

In vitro oxidant stress causes preferential loss of IRS proteins in rat skeletal muscle.

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Abstract

Oxidative stress, the imbalance of oxidant production and the antioxidant defenses in the cell, can impair the functionality of elements of the insulin-signaling pathway that regulates glucose metabolism in skeletal muscle. In this context, the primary purpose of the present investigation was to characterize the effects of shorter-term (2 hr) and longer-term (4 hr) oxidative stress on protein expression of critical insulin signaling elements in rat skeletal muscle. Soleus muscles from insulin-sensitive lean Zucker rats were exposed in vitro to an oxidant stress (60-90 μ M hydrogen peroxide produced by glucose oxidase) for 2 or 4 hr. The oxidant stress reduced ($p < 0.05$) insulin-stimulated glucose transport activity after shorter-term (50%) or longer-term (53%) exposure. Moreover, the diminution of insulin action in the presence of the oxidant stress after 4 hr was associated with decreased protein levels of the primary insulin receptor substrate (IRS) isoforms expressed in mammalian skeletal muscle, IRS-1 (59%) and IRS-2 (33%). In contrast, this longer-term oxidant stress did not alter the protein expression of other important insulin signaling factors (insulin receptor- β subunit, the p85 regulatory subunit of phosphatidylinositol-3-kinase, Akt, and glycogen synthase kinase-3). In addition, the stress-activated p38 mitogen-activated protein kinase (p38 MAPK) was activated after both 2 hr and 4 hr of oxidant exposure. The selective p38 MAPK inhibitor (10 μ M A304000) prevented a significant portion (40%) of the oxidant stress-induced loss of IRS-1, but not IRS-2. These data indicate that in vitro oxidative stress can lead to a selective loss of IRS-1 and IRS-2 proteins in rat skeletal muscle. The activation of p38 MAPK appears to be important in the oxidant-induced loss of IRS-1, but not IRS-2, protein in skeletal muscle.

Introduction

The insulin signaling pathway for the activation of glucose transport is a cascade that begins when insulin binds to the α -subunits of the insulin receptor (IR), which leads to an increase in the activity of tyrosine kinase of the β -subunits [12]. This results in the phosphorylation of IR substrates (IRS). There are numerous isoforms of IRS expressed in cells (IRS-1, 2, 3, and 4), with IRS-1 and IRS-2 being the primary isoforms expressed in skeletal muscle [12]. The tyrosine-phosphorylated IRS protein complexes with phosphoinositide-3 kinase (PI3-kinase) by docking at its 85-kd subunit (p85). The IRS-p85 complex then allows activation of the catalytic subunit (p110) of PI3-kinase. Lipid moieties produced by the activated PI3-kinase allosterically enhance the activity of phosphoinositide-dependent kinase (PDK). PDK is a serine/threonine kinase that can phosphorylate Protein Kinase B (Akt) and atypical forms of protein kinase C (PKC) and activate them. The activated Akt and atypical PKC isoforms, in turn, stimulate the intracellular movement of GLUT-4 to the plasma membrane [12].

Molecular regulation of the glucose transport system is also stimulated by contractions, and involve pathways that are independent of PI-3 kinase and include the action of 5'-AMP-activated kinase (AMPK) [9, 12].

The impairment of insulin-stimulated glucose transport in skeletal muscle (termed insulin resistance) plays a major role in the development of type 2 diabetes [13]. Defects in the activation of critical steps in the insulin signaling cascade, including IR and IRS-1, underlie the diminished ability of insulin to stimulate glucose transport in skeletal muscle [12]. Insulin resistance in skeletal muscle is often accompanied by the metabolic syndrome, a clustering of atherogenic risk factors in the same individual. The metabolic syndrome is characterized by

metabolic and cardiovascular abnormalities, such as obesity, hypertension, type 2 diabetes, hyperinsulinemia, dyslipidemia, and atherosclerosis [11].

