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Critical role of the transient activation of p38 MAPK in the etiology of skeletal muscle insulin resistance induced by low-level in vitro oxidant stress

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Abstract

Increased cellular exposure to oxidants may contribute to the development of insulin resistance and type 2 diabetes. Skeletal muscle is the primary site of insulin-dependent glucose disposal in the body; however, the effects of oxidative stress on insulin signaling and glucose transport activity in mammalian skeletal muscle are not well understood. We therefore studied the effects of a low-level in vitro oxidant stress (30–40 μM H_2O_2) on basal and insulin-stimulated (5 mU/ml) glucose transport activity and insulin signaling at 2, 4, and 6 hr in isolated rat soleus muscle. H_2O_2 increased basal glucose transport activity at 2 and 4 hr, but not at 6 hr. This lowlevel oxidant stress significantly impaired insulin-stimulated glucose transport activity at all time points, and was associated with inhibition of insulin-stimulated phosphorylation of Akt Ser^{473} and GSK-3β Ser^{9}. In the presence of insulin, H_2O_2 decreased total protein expression of IRS-1 at 6 hr and IRS-2 at 4 and 6 hr. Phosphorylation of p38 MAPK Thr^{180}/Tyr^{182} was transiently increased by H_2O_2 in the presence and absence of insulin at 2 and 4 hr, but not at 6 hr. Selective inhibition of p38 MAPK with A304000 partially rescued the H_2O_2-induced reduction in insulin-stimulated glucose transport activity. These results indicate that direct in vitro exposure of isolated mammalian skeletal muscle to a low-level oxidant stress impairs distal insulin signaling and insulin-stimulated glucose transport activity, at least in part, due to a p38 MAPK-dependent mechanism.

1. Introduction

Increased levels of oxidative stress are correlated with the development of whole-body insulin resistance and type 2 diabetes [1;2], and treatment of diabetic patients with the antioxidant α-lipoic acid improves whole body glucose handling [3]. In addition, exposure of insulin-sensitive 3T3-L1 adipocytes and L6 myotubes to low levels of an oxidant stress impairs insulin-stimulated glucose transport activity, a defect partially rescued by co-treatment with this antioxidant [4–6].

Skeletal muscle is the primary site of insulin-dependent glucose disposal, and insulin resistance in skeletal muscle is a major defect contributing to glucose dysregulation [7]. Oxidative stress may contribute to the development of skeletal muscle insulin resistance.

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Treatment of insulin-resistant obese Zucker rats [8] or soleus muscle strips isolated from obese Zucker rats [9] with antioxidants improves glucose transport activity. Additionally, in vitro exposure of isolated rat skeletal muscle to an oxidant stress impairs insulin-stimulated glucose transport activity [10–12].

Insulin stimulates glucose transport activity by binding to its receptor on the surface of insulin-responsive cells, inducing autophosphorylation of tyrosine residues of the β-subunit of the insulin receptor. The activated insulin receptor phosphorylates tyrosine residues of the insulin receptor substrate (IRS) proteins (IRS-1 and IRS-2 in skeletal muscle). Subsequent activation of downstream signaling elements, including phosphotidylinositol-3-kinase, phosphoinositide-dependent kinases, Akt, and the Akt-substrate protein AS160, ultimately induce translocation of vesicles containing the glucose transporter GLUT4 to the plasma membrane, where GLUT4 mediates glucose transport into the cell via facilitated diffusion [13;14]. Ex-vivo exposure of isolated rat soleus muscle to an oxidant stress suggests that the oxidative stress-induced disruption of insulin-stimulated glucose transport activity is associated with impairment of normal insulin signaling through this pathway [1;3;20].

Oxidative stress activates several stress-activated kinases, and these may play a role in the oxidant stress-induced impairment of insulin-stimulated glucose transport activity in skeletal muscle. Exposure of isolated rat skeletal muscle to a higher-level oxidant stress increases phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) [10–12] and decreases the inhibitory phosphorylation of glycogen synthase kinase 3β (GSK-3β) [10;11]. Furthermore, pharmacological inhibition of p38 MAPK [11] or GSK-3β [10] demonstrates a role for these kinases in mediating part of the negative effect of the oxidant stress on insulin-stimulated glucose transport activity.

