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# Contribution of the serine kinase c-Jun N-terminal kinase (JNK) to oxidant-induced insulin resistance in isolated rat skeletal muscle

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## Abstract

The specific and direct contribution of the stress-activated serine kinase c-Jun N-terminal kinase (JNK) in the development of oxidative stress-induced insulin resistance of the glucose transport system in mammalian skeletal muscle is not fully understood. We assessed the specific role of JNK in the development of insulin resistance caused by *in vitro* exposure of rat soleus muscle to low levels (30–40 μM) of the oxidant hydrogen peroxide ($H_2O_2$) for up to 6 h. Oxidant exposure caused significant ($p < 0.05$) decreases in insulin-stimulated glucose transport activity (up to 42%) and Akt $Ser^{473}$ phosphorylation (up to 67%), and increased (up to 74%) phosphorylation ($Thr^{183}$/$Tyr^{185}$) of JNK1 and JNK2/3 isoforms. Importantly, insulin-stimulated glucose transport activity in the presence of $H_2O_2$ was moderately improved with the selective JNK inhibitor SP600125. These results indicate that activation of the serine kinase JNK contributes, at least in part, to oxidative stress-induced insulin resistance in isolated mammalian skeletal muscle.

### Keywords

Hydrogen peroxide; soleus muscle; glucose transport; Akt serine phosphorylation; SP600125

## Introduction

A primary site of impaired insulin action leading to the development of type 2 diabetes is skeletal muscle, affecting mainly the glucose transport system (Abdul-Ghani and DeFronzo, 2010; DeFronzo, 1997, 2004, 2009; DeFronzo and Ferannini, 1991; Henriksen *et al.*, 2011; Zierath *et al.*, 2000). While the aetiology of insulin resistance in skeletal muscle is multi-factorial, the intracellular mechanisms underlying insulin resistance all seem to affect the translocation of GLUT-4-containing vesicles to the plasma membrane by impairing the functionality or protein expression of elements of the canonical insulin signalling pathway

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**Declaration of interest**

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(Abdul-Ghani and DeFronzo, 2010; DeFronzo 2004, 2009; Henriksen *et al.*, 2011; Holloszy and Hansen, 1996).

One important cause of insulin resistance in skeletal muscle is oxidative stress, the overproduction of reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$) (Davies, 1995; Diamond-Stanic *et al.* 2011; Evans *et al.*, 2002; Henriksen *et al.*, 2011; Sies, 1997). ROS are normally produced in cells by the mitochondria or by NADPH oxidase, but cellular compensatory mechanisms, such as superoxide dismutases and peroxidases, maintain them at low levels (Davies, 1995; Evans *et al.*, 2002; Sies, 1997). When these antioxidant mechanisms begin to falter, ROS levels increase and oxidative stress occurs. Excess ROS can lead to various cellular dysfunctions, including insulin resistance of the glucose transport system (Henriksen *et al.*, 2011).

Oxidants can engage various stress-activated serine kinases, such as glycogen synthase kinase-3 (GSK-3) and p38 mitogen-activated protein kinase (p38 MAPK), as demonstrated previously in cultured muscles cells (Blair *et al.*, 1999; Cirialdi *et al.*, 2007; Nikoulina *et al.,* 2002) and in isolated mammalian skeletal muscle, such as rat soleus (Diamond-Stanic *et al.*, 2011; Dokken *et al.*, 2008). An additional important stress-activated kinase, c-Jun N-terminal kinase (JNK), has also been shown previously to be associated with oxidative-stress induced insulin resistance (Hirosumi *et al.*, 2002; Solinas and Karin, 2010), but its direct contribution to insulin resistance in isolated mammalian skeletal muscle under highly defined incubation conditions of oxidant excess has not been quantified. When activated, these various serine kinases can phosphorylate numerous substrates, ultimately interfering with or deactivating critical insulin signalling elements, such as IRS-1 and Akt (Archuleta *et al.*, 2009; Blair *et al.*, 1999; Diamond-Stanic *et al.*, 2011; Dokken *et al.*, 2008; Evans *et al.*, 2002; Henriksen *et al.*, 2011).