One factor contributing to the multifactorial etiology of the insulin resistance is oxidative stress. Oxidative stress is the imbalance of oxidants and the antioxidant defenses in the cell [7]. The excess amount of oxidants in cells and tissues impairs the insulin signaling pathways that regulate glucose metabolism. Various studies using insulin-sensitive cell lines, such as 3T3-L1 adipocytes and L6 myocytes [1-3], have shown that exposure to oxidative stress decreases insulin-stimulated glucose transport and causes a selective degradation of the IRS-1. Moreover, when using a selective inhibitor of the stress-activated p38 MAPK in adipocytes, a significant portion of the stress-induced loss of IRS-1 protein is prevented [3].

Another cause for accelerated degradation of insulin signaling proteins and IRS proteins is the activation of the ubiquitin proteasome system. In rat models, catabolic conditions include increased ubiquitin conjugation to proteins, accumulation of ubiquitin conjugated proteins in the muscle, and inhibition of accelerated muscle proteolysis when proteasome activity is blocked [5]. Prolonged treatment with insulin-like growth factor-I and high concentrations of insulin can induce degradation of IRS-1 and IRS-2 via the ubiquitin proteasome pathway [4]. Thus, the ubiquitin proteasome system controls many cellular pathways by direct degradation of proteins via enzymatic reactions.

In the context of the foregoing information, the primary purpose of the present investigation was to characterize the short-term (2 hr) and long-term (4 hr) effects of oxidative stress on protein expression and functionality of insulin signaling factors in rat skeletal muscle. An associated purpose was to assess the role of p38 MAPK in the modulation of IRS proteins in skeletal muscle exposed to the oxidant stress. We provide evidence that in vitro oxidative stress

leads to a selective loss of IRS-1 and IRS-2 proteins in rat muscle. The activation of p38 MAPK appears to be important in the oxidant-induced loss of IRS-1, but not IRS-2, protein in skeletal muscle.

Methods

Animals

Female lean (Fa/-) Zucker rats (approximately 7-8 weeks of age) were obtained from Harlan (Indianapolis, IN) one week in advance of muscle incubations. A temperature-controlled room (20-22°C) with a 12:12-h light-dark cycle (lights on from 7AM to 7PM) at the University of Arizona Central Animal Facility was used to house the animals. The University of Arizona Animal Use and Care Committee approved all procedures involving the animals.

Skeletal muscle incubations and oxidant exposure

Food was restricted to 4 g of chow at 5 PM the evening before the experiment. The animals were deeply anesthetized using an intraperitoneal injection of pentobarbital sodium (50mg/kg body wt). Both soleus muscles were dissected and prepared for incubation [6]. Each muscle was split into two strips (~35-50 mg each). Muscles were incubated for 2 or 4 hours at 37°C in 3 ml of oxygenated (95% O₂-5% CO₂) in Krebs-Henseleit buffer (KHB) supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% BSA (radioimmunoassay grade, Sigma Chemical), in the absence or presence of a maximally effective concentration of insulin (5 mU/ml; Humulin, Eli Lilly, Indianapolis, IN), and in the absence or presence of 100 mU/ml glucose oxidase (MP Biomedicals, Inc., Solon, OH), which produced the oxidant hydrogen

peroxide (H₂O₂) at a concentration of 60-90 μM [8]. The incubation buffer was changed after each hour of treatment.

Determination of muscle glucose uptake

Following the 2-hr or 4-hr incubations, the muscles were rinsed for 10 min at 37°C in 3 ml of KHB containing 40 mM mannitol, 0.1% BSA, and insulin and glucose oxidase where appropriate. The muscles were then transferred to 2 ml of KHB containing 1 mM 2-deoxy-[1,2-³H]glucose (2-DG) (300 μCi/mmol; Sigma Chemical), 39 mM [U-¹⁴C]mannitol (0.8 mCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, and insulin and glucose oxidase if previously present. After this final 20-min incubation at 37°C, the muscles were removed, trimmed of excess fat and connective tissue, and quickly frozen between aluminum blocks cooled in liquid nitrogen. The frozen muscle was split into two pieces and the mass of each piece recorded. The specific intracellular accumulation of 2-DG was determined in one piece as described previously [6].