In each of these studies, however, the data and interpretation of the results were confounded by an oxidant stress-induced increase in basal glucose transport activity [11;15]. In the present study, we sought to identify an experimental condition in which an oxidant stress-induced impairment of insulin-stimulated glucose transport activity could be achieved without the confounding effect of the intervention on basal glucose transport activity. Here, we present data demonstrating that exposure to a low-level oxidant stress impairs insulin-stimulated glucose transport activity following a 6-hour incubation without increasing glucose transport activity under basal conditions. Under these conditions, the role of p38 MAPK in mediating this effect was assessed using the specific p38 MAPK inhibitor, A304000.

2. Methods

2.1. Animals

Procedures were approved by the Institutional Animal Care and Use Committee at the University of Arizona. Female lean (Fa/?) Zucker rats (Harlan, Indianapolis, IN) were used at 7–9 weeks of age. 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 experiment, animals were restricted to 4 g of chow, which was consumed immediately. Experiments began between 8 and 9 AM the next morning.

2.2. Muscle incubations and exposure to H_2O_2 and A304000

Animals were deeply anesthetized with pentobarbital sodium (50 mg/kg ip), and strips of soleus muscle (~25–35 mg) were prepared for in vitro incubation in the unmounted state. Muscles were initially incubated for 2–6 hr 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 hr of treatment. The H_2O_2 level in the medium was measured spectrophotometrically [4] and reached 30–40 μM. In a second set of experiments, muscle strips were incubated for up to 6 hr in the presence of insulin without or with glucose oxidase and/or the p38 MAPK inhibitor A304000 (10 μM; kindly provided by Abbott Laboratories, Abbott Park, IL). A304000 is selective for p38 MAPK, and does not affect the functionality of critical signaling factors affecting glucose transport activity, including Akt, GSK-3, and AMPK [15,18, and unpublished data].

2.3. 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 [16]. Briefly, after the initial incubation period, the muscles were rinsed for 10 minutes at 37°C in 3 ml of oxygenated KHB containing 40 mM mannitol, 0.1% BSA, and insulin, glucose oxidase, and/or A304000, 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 A304000, 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 [16;17].

2.4. Determination of signaling 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 8 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 7.5% or 10% polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated overnight with antibodies against phosphorylated Akt Ser^{473} (Cell Signaling Technology, Danvers, MA), phosphorylated glycogen synthase kinase-3β (GSK-3β) Ser$^{21/9}$ (Millipore, Billerica, MA), phosphorylated p38 MAPK Thr^{180}/Tyr^{182} (Cell Signaling)), or antibodies against total Akt (Cell Signaling), GSK-3β (Millipore), p38 MAPK (Cell Signaling), IRS-1 (Millipore) or IRS-2 (Millipore). Ser^{21} phosphorylation of GSK-3α in muscle from lean Zucker rats is very low (unpublished data), and all GSK-3 data in this study are therefore restricted to Ser^9 phosphorylation. 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 Western blot detection substrate (Pierce). Band density was quantified using the Bio-Rad Quantity One software.

2.5. Statistical analysis

Data are presented as mean ± SEM. Paired Student's t-tests were employed to determine statistically significant differences in group means when soleus splits derived from the same

muscle were used to assess the specific effects of H_2O_2 or the p38 MAPK inhibitor A304000. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of low-level oxidant stress on glucose transport activity

Soleus muscle was exposed to 30–40 μM H_2O_2 in the absence or presence of insulin for 2, 4, or 6 hr. H_2O_2 alone increased basal glucose transport activity at 2 and 4 hr ($P<0.05$), but this effect had disappeared by the 6-hr time point (Fig. 1), a critical difference from our previous investigations [10;11;15]. Importantly, this low-level oxidant stress decreased ($P<0.05$) insulin-stimulated glucose transport activity, reaching 40% inhibition by 6 hr.

3.2. Effect of low-level oxidant stress on insulin signaling

H_2O_2 had no effect on basal phosphorylation of Akt Ser^{473} at any time point (Fig 2, top panels). In contrast, insulin-stimulated phosphorylation of Akt Ser^{473} was inhibited (34–69%, $P<0.05$) by H_2O_2 at 2, 4, and 6 hr. H_2O_2 alone significantly increased phosphorylation of GSK-3β Ser^9 under basal conditions at 2 and 6 hr, but not at 4 hr (Fig 2, bottom panels). Insulin-stimulated phosphorylation of GSK-3β Ser^9 was significantly decreased by H_2O_2 at all time points (23–30%, $P<0.05$).

