Acute exposure of primary rat soleus muscle to zilpaterol HCl (β2 adrenergic agonist), TNFα, or IL-6 in culture increases glucose oxidation rates independent of the impact on insulin signaling or glucose uptake

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Abstract

Recent studies show that adrenergic agonists and inflammatory cytokines can stimulate skeletal muscle glucose uptake, but it is unclear if glucose oxidation is similarly increased. Thus, the objective of this study was to determine the effects of ractopamine HCl (β1 agonist), zilpaterol HCl (β2 agonist), TNFα, and IL-6 on glucose uptake and oxidation rates in unstimulated and insulin-stimulated soleus muscle strips from adult Sprague-Dawley rats. Effects on phosphorylation of Akt (phospho-Akt), p38 MAPK (phospho-p38), and p44/42 MAPK (phospho-p44/42) was also determined. Incubation with insulin increased (P < 0.05) glucose uptake by ~47%, glucose oxidation by ~32%, and phospho-Akt by ~238%. Insulin also increased (P < 0.05) phospho-p38, but only after 2 hours in incubation. Muscle incubated with β2 agonist alone exhibited ~20% less (P < 0.05) glucose uptake but ~32% greater (P < 0.05) glucose oxidation than unstimulated muscle. Moreover, co-incubation with insulin + β2 agonist increased (P < 0.05) glucose oxidation and phospho-Akt compared to insulin alone. Conversely, β1 agonist did not appear to affect basal or insulin-stimulated glucose metabolism, and neither β agonist affected phospho-p44/42. TNFα and IL-6 increased (P < 0.05) glucose oxidation by ~23% and ~33%, respectively, in the absence of insulin. This coincided with increased (P < 0.05) phospho-p38 and phospho-p44/42 but not phospho-Akt. Furthermore, co-incubation of muscle with insulin + either cytokine yielded glucose oxidation rates that were similar to insulin alone, despite lower (P < 0.05) phospho-Akt. Importantly, cytokine-mediated increases in glucose oxidation rates were not concomitant with greater glucose uptake. These results show that acute β2 adrenergic stimulation, but not β1 stimulation, directly increases fractional glucose oxidation in the absence of insulin and synergistically increases glucose oxidation when combined with insulin. The cytokines, TNFα and IL-6, likewise directly increased glucose oxidation in the absence of insulin, but were not additive in combination with insulin and in fact appeared to disrupt Akt-mediated insulin signaling. Rather, cytokines appear to be acting through MAPKs to elicit effects on glucose oxidation. Regardless,
stimulation of glucose oxidation by these key stress factors did not rely upon greater glucose uptake, which may promote metabolic efficiency during acute stress by increasing fractional glucose oxidation without increasing total glucose consumption by muscle.