Protein expression and functionality

At the end of the 2 or 4 hour incubations, muscles were trimmed, frozen, and weighed. Frozen 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 Na₃VO₄, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM MgCl₂, 1 mM CaCl₂, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mM PMSF). After homogenization, the homogenate was placed on ice for 20 min. The samples were then centrifuged at 13,000 X g for 20 min at 4°C. The supernatant was removed and stored at -80°C.

Protein concentration was determined using the BCA method (Sigma Chemical). Using 7.5% or 12% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), the insulin signaling proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The protein expression and function of relevant insulin signaling factors was determined as described previously [10]. The membranes were incubated with the appropriate dilution of commercially available antibodies to determine protein expression of IRS-1 (Upstate Biotechnology, Lake Placid, NY), IRS-2 (Upstate Biotechnology), the p85 regulatory subunit of PI3-kinase (Upstate Biotechnology) GSK-3 β (Upstate Biotechnology), p38 (H-147):sc-7149 (Santa Cruz Biotechnology, Santa Cruz, CA), Phospho-p38 MAP Kinase (Thr180/Tyr182) Antibody (Cell Signaling Technology, Beverly, MA), or insulin receptor β -subunit (IR- β) (Santa Cruz Biotechnology, Santa Cruz, CA). After the primary antibody treatment, the membranes were incubated in secondary goat anti-rabbit antibody conjugated with HRP (Chemicon, Temecula, CA). The nitrocellulose was then placed in an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia, Piscataway, NJ) in preparation for exposure to film. Kodak X-Omat AR films (Kodak, Rochester, NY) were then used to visualize the proteins. Densitometry was performed on the autoradiographs to determine the intensities of each protein band, using the Bio-Rad imaging densitometer (Model GS-800) with Quantity One software.

The incubated muscle strips were also used to evaluate the extent of Akt phosphorylation. The membranes were incubated with antibodies against ser⁴⁷³ on Akt (Cell Signaling Technology). Primary and secondary antibody incubations and protein band visualization were performed using the above-mentioned techniques.

Statistical Analysis

Data are presented as means \pm SE. Differences between groups were analyzed using Student's unpaired *t*-test. A *p*-value ≤ 0.05 was considered statistically significant.

Results

The effects of the oxidant H₂O₂ (2 hr vs. 4 hr exposure) on glucose transport activity in rat skeletal muscle in the absence or presence of insulin are shown in Figure 1. In the absence of insulin, there was a significant increase (23%, *P*<0.05) in glucose transport activity in the soleus muscle following a 2-hr oxidant exposure, and 38% increase (*P*<0.05) following the 4-hr exposure. In contrast, insulin-stimulated glucose transport was decreased by 50% (*P*<0.05) following a 2-hr oxidant exposure, and insulin-stimulated glucose transport was decreased by 53% (*P*<0.05) following a 4-hr exposure to the oxidant.

Figure 2 displays the effects of the oxidant H₂O₂ (2 hr vs. 4 hr exposure) on IRS-1 protein expression. In the absence of insulin, there was no significant alteration in IRS-1 protein expression after either a 2-hr or 4-hr exposure to the oxidant. In contrast, under insulin-stimulated conditions, IRS-1 protein expression tended to be decreased by 22% following a 2-hr H₂O₂ exposure, and decreased by 59% (*P*<0.05) following a 4-hr exposure to the oxidant.

Figure 3 demonstrates the effects of 2 hr vs. 4-hr exposure to the oxidant H₂O₂ on the protein expression of IRS-2. In the absence of insulin, there was no significant alteration in IRS-2 protein expression after either a 2-hr or 4-hr exposure to the oxidant. Under insulin-stimulated conditions, IRS-2 protein expression was decreased by 33% (*P*<0.05) following the 4-hr exposure to the oxidant.

The effects of the oxidant H₂O₂ (4 hr) on the protein expression of four critical elements of the insulin-signaling cascade (IR-β, the p85 regulatory subunit of PI3-kinase, Akt, and GSK3) are shown in Figure 4. In the absence or presence of insulin, there were no significant changes in the protein expression of these insulin-signaling proteins in response to the oxidant stress.