3.3. Effect of low-level oxidant stress on total protein levels of insulin signaling elements

Interestingly, we observed that in vitro insulin treatment decreased total Akt relative to basal levels following 2 hr (basal: 100 ± 4% vs. insulin: 35 ± 3%, $P<0.05$). A similar trend was observed at 4 hr, though this did not reach the level of significance (basal: 100 ± 22% vs. insulin: 44 ± 9%, $P=0.11$). This effect of insulin was lost by 6 hr (basal: 100 ± 8% vs. insulin: 71 ± 20%, $P=0.87$).

Insulin also significantly decreased total levels of GSK-3β at 2 and 4 hr (2 hr – basal: 100 ± 8% vs. insulin: 74 ± 5%; 4 hr – basal: 100 ± 5% vs. insulin: 60 ± 11%, both $P<0.05$). However, this effect had reversed by 6 hr (basal: 100 ± 3% vs. insulin: 93 ± 8%, $P=0.33$). While H_2O_2 had no additional effect on total GSK-3β levels in the presence of insulin at 2 and 4 hr, H_2O_2 significantly reduced GSK-3β protein expression in the presence of insulin following 6 hr (insulin: 93 ± 8% vs. insulin + H_2O_2: 59 ± 8%, $P<0.05$). H_2O_2 alone had no effect on GSK-3β expression levels at any time point (data not shown).

IRS-1 protein expression was significantly decreased by H_2O_2 in the presence of insulin at 6 hr (34%, $P<0.05$) (Fig. 3). H_2O_2 had no effect on IRS-1 protein expression in the presenceof insulin at 2 or 4 hr or in the absence of insulin at any time point (data not shown). H_2O_2 also significantly decreased IRS-2 protein expression in the presence of insulin at 4 and 6 hr (36% and 51%, respectively, both $P<0.05$) (Fig. 3), but not at 2 hr. H_2O_2 in the absence of insulin did impact IRS-2 protein expression at any time point (data not shown).

3.4. Effect of oxidant stress on engagement of p38 MAPK

At 2 hr, there was as trend towards an increase in p38 MAPK phosphorylation in the presence of H_2O_2 under basal conditions (Fig 4A). There was a significant increase in p38 MAPK phosphorylation by H_2O_2 in the presence of insulin ($P<0.05$). A similar trend in p38 MAPK phosphorylation was seen at 4 hr, with a significant ($P<0.05$) H_2O_2-induced increase in the absence of insulin, and a trend towards an H_2O_2-induced increase in the presence of insulin (Fig 4A). However, this effect of H_2O_2 exposure was transient, as p38 MAPK phosphorylation was no different from control by 6-hr time point (Fig 4A). Insulin alone had no effect on this parameter at any time point.

3.5. Role of p38 MAPK in oxidant stress-induced insulin resistance

To determine if engagement of p38 MAPK contributes mechanistically to this H_2O_2-induced insulin resistance, the selective p38 MAPK inhibitor, A304000 (10 μM) [10,15,18], was utilized in 6-hr incubations. A304000 had no affect on basal glucose transport activity, but significantly decreased insulin-stimulated glucose transport activity ($P<0.05$) (Fig 4B), consistent with our previous observations [11]. In muscle exposed to insulin, H_2O_2, and A304000 in combination, the glucose transport activity measured experimentally (217 ± 7 pmol/mg muscle/20 min) was substantially greater than the theoretical value (115 pmol/mg muscle/20 min) calculated by accounting for the decreases in insulin-stimulated glucose transport activity due to H_2O_2 or A304000 individually. This theoretical additive value assumed that H_2O_2 and A304000 impair insulin-stimulated glucose transport activity through separate pathways, and was calculated as the rate of insulin-stimulated glucose transport activity (500 ± 9 pmol/mg muscle/20 min) minus the individual decreases due to H_2O_2 (226 pmol/mg muscle/20 min) and A304000 (159 pmol/mg muscle/20 min). Because the actual experimental value is greater than this theoretical additive value, these data are consistent with the interpretation that p38 MAPK mediates, at least in part, the effects of the oxidant stress on insulin-stimulated glucose transport activity in skeletal muscle. Interestingly, the selective inhibition of p38 MAPK was associated with transient increases in IRS protein expression, as IRS-1 (70%) and IRS-2 (41%) ($P<0.05$) levels in the presence of insulin and H_2O_2 were increased by A304000 after 4 hr, but had returned to control levels by 6 hr. Therefore, the altered expression of these IRS proteins is not directly involved with the enhanced insulin action in the 6-hr oxidant-exposed skeletal muscle in which p38 MAPK has been inhibited.