**Graphical Abstract**

**Keywords**

β2 adrenergic agonist; glucose oxidation; IL-6; metabolic regulation; TNFα

**1. Introduction**

Skeletal muscle comprises about 40% of total body mass in humans, yet it accounts for greater than 85% of the body’s insulin-stimulated glucose utilization [1, 2]. The role of insulin in metabolic regulation is well-understood, but muscle metabolism can be influenced by additional factors including catecholamines and cytokines [3–5]. Catecholamines (i.e. epinephrine, norepinephrine) are released into circulation by the adrenal medulla and act by binding to a large class of G protein-coupled adrenergic receptors (α1A, α1B, α1C, α2A, α2B, α2D, β1, β2, and β3) [6] located throughout the body. Thus, the impact that adrenergic stimulation has on a specific tissue is a function of the specific receptor type or types that it expresses. In skeletal muscle, β2 adrenergic receptors are the most highly-expressed isoform, but β1 receptors and to a lesser extent β3 and α1D receptors are also present [7, 8]. Growth studies in animals [9] have led to the development of isoform-specific β adrenergic growth promoters that are used as feed additives in the livestock industry to increase meat yield per animal [10]. In addition, reports show that β agonists are commonly used by athletes to boost muscle growth and athletic performance, despite being restricted by the World Anti-Doping Agency [11]. However, far less is known about the effects of β agonists on muscle metabolism. The seemingly complex effects of inflammatory cytokines on metabolism are likewise only beginning to be understood. Inflammation is known to cause insulin resistance [12], yet recent studies show that two major inflammatory cytokines, TNFα and IL-6, may stimulate glucose metabolism in muscle independent of their actions on insulin signaling [4, 13, 14]. Insulin increases glucose uptake in skeletal muscle through a well-characterized signaling cascade that begins with binding of its transmembrane tyrosine kinase receptor followed by sequential activation of the downstream targets, IRS1, PI3K, and Akt via phosphorylation [15]. This canonical pathway ultimately stimulates
translocation of glucose transporter, Glut4, to the cell membrane where it is imbedded and facilitates greater glucose passage into the muscle cell [16]. Phosphorylation of Akt appears to be a critical step in most insulin-regulated events, and thus the ratio of phosphorylated Akt to total Akt is considered to be a reliable indicator of insulin signaling [17]. In addition to the canonical PI3K/Akt-mediated signal transduction pathway, mitogen-activated protein kinases (MAPKs) have also been identified as activators of Glut4-mediated glucose uptake [18, 19]. Insulin-stimulated glucose oxidation rates have long been presumed to be proportional with glucose uptake rates [20], but we postulate that this relationship may not be maintained with the additional influence of adrenergic or inflammatory factors. Moreover, it is unclear whether these factors would regulate skeletal muscle glucose metabolism through direct effects or by altering insulin signaling. Thus, the objective of this study was to determine the respective impacts of β1 and β2 adrenergic agonists, TNFα, and IL-6 on glucose uptake and oxidation rates in intact soleus muscle strips isolated from adult rats. Furthermore, we sought to determine whether these effects were insulin-associated or insulin-independent by incubating muscle strips with each factor alone or in combination with insulin.

2. Materials and Methods

2.1. 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 collected tendon-to-tendon from adult Sprague-Dawley rats (females 252.86 ± 14.93 g, males 378 ± 15.72 g) after decapitation under heavy isoflurane anesthesia were used to measure glucose uptake (n=10), glucose oxidation (n=9), and protein expression (n = 8). Males and females were spread evenly across groups. Isolated soleus muscles were washed in ice-cold phosphate buffered saline (PBS; pH 7.4), and each muscle was dissected longitudinally (tendon to tendon) into 25–45 mg strips. For all experiments, muscle strips were pre-incubated for 1 hour at 37°C in gassed (95% O₂, 5% CO₂) Krebs-Henseleit bicarbonate buffer (KHB, pH 7.4; 0.1% bovine serum albumin; Sigma-Aldrich, St. Louis, MO) spiked with respective treatment (Table 1) and 5 mM D-glucose (Sigma-Aldrich, St. Louis, MO). Treatment concentrations were consistent with those previously described [4, 21–25]. Muscle strips were then washed for 20 minutes in treatment-spiked KHB with no glucose. Pre-incubation and wash media for glucose uptake (but not glucose oxidation) experiments also contained 35 mM and 40 mM mannitol (Sigma-Aldrich), respectively. Glucose uptake and glucose oxidation rates were determined as described below. Protein analysis was performed in soleus strips from parallel incubations.

2.2. Glucose uptake

Glucose uptake rates were determined from intracellular accumulation of [³H]2-deoxyglucose as previously described [26], with some modifications. After being pre-incubated and washed, soleus strips were incubated at 37°C for 20 minutes in treatment-
spiked KHB containing 1 mM [3H]2-deoxyglucose (300 μCi/mmol) and 39 mM [1,14C] mannitol (1.25 μCi/mmol). Muscle strips were then removed, thrice washed in ice-cold PBS, weighed, and lysed in 2M NaOH (Sigma-Aldrich) at 37°C for 1 hour. Lysates were vortexed and mixed with UltimaGold scintillation fluid, and specific activity of 3H and 14C 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 10-μl aliquots mixed with 500 μl distilled water and scintillation fluid. Mannitol concentrations were used to estimate the amount of extracellular fluid in each lysate, and intracellular accumulation of 2-deoxyglucose was calculated as total 2-deoxyglucose in the lysate less the extracellular concentration. All radioactive compounds and scintillation fluids were purchased from Perkin-Elmer (Waltham, MA).