The stress-activated p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation was activated after both 2 hr (49%, P<0.05) and 4 hr (37%, P<0.05) of oxidant exposure (Figure 5). The selective inhibition of p38 MAPK (using 10 μM A304000) prevented a significant portion (40%, P<0.05) of the oxidant stress-induced loss of IRS-1, but not IRS-2, after a 4-hr exposure.

Discussion

The primary finding of the present study was that oxidative stress (2-4 hr exposure to the oxidant H₂O₂) adversely affects insulin-stimulated glucose transport activity in rat skeletal muscle (Fig. 1). Moreover, this diminution of insulin action in the presence of an oxidant stress was associated with decreased protein expression of the primary IRS isoforms expressed in mammalian skeletal muscle, IRS-1 and IRS-2 (Figs. 2 and 3). The loss of IRS-1 and IRS-2 proteins was much greater under insulin-stimulated conditions following a 4-hr exposure to the oxidant (59% and 33% respectively) relative to the 2-hr time point (Figs. 2 and 3).

In addition, we have demonstrated in this study that long-term (4 hr) exposure in vitro to an oxidant stress caused a preferential loss of IRS-1 and IRS-2 proteins in mammalian skeletal muscle, with the preservation of other important insulin signaling factors (IR-β, the p85 regulatory subunit of PI3-kinase, Akt, and GSK3) (Fig. 4).

The exposure of mammalian skeletal muscle to the oxidant stress caused a significant increase in the phosphorylation of the stress-activated p38 MAPK (Fig. 5). Using a selective p38 MAPK inhibitor (10 μ M A304000), a significant portion of the oxidant stress-induced loss of IRS-1, but not IRS-2, was prevented (Fig. 6). These data indicate that in vitro oxidative stress can lead to a selective loss of IRS-1 and IRS-2 proteins in rat skeletal muscle. Interestingly, these data show that a significant portion of the oxidant-induced loss of IRS-1 involved a p38 MAPK-dependent mechanism, whereas the loss of IRS-2 in response to the oxidant stress was independent of the activation of p38 MAPK. Additional experimental work is needed to define the exact role of p38 MAPK in this oxidant-induced loss of IRS-1.

The present study was undertaken to examine the effects of long-term oxidative stress on glucose transport activity and insulin resistance in mammalian skeletal muscle. We have shown that oxidative stress adversely affects insulin-stimulated glucose transport. The oxidant dramatically decreased the protein expression of IRS-1 and IRS-2 signaling proteins after 4 hours. In addition, the oxidant stress caused a significant increase in the phosphorylation of the stress-activated p38 MAPK. Using a selective p38 MAPK inhibitor (10 μ M A304000), a significant portion of the oxidant stress-induced loss of IRS-1, but not IRS-2, was prevented. These data indicate that in vitro oxidative stress can lead to a selective loss of IRS-1 and IRS-2 proteins in rat skeletal muscle. The activation of p38 MAPK appears to be important in the oxidant-induced loss of IRS-1, but not IRS-2, protein in skeletal muscle. Future studies will focus on the characterization of long-term (4 hours) effects of oxidative stress on phosphorylation of GSK3 and Akt in skeletal muscle.