4. Discussion

In the present study, we have demonstrated in mammalian skeletal muscle that a low-level oxidant stress (30–40 μM H_2O_2) impairs insulin-stimulated glucose transport activity following a 6-hr exposure without a confounding increase in basal glucose transport activity. Furthermore, we have demonstrated that this impairment is associated with reduced engagement of important distal insulin signaling elements (including Akt and GSK-3) and with decreases in IRS-1 and IRS-2 protein expression. In addition, our data provide further support for a critical role of p38 MAPK in mediating oxidant-induced insulin resistance in muscle.

While we have previously demonstrated that exposure of isolated soleus muscle to a higher oxidant level (60–90 μM H_2O_2) results in insulin resistance following 2 and 4 hr exposures [10;11], interpretation of these results was confounded by an increase in basal glucose transport activity caused by H_2O_2 [10;11;15]. In the present study, the incubation period was extended to 6 hr, and a lower oxidant level was used (30–40 μM H_2O_2). This lower oxidant level significantly inhibited insulin-stimulated glucose transport activity at 2, 4, and 6 hr, and was associated with similarly decreased phosphorylation of Akt Ser^{473}. Importantly, this oxidant exposure did not cause a sustained increase in basal glucose transport activity or an increase in basal Akt Ser^{473} phosphorylation. Therefore, the present study demonstrates for the first time an experimental design utilizing mammalian skeletal muscle in which exposure to an oxidant stress impairs insulin-stimulated insulin signaling and glucose transport activity without a confounding effect on these parameters in the absence of insulin.

Insulin alone significantly suppressed total protein expression of both Akt and GSK-3β at 2 and 4 hr, and exposure to the oxidant stress had no additional impact on these parameters. Protein expression of Akt and GSK-3β both recovered to basal levels by the 6-hr time point in the presence of insulin alone. Interestingly, H_2O_2 inhibited the recovery of GSK-3β protein expression at the 6-hr time point, although it had no effect on Akt expression. To the

best of our knowledge, this is the first report of a significant decrease (albeit transient) in Akt and GSK-3β protein expression in response to insulin in isolated skeletal muscle. We speculate that the decrease in protein expression is likely due to increased rates of protein degradation, not decreased rates of protein synthesis, as the effect is rapid, and seen after only 2 hr of incubation. In cultured bovine adrenal chromaffin cells, LiCl and SB216763, inhibitors of GSK-3β, increased GSK-3β phosphorylation and decreased total Akt protein expression via a proteosome-independent mechanism over a 24-hr period [19]. This finding suggests that the insulin-induced inhibition of GSK-3β, evidenced by the increase in GSK-3β phosphorylation, may feed back to increase degradation of Akt.

Oxidant exposure decreased protein expression of IRS-1 at 6 hr and IRS-2 at 4 and 6 hr in the presence of insulin, but not in the absence of insulin. We have previously demonstrated that exposure of soleus muscle to a higher oxidant level decreased IRS-1 and IRS-2 protein expression at 4 hr [11]. It is likely that the lack of a decrease in IRS-1 at the 4-hr time point in the present study was due to the lower level of oxidant employed. Other groups have demonstrated decreased IRS-1 expression in rat hepatoma cells and 3T3-L1 adipocytes in response to insulin via a proteosome-dependent mechanism, or in response to an oxidant stress via a proteosome-independent mechanism [20;21]. The effect of insulin and H_2O_2 in combination was not tested in those investigations. Interestingly, in the present study, insulin or H_2O_2 individually did not alter IRS-1 or IRS-2 protein expression; rather, the combination of the two was required to decrease IRS-1 and IRS-2 expression levels. This discrepancy between our study and previous studies may be due to the type of tissue studied or the model system used (whole tissue versus cultured immortalized cells), and certainly requires further investigation.

We have previously demonstrated that a higher level of oxidant stress exposure causes phosphorylation of p38 MAPK at 2 and 4 hr, and this effect is insulin-independent [11]. In the present study, we have extended and elaborated upon these previous findings by employing a lower oxidant exposure and extending the incubation time to 6 hr. The lower level of oxidant stress increased p38 MAPK phosphorylation at 2 and 4 hr, similar to what was previously observed with the higher level of oxidant exposure. Interestingly, this effect was transient, and had disappeared by the 6-hr time point. These p38 MAPK data correlate well with the glucose transport data, which showed a small increase in basal glucose transport activity at 2 and 4 hr, but not at 6 hr. These data provide further support for the concept that the oxidant-induced increase in glucose transport activity under basal conditions is due to activation of p38 MAPK [15].