2.3. Glucose oxidation

Glucose oxidation rates were determined by oxidation of [14C-U] D-glucose as previously described [27], with some modifications. After being pre-incubated and washed, muscle strips were placed in one side of a sealed dual-well chamber and incubated at 37°C for 2 hours in treatment-spiked KHB containing 5 mM [14C-U] D-glucose (0.25 μCi/mmol). NaOH (2M) was placed in the adjacent well to capture CO2. After 2 hours, chambers were cooled at −20°C for 2 minutes. HCl (2M; Sigma-Aldrich) was added to the media through a rubber seal on the top of the chamber, and the chambers were then incubated at 4°C for 1 hour to release bicarbonate-bound CO2 from the media. Finally, chambers were unsealed and each muscle strip was washed and weighed. NaOH was collected from each chamber, mixed with UltimaGold scintillation fluid, and analyzed by liquid scintillation to determine specific activity of captured 14CO2. Specific activity of media was determined as described above.

2.4. Western Immunoblot

The respective activities of Akt and MAPKs were estimated by the proportions of phosphorylated target protein to total target protein as previously described [28–31], with minor modifications. Target protein concentrations were determined in soleus strips that were incubated in treatment-spiked KHB for 1 or 2 hours and then snap-frozen and stored at −80°C. Each muscle strip was thoroughly homogenized in 200 μl of radioimmunoprecipitation buffer containing manufacturer-recommended concentrations of Protease and Phosphatase Inhibitor (Thermo Fisher, Carlsbad, CA). Homogenates were then sonicated and centrifuged (14,000 × g for 5 minutes at 4°C), and supernatant was collected. Total protein concentrations were determined by Pierce BCA Protein Assay Kit (Thermo Fisher). Protein samples (35 μg) were boiled for 5 minutes at 95°C in BioRad 4x Laemmli Sample Buffer (BioRad, Hercules, CA) and then separated by SDS-polyacrylamide. Gels were transferred to polyvinylidene fluoride low fluorescence membranes (BioRad), which were incubated in Odyssey block solution (Li-Cor Biosciences, Lincoln, NE) for 1 hour 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 4°C with one of the following rabbit antibodies diluted in Odyssey block solution + 0.05% Tween-20: anti-Akt (1:1,000), anti-phospho-Akt (Ser473) (1:2,000), p44/42 MAPK (1:2,000), phospho-p44/42 MAPK (Thr202/Tyr204) (1:1,000), p38 MAPK (1:1,000), or phospho-p38 MAPK.
(Thr180/Tyr182) (1:1,000) (Cell Signaling, Danvers, MA). An IR800 goat anti-rabbit IgG secondary antibody (1:10,000 for Akt; 1:5,000 for MAPKs; Li-Cor) diluted in Odyssey block solution with 0.05% Tween-20 and 0.01% SDS was applied for 1 hour at room temperature. Blots were scanned on an Odyssey Infrared Imaging System and analyzed with Image Studio Lite Software (Li-Cor). For each protein of interest, phosphorylation rates were estimated by the proportions of phosphorylated protein to total protein (phospho-Akt, phospho-p38, phospho-p44/42, respectively).

2.5. Statistical analysis

All data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, Cary, NC). Each data point was derived from the average of 2 technical replicates (i.e. in each experiment, each condition was studied in 2 muscle strips from each rat). Four separate metabolic experiments were performed, each with its own non-stimulated and insulin-only conditions as well as its own group of rats: glucose uptake with adrenergic agonists (n = 10), glucose uptake with cytokines (n = 10), glucose oxidation with adrenergic agonists (n = 9), and glucose oxidation with cytokines (n = 9). Likewise, separate groups of rats were used to determine protein concentrations in soleus muscle strips after 1 hour (n = 8) or 2 hours (n = 4) in incubation. Sex of the donating rat was initially included as a covariate but did not affect any of the measured variables and was removed from the model. Data are presented as mean ± standard error.