While the direct contributions of GSK-3 and p38 MAPK to the aetiology of oxidant-induced insulin resistance in mammalian skeletal muscle have been previously demonstrated and quantified (Dokken *et al.*, 2008; Diamond-Stanic *et al.*, 2011), the direct effects of JNK in the development of oxidative stress-induced insulin resistance in mammalian skeletal muscle are not fully understood. Therefore, this study was designed to assess the specific impact of JNK in the development of insulin resistance caused by *in vitro* exposure of mammalian skeletal muscle to low levels of a known oxidant, $H_2O_2$. In the present study, isolated soleus strips from lean Zucker rats with normal insulin sensitivity were used as the model of mammalian skeletal muscle. The effect of *in vitro* exposure to low levels (30–40 μM) of $H_2O_2$ on basal and insulin-stimulated glucose transport activity and Akt functionality were performed to measure the degree of insulin resistance induced by this oxidant. Moreover, the impact of this oxidant stress on the activation of JNK isoforms (JNK1 and JNK2/3) was determined. Finally, a selective JNK inhibitor, the anthrapyrazolone SP600125 (Bennett *et al.*, 2001; Li *et al.*, 2005), was used to assess the specific role of JNK in the development of this oxidant-induced insulin resistance in mammalian skeletal muscle.

## Methods

### Animals

All procedures used were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Female lean Zucker rats (Harlan, Indianapolis, IN) were used at 6–8 weeks of age (body weights of 130–150 g). Animals were housed in a temperature-controlled (20–22°C) room with a 12:12 h light–dark cycle, and the animals had free access to chow (Teklad 7001, Madison, WI) and water. At 5 pm the evening before each

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experiment, animals were restricted to 4 g of chow, which was consumed immediately. Experiments commenced the next morning between 8 and 9 am.

## Muscle incubations and exposure to $H_2O_2$ and SP600125

Animals were deeply anaesthetized with pentobarbital sodium (50 mg/kg), and soleus muscle strips (~25–35 mg) were prepared for *in vitro* incubation in the unmounted state. Muscles were initially incubated for 2–6 h at 37°C in oxygenated (95% $O_2$/5% $CO_2$) Krebs–Henseleit buffer (KHB) containing 8 mM glucose, 32 mM mannitol, and 0.1% bovine serum albumin (Sigma Chemical, St Louis, MO), with or without 5 mU/ml insulin (Humulin, Eli Lilly, Indianapolis, IN) and/or 50 mU/ml glucose oxidase (MP Biomedicals, Solon, OH). The incubation medium was changed after every 2 h of treatment. The $H_2O_2$ level in the medium was measured spectrophotometrically (Diamond-Stanic *et al*., 2011; Kim *et al*., 2006) and reached 30–40 μM. In a second set of experiments, muscle strips were incubated for 6 h in the presence or absence of insulin with or without glucose oxidase and/or the selective JNK inhibitor SP600125 (Bennet *et al.*, 2001; Li *et al.*, 2005) (10 μM; EMD Chemicals, Gibbtown, NJ).

## Assessment of glucose transport activity

Glucose transport activity was assessed by determination of the intracellular accumulation of 2-deoxyglucose (2-DG, 1 mM) as described previously (Henriksen and Jacob, 1995). Briefly, after the initial incubation period, the muscles were rinsed for 10 min at 37°C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1% BSA, and insulin, glucose oxidase, and/or SP600125, if present previously. Following the rinse period, muscles were transferred to 2 ml of KHB containing 1 mM 2-deoxy-[1,2-$^{3}$H]glucose (0.3 mCi/mmol; Sigma Chemical), 39 mM [U-$^{14}$C]mannitol (0.8 mCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, and insulin, glucose oxidase, and/or SP600125, if previously present, and incubated for 20 min at 37°C. At the end of this final incubation period, muscles were removed and quickly frozen in liquid nitrogen, weighed, and placed in 0.5 ml of 0.5 mM NaOH. After the muscles were completely solubilized, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of 2-[$^{3}$H]DG was determined as described previously (Hansen *et al.*, 1994).