We previously used the selective p38 MAPK inhibitor, A304000, in 4-hr experiments that support a role for p38 MAPK in mediating oxidant-induced insulin resistance [11]. Importantly, in the present investigation we have demonstrated that early inhibition of p38 MAPK with A304000 is important in preventing a substantial portion of the insulin resistance caused by a longer-term exposure to a low-level oxidant stress, even at a time point (6 hr) when the initial increase in p38 MAPK phosphorylation had disappeared. In our previous study [11], interpretation of the data of the effect of the p38 MAPK inhibitor A304000 on glucose transport activity was confounded by the oxidant-induced increase in basal glucose transport activity [11]. This was certainly not an issue in the present study, and the present data clearly demonstrate a role for p38 MAPK in mediating, at least in part, the effect of the oxidant stress on insulin-stimulated glucose transport activity in mammalian skeletal muscle.

In conclusion, levels of oxidative stress are increased in insulin-resistant humans [1;2]. In the present study, we have demonstrated that low levels of an oxidant stress in vitro induce insulin resistance of insulin signaling and glucose transport activity in mammalian skeletal

muscle, and underscore the critical role of the transient enhancement of p38 MAPK activity in the mechanism responsible for this action of H_2O_2 on the insulin-dependent glucose transport system. Further research is necessary to determine the exact biochemical mechanisms by which this stress-activated serine kinase mediates the deleterious effects of low-level oxidative stress on the insulin-dependent glucose transport system in mammalian skeletal muscle.

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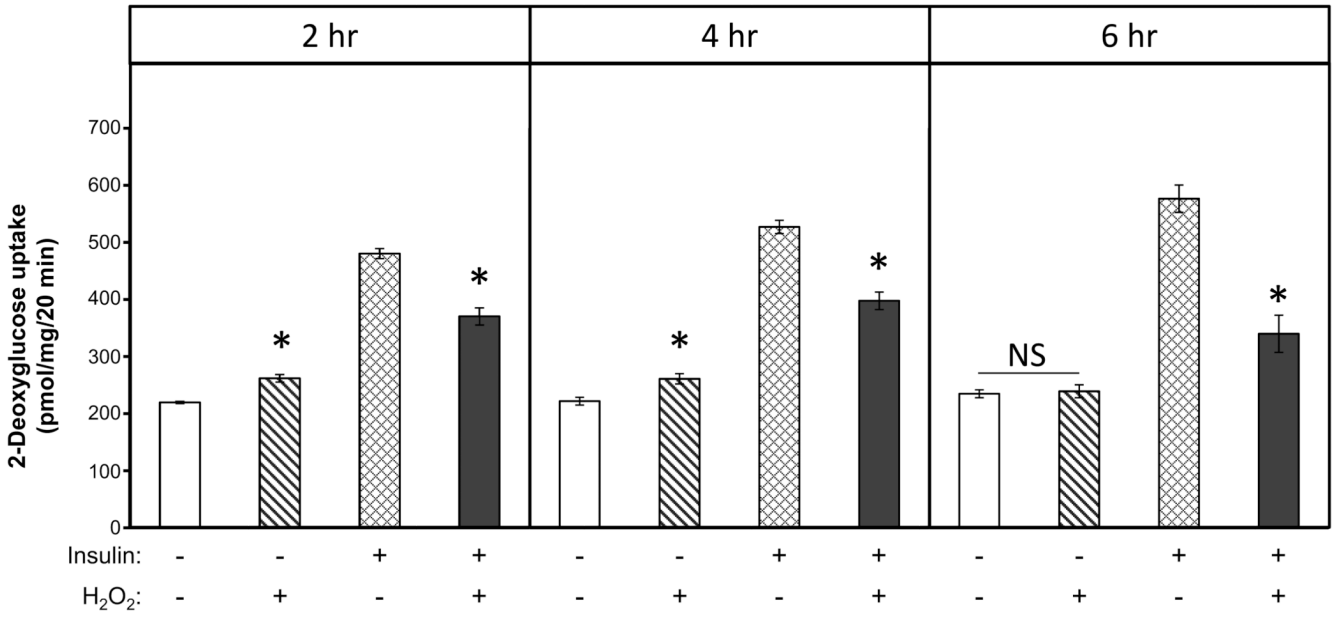

Fig. 1.