3. Results

3.1. Glucose uptake and oxidation rates

3.1a. Adrenergic agonist stimulation—Glucose uptake and oxidation rates were greater (P < 0.05) for muscle incubated in media spiked with insulin compared to muscle incubated in un-spiked (basal) media (Figure 1). When muscle was incubated in media spiked with β1 agonist alone, glucose uptake and oxidation rates did not differ from unstimulated muscle (incubated in basal media). Moreover, when muscle was co-incubated with insulin + β1 agonist, glucose uptake and oxidation did not differ from muscle incubated with insulin alone. Conversely, muscle incubated with β2 agonist alone exhibited decreased (P < 0.05) glucose uptake and increased (P < 0.05) glucose oxidation compared to unstimulated muscle. Moreover, glucose uptake rates did not differ between muscle incubated with insulin alone or with insulin + β2 agonist, but glucose oxidation rates were greater (P < 0.05) for muscle incubated with insulin + β2 agonist compared to muscle incubated with either insulin alone or β2 agonist alone.

3.1b. Inflammatory cytokine stimulation—As with the adrenergic agonist studies, glucose uptake and oxidation rates were greater (P < 0.05) for muscle incubated with insulin compared to unstimulated muscle (Figure 2). Glucose uptake rates for muscle incubated with TNFα alone or IL-6 alone did not differ from rates in unstimulated muscle. Likewise, glucose uptake for muscle incubated with insulin + TNFα or insulin + IL-6 did not differ from muscle incubated with insulin alone. However, glucose oxidation rates for muscle incubated with TNFα alone or IL-6 alone were greater (P < 0.05) than for unstimulated muscle and, in fact, were similar to muscle incubated with insulin alone. Interestingly,
glucose oxidation rates for muscle incubated with insulin + TNFα, or insulin + IL-6 did not differ from muscle incubated with insulin alone, TNFα alone, or IL-6 alone.

### 3.2. Akt phosphorylation

Muscle incubated with insulin exhibited greater ($P < 0.05$) phospho-Akt than unstimulated muscle after 1 and 2 hours in incubation (Figure 3). Conversely, phospho-Akt did not differ between unstimulated muscle and muscle incubated with β1 agonist, β2 agonist, TNFα, or IL-6 alone at either time point. After 1 hour, phospho-Akt did not differ between muscle incubated with insulin alone or insulin + β1 agonist but was greater ($P < 0.05$) in muscle incubated with insulin + β2 agonist. After 2 hours, phospho-Akt was greater ($P < 0.05$) in muscle incubated with either insulin + β1 agonist or insulin + β2 agonist than in unstimulated muscle but was less ($P < 0.05$) than in muscle incubated with insulin alone. Conversely, phospho-Akt was less ($P < 0.05$) in muscle incubated with insulin + TNFα or with insulin + IL-6 than in muscle incubated with insulin alone. In fact, phospho-Akt did not differ between unstimulated muscle and muscle incubated with insulin + IL-6.

### 3.3. MAPK phosphorylation

#### 3.3a. p38 MAPK—

After 1 hour in incubation, phospho-p38 did not differ between unstimulated muscle and muscle incubated with insulin alone, β1 agonist alone, β2 agonist alone, insulin + β1 agonist, insulin + β2 agonist, or insulin + IL-6 (Figure 4). However, muscle incubated with TNFα alone, IL-6 alone, or insulin + TNFα exhibited greater ($P < 0.05$) phospho-p38 after 1 hour than unstimulated muscle or muscle incubated with insulin alone. After 2 hours, muscle incubated with insulin alone exhibited greater ($P < 0.05$) phospho-p38 than unstimulated muscle, but no other groups differed from unstimulated muscle.

#### 3.3b. p44/42 MAPK—

After 1 hour, phospho-p44/42 did not differ between unstimulated muscle and muscle incubated with insulin alone, β1 agonist alone, β2 agonist alone, or insulin + β1 agonist (Figure 5). However, phospho-p44/42 was lower ($P < 0.05$) in muscle incubated with insulin + β2 agonist compared to unstimulated muscle. Conversely, muscle incubated with TNFα alone, IL-6 alone, insulin + TNFα, or insulin + IL-6 exhibited greater ($P < 0.05$) phospho-p44/42 than unstimulated muscle or muscle incubated with insulin alone. No difference in phospho-p44/42 was observed among any groups after 2 hours in incubation.