## Determination of signalling protein expression and functionality

In some experiments, muscles were frozen after the initial incubation period, weighed, and stored at −80°C until analysis. Muscles were homogenized in eight volumes of ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM Na pyrophosphate, 20 mM *β*-glycerophosphate, 10 mM NaF, 2 mM $Na_3VO_4$, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mM PMSF). Homogenates were incubated on ice for 20 min and then centrifuged at 13,000 *g* for 20 min at 4°C. Total protein concentration was determined using the BCA method (Pierce, Rockford, IL). Samples containing equal amounts of total protein were separated by SDS–PAGE on 10% or 12% polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated overnight with antibodies against phosphorylated Akt Ser$^{473}$ (Cell Signaling Technology, Danvers, MA), for 72 h with antibodies against phosphorylated JNK Thr$^{183}$/Tyr$^{185}$ (Cell Signaling), or overnight with antibodies against total Akt or total JNK (Cell Signaling). The membranes were then incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (Chemicon, Temecula, CA) or anti-mouse antibody conjugated with HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized using a Bio-Rad Chemidoc XRS instrument (Bio-Rad Laboratories, Hercules, CA) using the SuperSignal West Femto Maximum Sensitivity

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Western blot detection substrate (Pierce). Band density was quantified using the Bio-Rad Quantity One software.

### Statistical analysis

All values are expressed as means ± SEM for 4–5 muscles/group. Paired Student's *t*-tests were used to assess the specific effects of $H_2O_2$ or the JNK inhibitor SP600125 on group means. A *p*-value of $p < 0.05$ was considered to be statistically significant.

## Results

### Effects of low-level oxidant stress on glucose transport activity

Soleus muscle strips were incubated in 30–40 $\mu$M $H_2O_2$ in the absence or presence of insulin for 2, 4 or 6 h. In the absence of insulin, the $H_2O_2$ significantly ($p < 0.05$) increased basal glucose transport activity at 2 and 4 h, but not at 6 h (Figure 1). However, oxidant-induced decreases in insulin-stimulated glucose transport occurred at 2 h (23%), 4 h (25%) and 6 h (42%) (all $p < 0.05$).

### Effect of low-level oxidant stress on insulin signalling

The $H_2O_2$ had no effect on the basal phosphorylation of Akt $Ser^{473}$ at any time point, but did inhibit insulin-stimulated phosphorylation of Akt $Ser^{473}$ by 37, 57, and 67% ($p < 0.05$) at 2, 4, and 6 h, respectively (Figure 2).

### Effect of low-level oxidant stress on engagement of JNK

The responses to the oxidant intervention for phosphorylation of JNK are shown in Figure 3. For the final analysis, data from the JNK1 and JNK2/3 isoforms were pooled. At 2 and 4 h, there were significant (46% and 86%, respectively, both $p < 0.05$) overall increases in JNK phosphorylation in the presence of $H_2O_2$ under basal conditions. This effect, however, had disappeared by 6 h. At all time points, there were significant increases in JNK phosphorylation induced by $H_2O_2$ in the presence of insulin: 35% at 2 h, 74% at 4 h, and 55% at 6 h (all $p < 0.05$). Insulin alone had no effect on this parameter at any time point.

### Specific role of JNK in oxidant-stress-induced insulin resistance

To determine if the activation of JNK contributes to this $H_2O_2$-induced insulin resistance in isolated mammalian skeletal muscle, the selective JNK inhibitor, SP600125 (10 μM), was utilized in 6-h incubations. Treatment with SP600125 significantly ($p < 0.05$) decreased basal glucose transport activity (Figure 4, first and second bars from left), and this effect of SP600125 was maintained in the presence of $H_2O_2$ alone (Figure 4, third and fourth bars from left). SP600125 caused a small, but statistically insignificant, decrease in insulin-stimulated glucose transport activity (Figure 4, fifth and sixth bars from the left), likely a carryover from the effect of SP600125 on basal glucose transport activity. In muscles that were incubated with insulin, $H_2O_2$, and SP600125 in combination, the rate of glucose transport activity measured experimentally (332 ± 15 pmol/mg muscle/20 min) (Figure 4, ninth bar from left) was considerably greater than the theoretical value (261 pmol/mg muscle/20 min) (Figure 4, eighth bar from left) calculated by accounting for the decreases in insulin-stimulated glucose transport activity due to $H_2O_2$ or SP600125 individually. This theoretical additive value assumes that $H_2O_2$ and SP600125 impaired insulin-stimulated glucose transport activity through separate pathways and that JNK did not have a direct effect on oxidative-stress induced insulin resistance. The theoretical additive value was calculated as the rate of insulin-stimulated glucose transport activity (440 pmol/mg muscle/20 min) minus the effect of $H_2O_2$ on insulin-stimulated glucose transport activity (137 pmol/mg muscle/20 min) and also minus the decrease in basal glucose transport due to SP600125

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(42 pmol/mg muscle/20 min). Since the actual experimental value is greater than this theoretical additive value, these data are consistent with the interpretation that JNK mediates, at least in part (~30%), the effects of the oxidant stress on insulin-stimulated glucose transport activity in skeletal muscle.