Effect of 2-, 4-, or 6-hr exposure to low-level oxidant stress on insulin-stimulated glucose transport activity in skeletal muscle. Values are mean ± SEM for 4 muscles per group. *P<0.05 vs. paired muscle in the presence of insulin without H_2O_2.

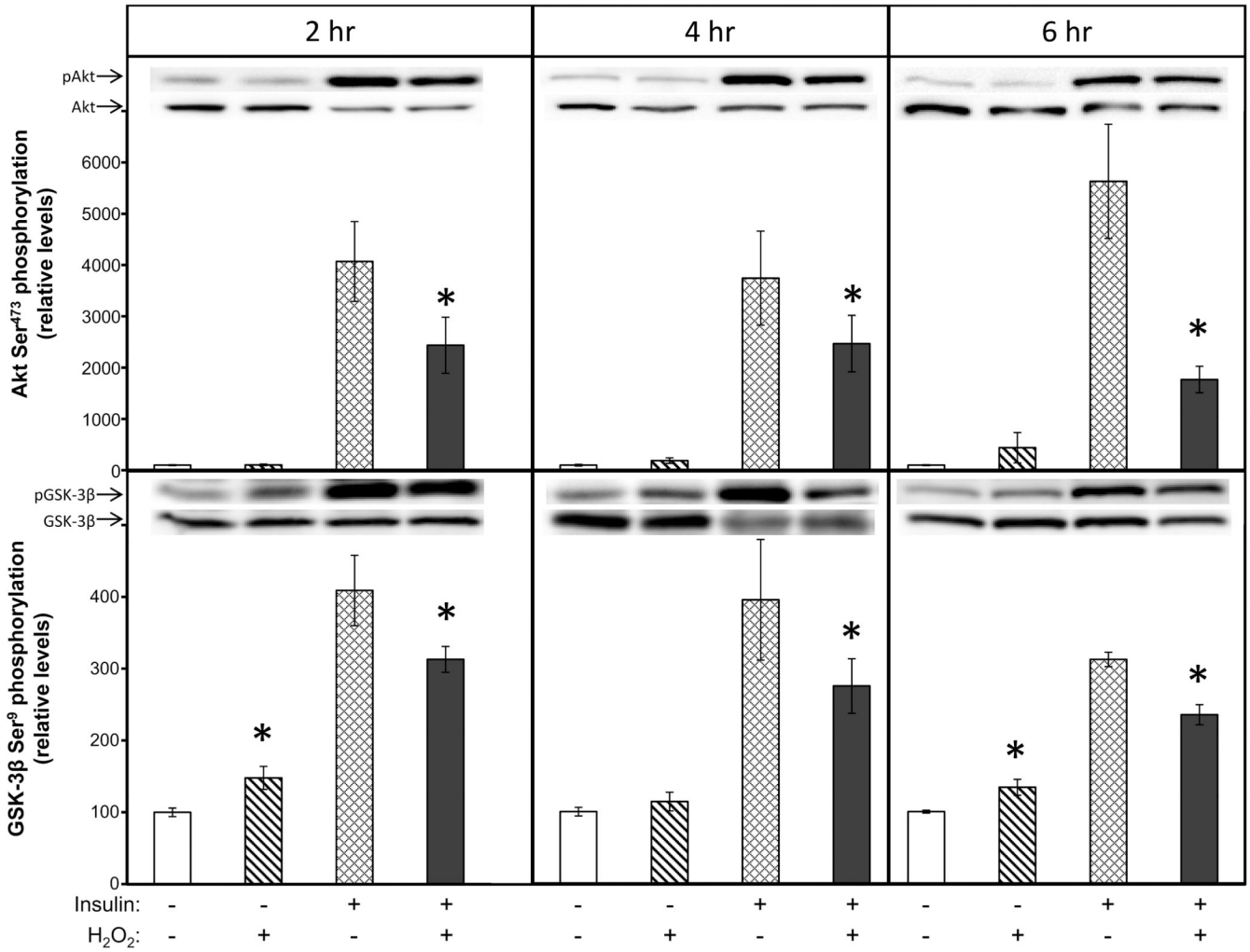

Fig. 2.