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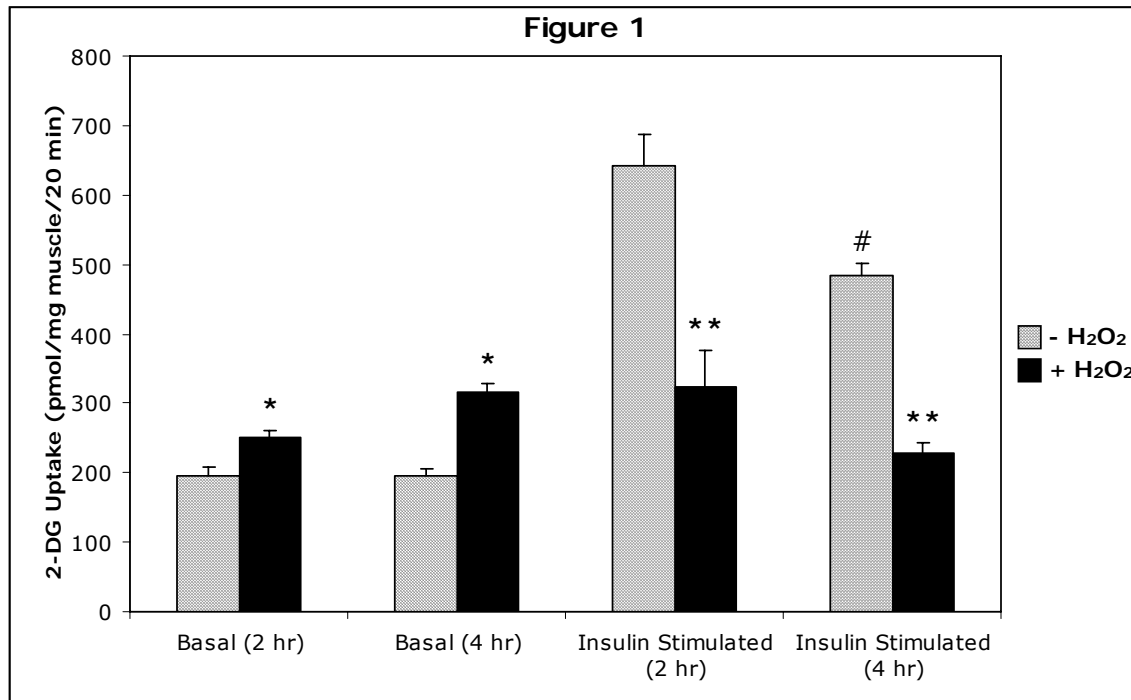
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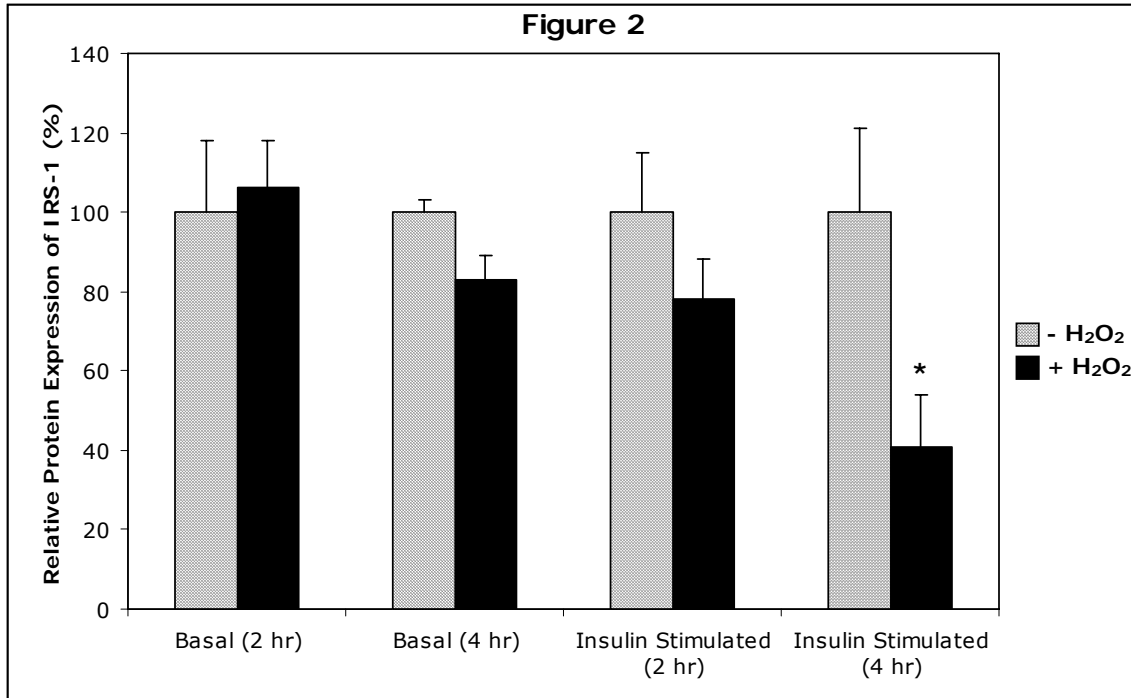
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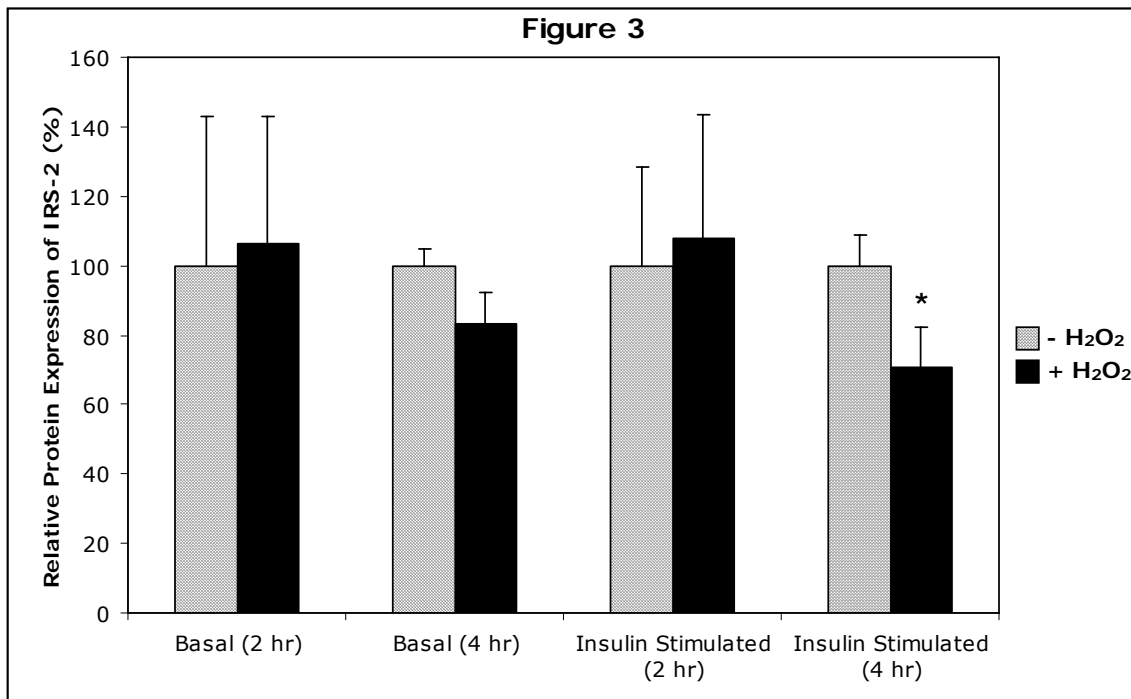
Figures