### 4. Discussion

In this study, we show that acute stimulation of skeletal muscle with either β2 adrenergic agonist or inflammatory cytokines can increase glucose oxidation rate independent of insulin activity. Moreover, we show that these increases in glucose oxidation are not dependent upon concomitant increases in glucose uptake. Adrenergic effects on glucose metabolism were isoform-specific, as β2 agonist reduced basal glucose uptake, increased basal glucose oxidation, and synergistically enhanced insulin-stimulated glucose oxidation and Akt phosphorylation, but β1 agonist had no discernable effects on glucose metabolism or insulin signaling. Inflammatory cytokines antagonized insulin signaling but simultaneously
stimulated skeletal muscle glucose oxidation, seemingly via MAPK-mediated signaling pathways. Together, our findings indicate that acute exposure to these stress factors can increase glucose oxidation independently of changes in glucose uptake and insulin signaling. This demonstrates a role for stress mediators in regulation of energy homeostasis that may be important in both stressed and unstressed states.

Acute β2-specific adrenergic stimulation in the absence of insulin had contrasting effects on skeletal muscle glucose uptake and oxidation rates, as glucose uptake was reduced but glucose oxidation was improved. Previous studies in humans and animals have indicated that glucose oxidation rates are typically proportional to glucose utilization rates across a variety of physiological conditions [32, 33], yet our findings show that fractional glucose oxidation by soleus muscle grew substantially during acute β2 adrenergic stimulation, independent of the rate of glucose uptake. This may help to explain previously reported reductions in (whole-body) fractional glucose oxidation rates in growth-restricted fetal sheep [34], which exhibit reduced expression of β2 receptors in muscle, fat, and other tissues [35–38]. In addition to increasing basal glucose oxidation rates, acute β2 adrenergic stimulation synergistically enhanced the effects of insulin on skeletal muscle glucose oxidation, as the two factors together elicited a greater combined impact than either factor individually. The additive effect of β2 adrenergic agonist and insulin appeared to be mechanistically facilitated by greater activation of the canonical signaling component, Akt, which is a key mediator for most of insulin’s intracellular effects [39]. Conversely, β1 adrenergic stimulation produced no such additive effect with insulin for glucose uptake or oxidation and did not appear to have any direct impact on the metabolic outputs measured in this study. One possible explanation for the distinctly different impacts of the respective β agonists in the present study is the substantially lower expression of β1 adrenergic receptors in muscle relative to β2 receptors. Kim et al. [7] showed that the β1 isoform comprises only about 15–21% of the total β receptor population expressed by the soleus muscle of the adult rat and that the β2 isoform can account for essentially all β receptors in some muscles. Similar β receptor populations were found in human muscle biopsies [40, 41]. The present findings also help explain greater muscle growth efficiency in food animals supplemented with dietary β2 agonist compared to β1 agonist [42–44].

Acute exposure of muscle to the inflammatory cytokines, TNFα and IL-6, antagonized insulin signaling, as expected, but stimulated glucose oxidation directly. Inflammation has long been linked to insulin resistance [45–47], and this effect was evident in our study by the reduction of insulin-stimulated Akt phosphorylation after 2-hour exposure to either TNFα or IL-6. Despite this apparent impairment of insulin signaling, glucose uptake and oxidation rates were nonetheless similar between muscle stimulated with insulin alone or co-stimulated with insulin and cytokines, indicating direct stimulation by cytokines. Moreover, cytokines were able to stimulate glucose oxidation in the absence of insulin. Together, these results show that inflammatory cytokines increase glucose metabolism in skeletal muscle independently of their impact on canonical insulin signaling. Furthermore, we show that the direct effects on glucose oxidation are not dependent upon parallel changes in glucose uptake. Previous studies have shown that short-term exposure to IL-6 acutely increases glucose uptake in humans and in human muscle biopsies via AMPK-mediated signaling pathways rather than PI3K or Akt-mediated pathways [4, 48, 49]. Acute exposure of culture-
derived C2C12 myotubes to TNFα likewise increased glucose uptake, as well as glucose utilization [14]. It is unclear why cytokines did not increase basal glucose uptakes rates in the present study, but one possible explanation is the relatively short exposure time, as other studies have found that similar durations of IL-6 stimulation required co-incubation with the IL-6 receptor in order to observe increased glucose uptake [48, 50]. Nevertheless, it is noteworthy that greater glucose oxidation was independent of glucose uptake rates in the present study. Glund et al. [4] previously found a moderate increase in glucose oxidation rates in IL-6 stimulated human muscle biopsies that was attributed to a concomitant increase in glucose uptake. However, we observed an ~36% increase in glucose oxidation with no increase in glucose uptake. Cytokine-stimulated increases in glucose oxidation were paralleled by greater phosphorylation of p38 and p44/42 MAPKs. Greater MAPK activity has been associated with increased glucose oxidation in cardiac myocytes [51] and may likewise represent an Akt-independent mechanism for increasing glucose oxidation in skeletal muscle, much the same way that greater AMPK activation is thought to increase glucose uptake [4, 48, 50].

