each rat was exposed to each condition in the experiment. Data are presented and means ± standard error.

RESULTS

Glucose uptake and oxidation

Incubation of muscle with insulin increased ($P < 0.05$) glucose uptake in both the adrenergic (Figure 1) and cytokine (Figure 2) experiments. Likewise, glucose oxidation was increased ($P < 0.05$) by insulin compared to basal in adrenergic (Figure 3) and cytokine (Figure 4)

experiments. When muscle was incubated with β1 agonist alone, glucose uptake and oxidation did not differ from basal. Moreover, when muscle was incubated with β1+insulin, glucose uptake and oxidation did not differ from incubation with insulin alone. Incubation of muscle with β2 agonist decreased ($P < 0.05$) glucose uptake but increased ($P < 0.05$) glucose oxidation compared to basal. Glucose uptake did not differ between muscle incubated with β2+insulin and insulin alone, but glucose oxidation was greater ($P < 0.05$) in muscle incubated with β2+insulin than insulin alone or β2 agonist alone. Glucose uptake in muscle incubated with TNFα or with IL-6 did not differ from basal, and in muscle incubated with TNFα+insulin or IL-6+insulin did not differ from insulin alone. However, glucose oxidation in muscle incubated with TNFα or with IL-6 was greater ($P < 0.05$) than basal and in fact was not different from insulin alone. Interestingly, glucose oxidation in muscle incubated with TNFα+insulin or with IL-6+insulin did not differ from insulin alone, TNFα alone, or IL-6 alone.

Akt phosphorylation

Muscle incubated with insulin exhibited 4.26-fold greater ($P < 0.05$) p-Akt/Akt than basal (Figure 5), but muscle incubated with β1, β2, TNFα, or IL-6 alone did not differ from basal. Muscle incubated with β1+insulin did not differ in p-Akt/Akt from muscle incubated with insulin alone, but muscle incubated with β2+insulin was greater ($P < 0.05$) in p-Akt/Akt than insulin alone. Conversely, p-Akt/Akt in muscle incubated with TNFα+insulin or with IL-6+insulin was less ($P < 0.05$) than insulin alone and, in fact, IL-6+insulin did not differ from basal.

DISCUSSION

In this study, we show that both adrenergic and pro-inflammatory stimulation can acutely impact basal and insulin-stimulated glucose metabolic rates in skeletal muscle. Adrenergic effects were isomer-specific, as β1 stimulation had no discernable effect, but β2 stimulation impaired basal glucose uptake, increased basal oxidation, and was synergistic with insulin. Cytokines appeared to directly stimulate glucose oxidation and impair insulin-stimulated Akt phosphorylation, but had no effect on glucose uptake rates. These data indicate that stress factors may initially benefit glucose metabolism, particularly glucose oxidation, despite apparent changes in insulin sensitivity.

Acute β2-specific adrenergic stimulation had differing effects on glucose uptake and oxidation rates in the absence of insulin, as uptake was impaired but oxidation was enhanced. Total oxidative metabolic rates are believed to remain static but individual substrate oxidation may vary (Hay et al., 1983), and our findings show that fractional oxidation of glucose increases substantially during β2 stimulation. Indeed, this may help to explain smaller fractional glucose oxidation rates in growth-restricted fetuses (Limesand et al., 2007), which we previously found to have reduced skeletal muscle β2 receptors (Yates et al., 2012). In addition to its own direct effects, β2 stimulation appeared to synergistically enhance glucose oxidation with insulin, as the two factors together had a greater impact than either individually. The additive effect may be explained mechanistically by greater p-Akt/Akt in muscle incubated with both, as Akt phosphorylation is a major step in insulin signaling (Saltiel and Kahn, 2001). However, no such synergistic effect was observed for glucose uptake rates despite enhanced Akt phosphorylation. Unlike β2, β1 stimulation did not appear to affect basal or insulin-stimulated glucose uptake or oxidation, possibly due to the relatively low expression of the β1 isomer compared to β2 (Kim et al., 1991).