Effect of 2-, 4-, or 6-hr exposure to low-level oxidant stress on basal and insulin-stimulated phosphorylation of Akt Ser^{473} and GSK-3β Ser_9 in skeletal muscle. Values are the ratio of phosphorylated protein to total protein and are expressed as mean ± SEM for 3–8 muscles per group. *P<0.05 vs. paired muscle in the presence of insulin without H_2O_2.

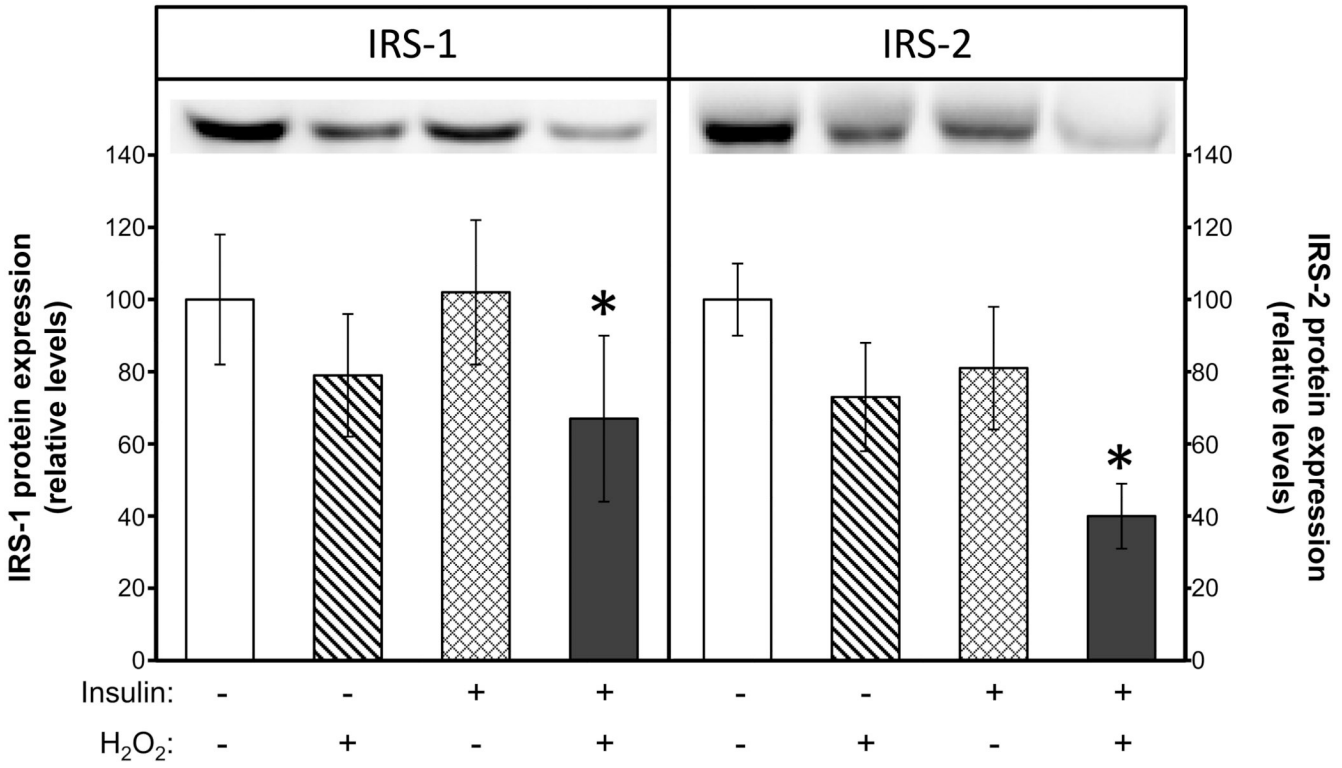

Fig. 3.

Effect of 6-hr exposure to low-level oxidant stress on IRS-1 and IRS-2 protein expression in skeletal muscle. Values are mean ± SEM for 5–9 muscles per group. *P<0.05 vs. paired muscle in the presence of insulin without H_2O_2.