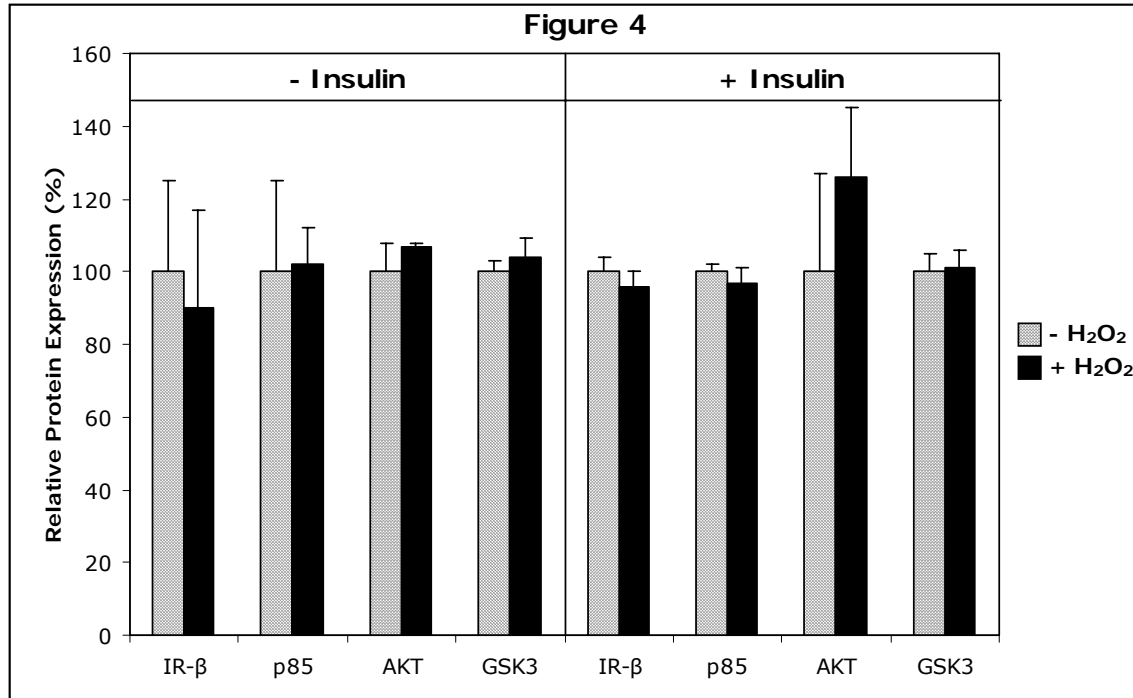
Effects of the oxidant H₂O₂ (2 hr vs. 4hr) on glucose transport activity. Values are means \pm SE for 4-5 animals per group. *P<0.05 vs. basal conditions in the absence of H₂O₂. **P<0.05 vs. insulin-stimulated conditions in the absence of H₂O₂. # P<0.05 vs. 2 hr insulin-stimulated condition in the absence of H₂O₂.