5. Conclusions

Our findings show that acute exposure of adult rat soleus muscle to β2 adrenergic agonist or to inflammatory cytokines stimulates greater glucose oxidation. Furthermore, we found that this effect was independent of insulin action and was not contingent upon increases in glucose uptake. In fact, muscle stimulated with TNFα and IL-6 showed evidence of decreased insulin signaling but greater MAPK activity, indicating that MAPKs may be important mediators of cytokine-induced glucose oxidation. Conversely, β2 adrenergic stimulation enhanced basal and insulin-stimulated glucose oxidation without affecting Akt or MAPK activity. The increase in fractional glucose oxidation rates appears to indicate greater skeletal muscle metabolic efficiency at the onset of the stress response, which may help to improve outcomes of acute adverse events.

Acknowledgments

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>phopho-Akt</td>
<td>phosphorylated Akt</td>
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<tr>
<td>phopho-p38</td>
<td>phosphorylated p38 MAPK</td>
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<tr>
<td>phopho-p44/42</td>
<td>phosphorylated p/44/42 MAPK</td>
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References


Highlights

- Inflammatory cytokines and β2 agonists stimulate skeletal muscle glucose oxidation.
- Greater oxidation is independent of glucose uptake rates or insulin activity.
- β2 agonists also enhance insulin-stimulated glucose oxidation and Akt activation.
- TNFα and IL-6 impaired insulin action but increased glucose oxidation directly.
Figure 1.
Glucose uptake and oxidation rates in primary rat soleus muscle during 20-minute (n = 10) or 2-hour incubation (n = 9), respectively, with isoform-specific adrenergic agonists. a,b,c,d means with different superscripts differ (P < 0.05).
Figure 2.
Glucose uptake and oxidation rates in primary rat soleus muscle during 20-minute (n = 10) or 2-hour incubation (n = 9), respectively, with inflammatory cytokines. \(^{a,b}\) means with different superscripts differ (\(P < 0.05\)).
Figure 3.
Akt phosphorylation in primary rat soleus muscle after 1 hour (n = 8) or 2 hours (n = 4) in incubation with isoform-specific adrenergic agonists or cytokines. a,b,c,d,e,f means with different superscripts differ (P < 0.05).
Figure 4.
p38 MAPK phosphorylation in primary rat soleus muscle after 1 hour ($n = 8$) or 2 hours ($n = 4$) in incubation with isoform-specific adrenergic agonists or cytokines. $a,b$ means with different superscripts differ ($P < 0.05$).
Figure 5. p44/42 MAPK phosphorylation in primary rat soleus muscle after 1 hour (n = 8) or 2 hours (n = 4) in incubation with isoform-specific adrenergic agonists or cytokines. \textsuperscript{a,b,c} means with different superscripts differ ($P < 0.05$).
Table 1

Components of each treatment media.

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<th>Additive3</th>
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<td>b. Insulin</td>
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<td>c. β1 Agonist</td>
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<td>d. β2 Agonist</td>
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<td>e. Insulin + β1 Agonist</td>
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<td>f. Insulin + β2 Agonist</td>
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<td>g. TNFα</td>
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</table>

1 Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 0.1% bovine serum albumin, gassed with 95% O2 + 5% CO2.

2 All additives were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of Humulin R, which was purchased from Eli Lilly (Indianapolis, IN).