## Discussion

The purpose of the present study was to assess, for the first time, the direct role of the stress-activated serine kinase JNK in the development of insulin resistance in mammalian skeletal muscle in response to an *in vitro* oxidant stress under highly defined incubation conditions (Figures 1 and 2). The results of this study show that JNK activation (Figure 3) is necessary, at least in part, for the ability of low levels (30–40 μM) of the oxidant $H_2O_2$ to induce insulin resistance of glucose transport activity in an isolated rat skeletal muscle preparation (Figure 4).

While previous studies have shown that JNK activation is associated with oxidative stress-induced insulin resistance (Blair *et al.*, 1999; Hirosumi *et al.*, 2002; Solinas and Karin, 2010), we have demonstrated this relationship in isolated mammalian skeletal muscle (Figure 3), with direct exposure of this tissue to the oxidant $H_2O_2$ causing an increase in JNK phosphorylation and therefore activation. Indeed, all three JNK isoforms present in skeletal muscle (JNK1, JNK2, and JNK3) were engaged by this low-level oxidant stress (Figure 3). Our findings are consistent with those from a previous study using the L6 myotube cell line, in which JNK activation was increased as much as 8-fold in the presence of high concentrations of $H_2O_2$ (Blair *et al.*, 1999). Moreover, the selective inhibition of JNK using the compound SP600125 (Bennett *et al.*, 2001; Li *et al.*, 2005) in the presence of both insulin and $H_2O_2$ resulted in a partial reversal of the insulin resistance of glucose transport activity (Figure 4). These latter results provide solid evidence supporting an important role of JNK in oxidative stress-induced insulin resistance in mammalian skeletal muscle.

It is important to note that JNK is not the only serine kinase that is activated by oxidative stress or that can have an effect on insulin resistance in mammalian skeletal muscle. The activation of other serine kinases, such as GSK-3 (Archuleta *et al.*, 2009; Dokken *et al.*, 2008) and p38 MAPK (Archuleta *et al.*, 2009; Blair *et al.*, 1999; Diamond-Stanic *et al.*, 2011; Kim *et al.*, 2006), by oxidative stress is also mechanistically connected with the development of insulin resistance of glucose transport acivity. Interestingly, none of these serine kinases, including JNK, can account for more than 25–30% of the insulin resistance caused by $H_2O_2$ (Figure 4; Archuleta *et al.*, 2009; Diamond-Stanic *et al.*, 2011; Dokken *et al.*, 2008; Henriksen, 2010; Henriksen *et al.*, 2011). It is possible that oxidative stress induces insulin resistance in mammalian skeletal muscle by simultaneously engaging all of these pathways, and maybe others that have not been extensively studied in this context, such as p70S6 kinase and IKK-$\beta$. Previous studies that have shown the direct roles of GSK-3 and p38 MAPK (Archuleta *et al.*, 2009; Diamond-Stanic *et al.*, 2011; Dokken *et al.*, 2008) by using selective inhibitors, including the present study (Figure 4), suggest that using multiple serine kinase inhibitors in combination could potentially normalize insulin action under conditions of oxidative stress; this approach should be evaluated in future investigations.

While the kinase inhibitor used in this study, SP600125, as well as other kinase inhibitors used in previous studies (Archuleta *et al.*, 2009; Diamond-Stanic *et al.*, 2011; Dokken *et al.*, 2005, 2008; Kim *et al.*, 2006), have been shown to have metabolic benefits, caution should be expressed before fully accepting their usefulness. Whereas the benefits seem obvious for skeletal muscle, it is very possible that these inhibitors, when administered systemically

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(Dokken and Henriksen, 2006), will act on many other tissues and cells in the experimental subject. It is possible that these same serine kinases that contribute to oxidative stress-induced insulin resistance in skeletal muscle could have normal regulatory functions in other cells. Inhibiting these regulatory functions could be detrimental overall and the costs might outweigh the benefits. The *in vivo* use of these kinase inhibitors should be carefully studied before approving their use as a treatment for any disease state.