Acute stimulation with the pro-inflammatory cytokines TNFα and IL-6 increased glucose oxidation independently of insulin and, in fact, appeared to antagonize insulin when incubated together, as muscle co-incubated with insulin and either cytokine had lower Akt phosphorylation than muscle incubated with insulin alone. Pro-inflammatory cytokines have long been linked to insulin resistance (Heliövaara et al., 2005; Lazar, 2005) which is evident by the decrease in insulin-stimulated Akt phosphorylation in our study. Yet, our results indicate that cytokines may also initially be assuming insulin's role of stimulating glucose oxidation via a non-Akt mechanism, as previously postulated in humans (Saini et al., 2014).

IMPLICATIONS

Metabolic changes that result from acute stress factors may initially be more beneficial than previously understood and may include compensation for reduced insulin action. Conversely, other stress factors like adrenergic stimulation appear to boost insulin's metabolic actions. Together, our findings show that components of the stress response may help to maintain glucose metabolism during the initial onset of stress.

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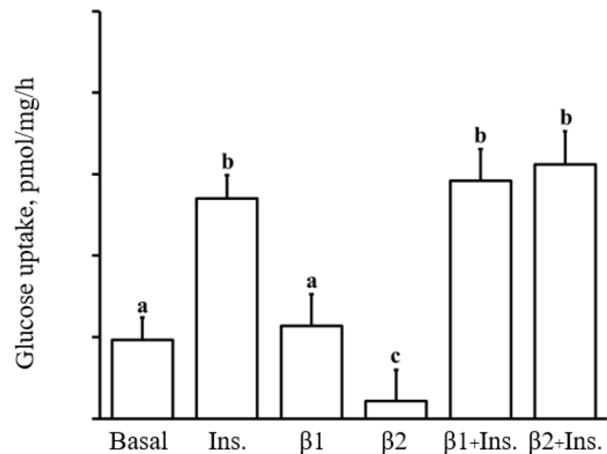

Figure 1. Glucose uptake in primary rat soleus strips (25-45 mg) during a 20-min incubation with the following treatments: none (basal), insulin (5 mU/ml Humulin-R), β1 agonist (10 µM ractopamine HCl), β2 agonist (0.5 µM zilpaterol HCl), β1+insulin, or β2+insulin. (n = 10 rats). [a,b,c] means with different superscripts differ ($P < 0.05$).

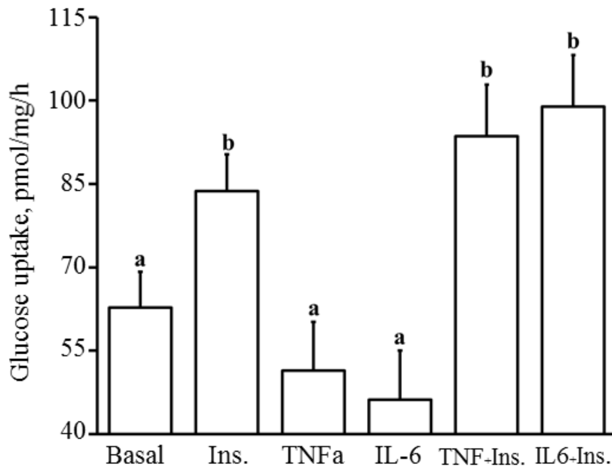

Figure 2. Glucose uptake in primary rat soleus strips (25-45 mg) during a 20-min incubation with the following treatments: none (basal), insulin (5 mU/ml Humulin-R), TNFα (20 ng/ml hTNFα), IL-6 (1 ng/ml rIL-6), TNFα+insulin, and IL-6+insulin. (n = 10 rats). [a,b] means with different superscripts differ ($P < 0.05$).