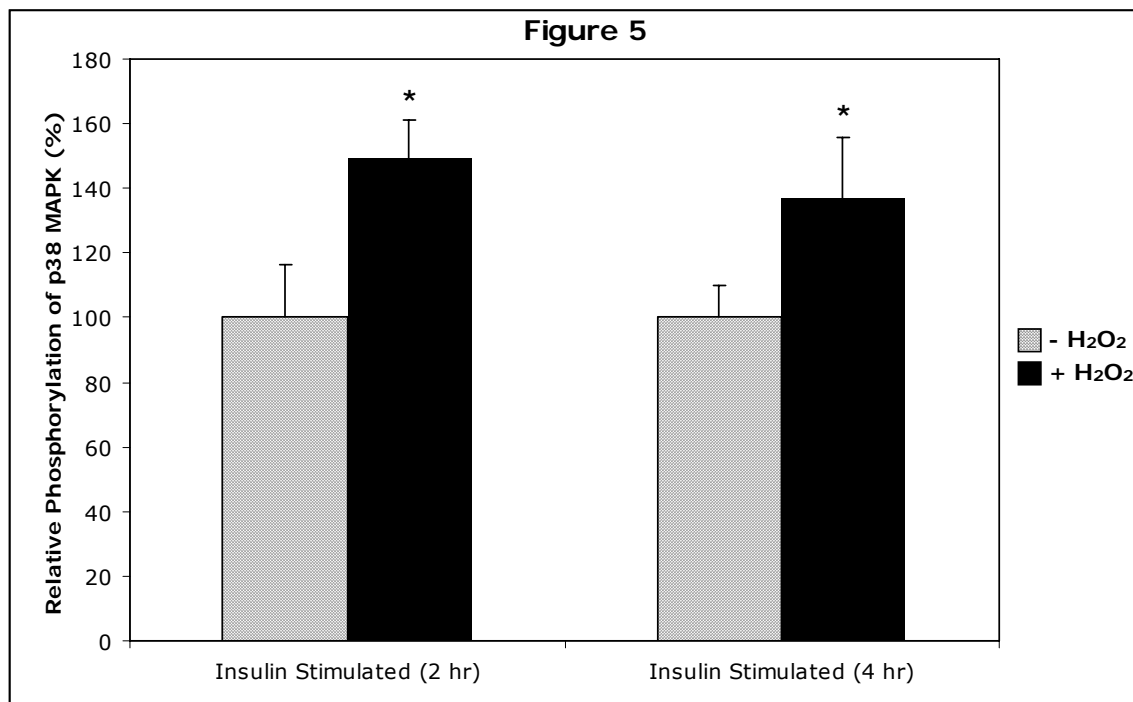
Effects of the oxidant H₂O₂ (2 hr vs. 4 hr) on IRS-1 protein expression. Values are means ± SE for 4-5 animals per group. * p<0.05 vs. basal.