In conclusion, the results of the present study indicate that activation of the serine kinase JNK does directly contribute to oxidative stress-induced insulin resistance in isolated slow-twitch skeletal muscle. However, like other stress-activated serine kinases studied under these same experimental conditions, such as GSK-3 and p38 MAPK, the engagement of JNK alone cannot account completely for the induction of insulin resistance by a low-level oxidant stress ($H_2O_2$) in this rat skeletal muscle preparation. It is likely that the simultaneous engagement of numerous stress-activated serine kinases (including JNK, GSK-3, and p38 MAPK, as well as others) must be stimulated by this oxidant stress in order to fully induce insulin resistance of the glucose transport system in mammalian skeletal muscle. This latter speculation should be addressed in a future investigation.

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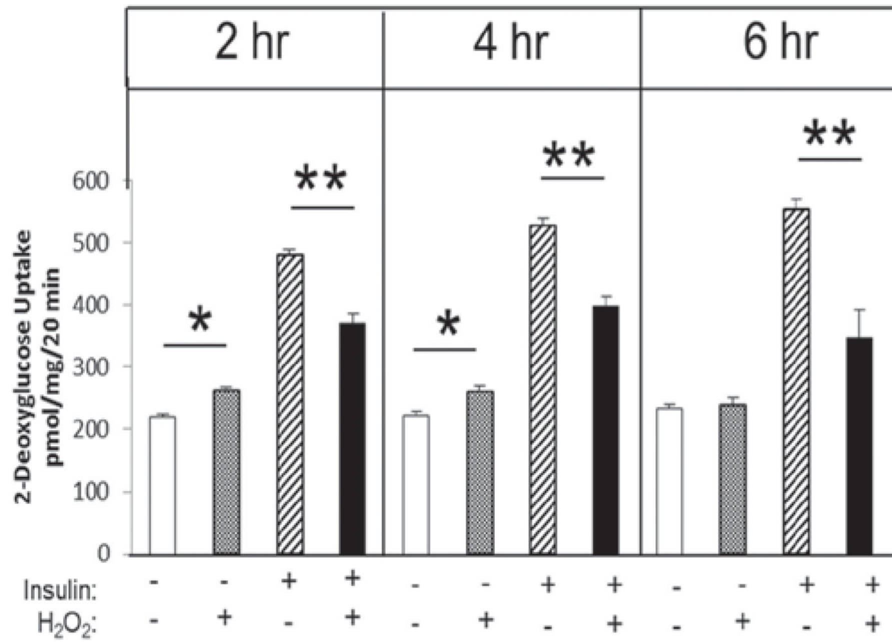


**Figure 1.**

Time course of the effect of low-level $H_2O_2$ on glucose transport activity in the absence or presence of insulin in isolated rat soleus muscle. *$p < 0.05$ vs. no $H_2O_2$; **$p < 0.05$ vs. insulin without $H_2O_2$.

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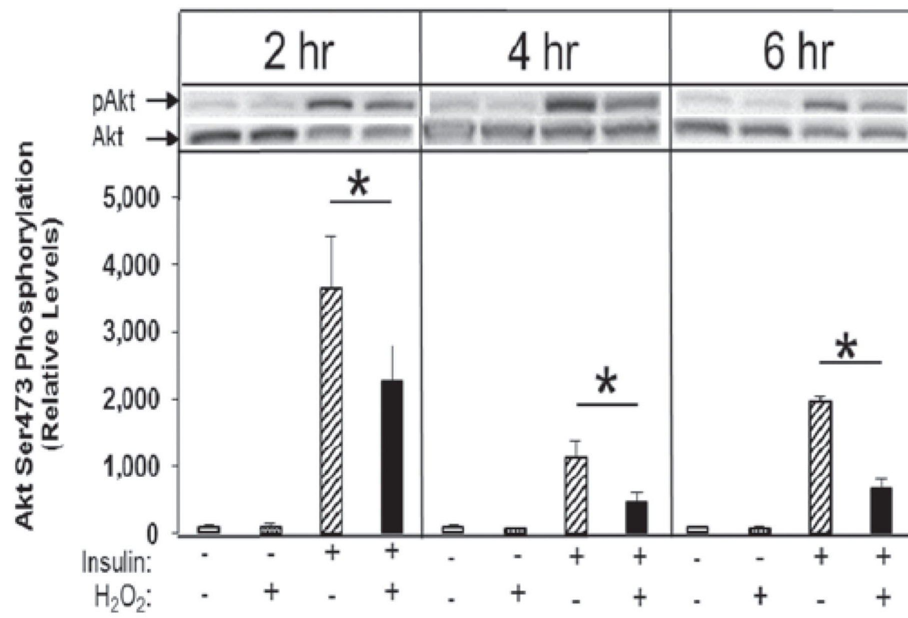


**Figure 2.**

Time course of the effect of low-level $H_2O_2$ on basal and insulin-stimulated Akt $Ser^{473}$ phosphorylation in isolated rat soleus muscle. $*p < 0.05$ vs. insulin without $H_2O_2$.