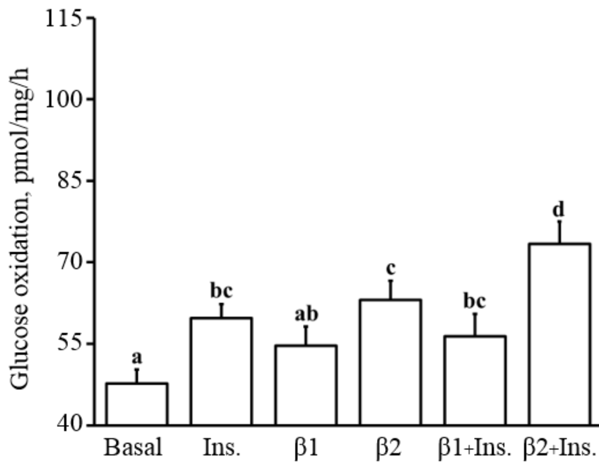

Figure 3. Glucose oxidation in primary rat soleus strips (25-45 mg) during a 2-h incubation with the following treatments: none (basal), insulin (5 mU/ml Humulin-R), β1 agonist (10 μM ractopamine HCl), β2 agonist (0.5 μM zilpaterol HCl), β1+insulin, or β2+insulin. (n = 9 rats). [a,b,c,d] means with different superscripts differ ($P < 0.05$).

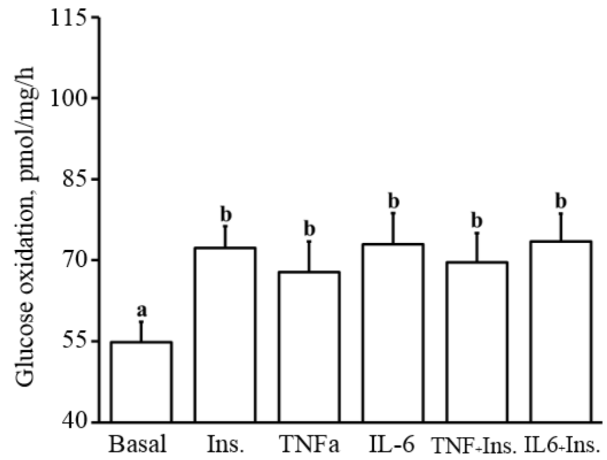

Figure 4. Glucose oxidation in primary rat soleus strips (25-45 mg) during a 2-h incubation with the following treatments: none (basal), insulin (5 mU/ml Humulin-R), TNFα (20 ng/ml hTNFα), IL-6 (1 ng/ml rIL-6), TNFα+insulin, and IL-6+insulin. (n = 9 rats). [a,b] means with different superscripts differ ($P < 0.05$).

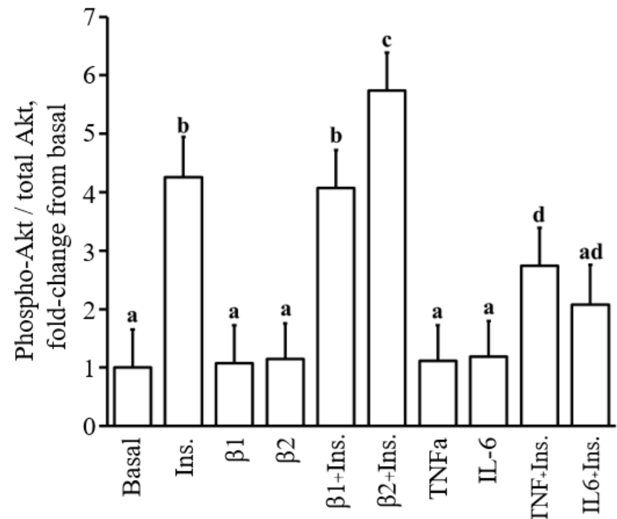

Figure 5. Akt phosphorylation in primary rat soleus strips (25-45 mg) during a 1-h incubation with the following treatments: none (basal), insulin (5 mU/ml Humulin-R), β1 agonist (10 μM ractopamine HCl), β2 agonist (0.5 μM zilpaterol HCl), β1+insulin, β2+insulin, TNFα (20 ng/ml hTNFα), IL-6 (1 ng/ml rIL-6), TNFα+insulin, and IL-6+insulin. (n = 8 rats). [a,b,c,d] means with different superscripts differ ($P < 0.05$).