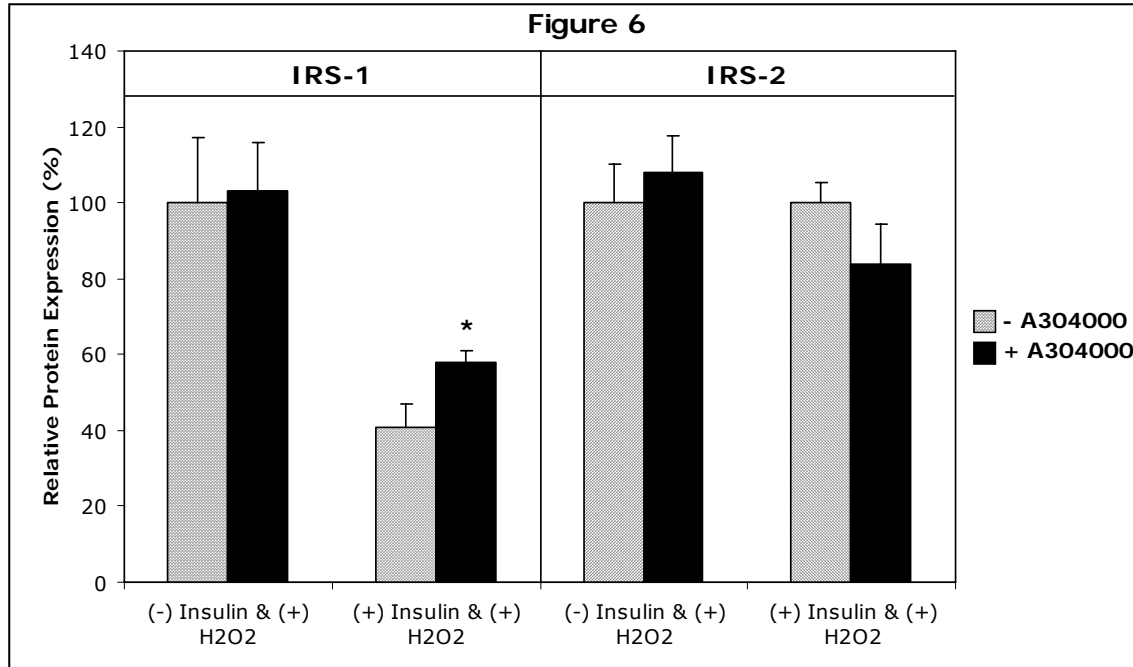
Effects of the oxidant H₂O₂ (2 hr and 4 hr) on IRS-2 protein expression. Values are means ± SE for 4-5 animals per group. * p<0.05 vs. minus H₂O₂.



Effects of the oxidant H₂O₂ (4 hr) on the protein expression of IR-β, the p85 regulatory subunit of PI3-kinase, Akt, and GSK3. No significant differences in protein expression were observed. Values are means ± SE for 4-5 animals per group.



Effects of the oxidant H₂O₂ (2 hr and 4 hr) on thr180/tyr182 phosphorylation of p38 MAP kinase. Values are means ± SE for 4-5 animals per group. * p<0.05 vs. minus H₂O₂.



Effects of the selective p38 MAP kinase inhibitor A304000 (10 μ M) on the loss of IRS-1 and IRS-2 induced by the oxidant H₂O₂ (4 hr). Values are means \pm SE for 4-5 animals per group. * $p < 0.05$ vs. absence of p38 MAPK inhibitor under insulin-stimulated conditions plus H₂O₂.