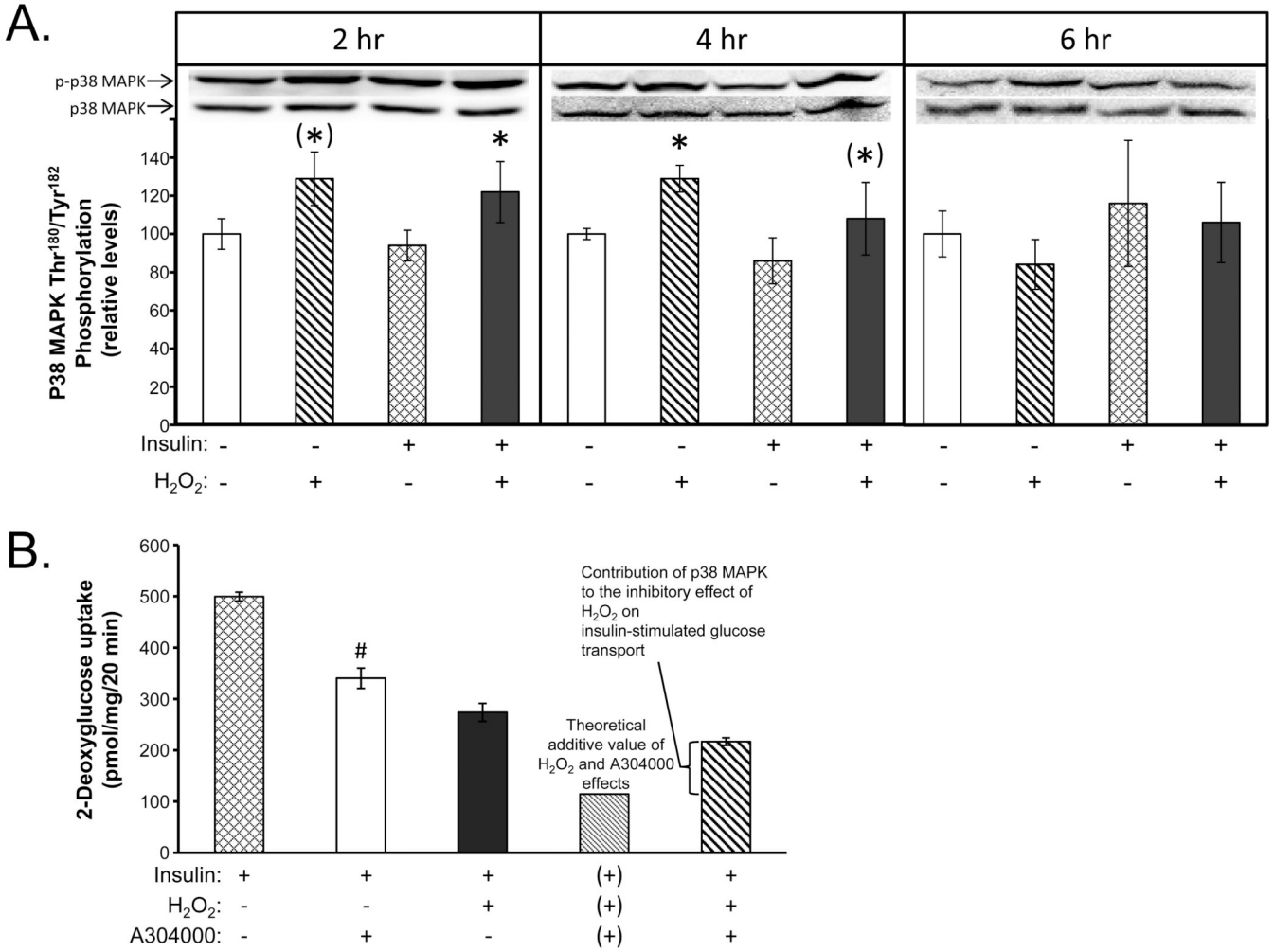

Fig. 4.

Panel A. Effect of 2-, 4-, or 6-hr exposure to low-level oxidant stress on phosphorylation of MAPK Thr^{180}/Tyr^{182} in skeletal muscle in the absence or presence of insulin. Values are the ratio of phosphorylated protein to total protein and are expressed as mean ± SEM for 4–8 muscles per group. *P<0.05, (*) P<0.1 vs. paired muscle in the presence of insulin without H_2O_2; Panel B. Effect of the p38 MAPK inhibitor, A304000 (10 μM), on basal and insulin-stimulated glucose transport activity following a 6-hr incubation in skeletal muscle in the absence or presence of insulin and/or H_2O_2. Values are mean ± SEM for 4 muscles per group. # P<0.05 vs. paired muscle in the presence of insulin without A304000.