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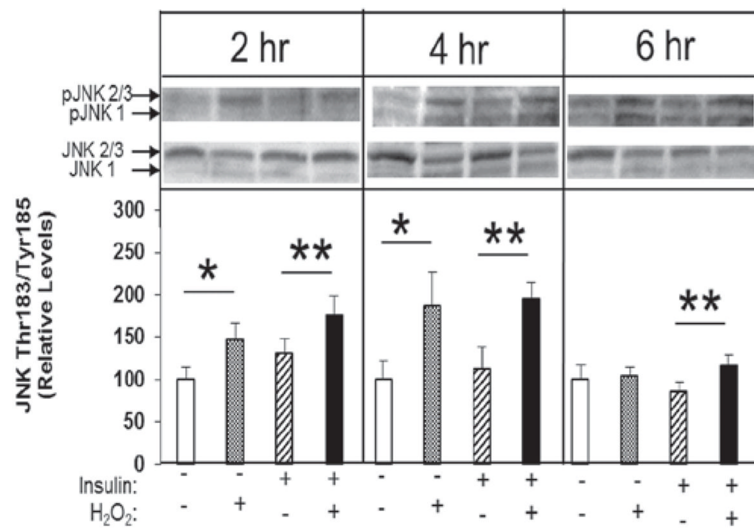


**Figure 3.**

Time course of the effects of low-level $H_2O_2$ on Thr[183]/Tyr[185] phosphorylation of JNK 1 and JNK 2/3 isoforms in the absence or presence of insulin in isolated rat soleus muscle. *$p < 0.05$ vs. no $H_2O_2$; **$p < 0.05$ vs. insulin without $H_2O_2$.

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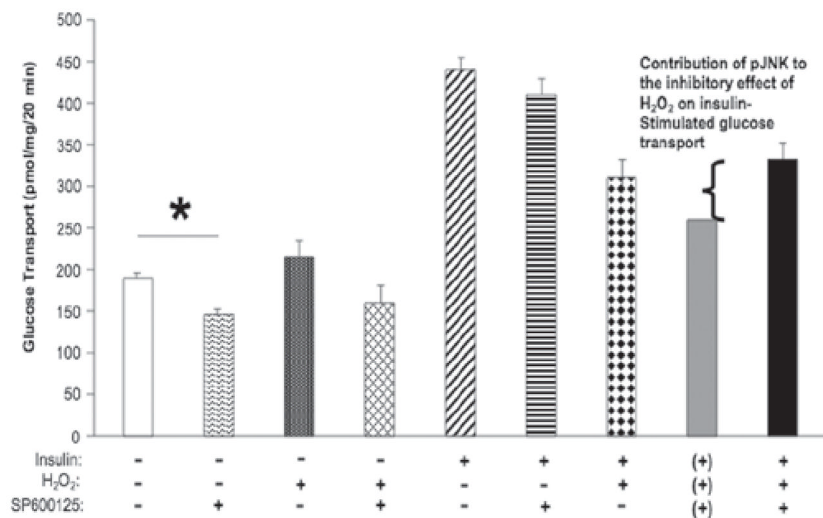


**Figure 4.**

Effects of JNK inhibitor SP600125 on $H_2O_2$-induced inhibition of insulin-stimulated glucose transport activity at 6 h in isolated rat soleus muscle. The grey bar indicates the theoretic value due to the additive effects of SP600125 and $H_2O_2$ on insulin-stimulated 2DG uptake (the theoretical conditions are indicated by the (+) symbols below the grey bar). The theoretical additive value (261 pmol/mg muscle/20 min) was calculated as the rate of insulin-stimulated glucose transport activity (440 pmol/mg muscle/20 min) minus the effect of $H_2O_2$ on insulin-stimulated glucose transport activity (137 pmol/mg muscle/20 min) and also minus the decrease in basal glucose transport due to SP600125 (42 pmol/mg muscle/20 min). *$p < 0.05$ vs. no additions.