Acute Oxidative Stress Can Reverse Insulin Resistance by Inactivation of Cytoplasmic JNK*

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Chronic oxidative stress results in decreased responsiveness to insulin, eventually leading to diabetes and cardiovascular disease. Activation of the JNK signaling pathway can mediate many of the effects of stress on insulin resistance through inhibitory phosphorylation of insulin receptor substrate 1. By contrast, exercise, which acutely increases oxidative stress in the muscle, improves insulin sensitivity and glucose tolerance in patients with Type 2 diabetes. To elucidate the mechanism underlying the contrasting effects of acute versus chronic oxidative stress and investigate their effects on insulin and JNK signaling, Chronic oxidative stress resulted in increased levels of phosphorylated (activated) JNK in the cytoplasm, whereas acute oxidative stress led to redistribution of JNK-specific phosphatase MKP7 from the nucleus into the cytoplasm, reduction in cytoplasmic phospho-JNK, and a concurrent accumulation of phospho-JNK in the nucleus. Acute oxidative stress restored normal insulin sensitivity and glucose uptake in insulin-resistant muscle cells, and this effect was dependent on MKP7. We propose that the contrasting effects of acute and chronic stress on insulin sensitivity are driven by changes in subcellular distribution of MKP7 and activated JNK.

Chronic oxidative stress is one of the major sources of metabolic abnormalities associated with Type 2 diabetes (1–3). High glucose and fatty acid levels lead to increased production of reactive oxygen species (ROS), which can cause insulin resistance in peripheral metabolic tissues. This leads to decreased glucose uptake in muscle and adipose tissue, and eventually, pancreatic β cell failure, glucose intolerance, and frank diabetes (4–8). The mechanistic link between increased ROS levels and insulin resistance is activation of several signaling pathways, primarily mitogen-activated protein kinases (MAPK) pathways. JNK (Jun N-terminal kinases) are MAP kinases activated by cellular stresses, including oxidative stress, and play a role in apoptosis and survival, stress resistance, and immune response (9). Upstream signaling leading to JNK activation involves stress-induced MAPK kinases MEKK4 and MEKK7, as well as scaffold protein JIP (JNK-interacting protein) (10). Activation of JNK leads to dimerization followed by translocation into the nucleus, where it can phosphorylate its downstream target c-Jun, leading to activation of stress response and apoptotic pathways. JNKs are specifically dephosphorylated and inactivated by MAP kinase phosphatase 7 (MKP7), which also acts as a shuttle protein and was proposed to be involved in JNK nucleocytoplasmic translocation (11). Obesity increases JNK activation in muscle and adipose tissue in mice. Genetic ablation or pharmacological inhibition of JNK results in marked improvement of insulin sensitivity in mouse models of diet-induced obesity and insulin resistance (6, 12, 13). Mechanistically, JNK has been shown to phosphorylate IRS1 (insulin receptor substrate 1) at multiple serine residues, targeting IRS1 to degradation by the proteasome machinery (12). Inhibition of JNK activation prevents IRS1 degradation and promotes downstream insulin signaling and insulin-dependent glucose uptake. JNK activation is a key mediator of ROS-induced insulin resistance (14).

As skeletal muscle is responsible for over 80% of the peripheral glucose uptake, chronic oxidative stress in this tissue can result in particularly devastating effects on peripheral insulin sensitivity. Exercise is beneficial to patients with metabolic syndrome, and can markedly increase glycemic control (15, 16). Exercise stimulates glucose uptake and increases insulin sensitivity in the muscle and other peripheral tissues (16). Muscle contraction during exercise results in elevated oxidative phosphorylation, ROS production, and activation of MAPK cascades, including JNK signaling (15, 17–19). Paradoxically, exercise-activated JNK does not lead to impaired insulin sensitivity. These contrasting effects of acute oxidative stress during exercise and chronic oxidative stress in metabolic syndrome are not well understood. Indeed, some reports indicate that oxidative stress impairs glucose uptake in the muscle by inhibiting translocation of glucose transporter GLUT4 to plasma membrane (20). Other reports indicate that positive effects of oxidative stress on muscle glucose uptake involve activation of phosphatidylinositol 3-kinase signaling (21, 22). It is unclear what mechanistic differences lead to opposite effects of acute and chronic stress on muscle insulin sensitivity and glucose uptake, or why activation of MAPK/JNK signaling causes insulin resistance in the case of chronic, but not acute, oxidative stress.
To answer these fundamental questions we used a cellular model of muscle insulin resistance based on mouse C2C12 myocytes, which can be made insulin-resistant by culturing in high glucose- and high insulin-containing media, mimicking hyperglycemia and hyperinsulinemia that cause insulin resistance in the pre-diabetic state. We induced acute or chronic stress in insulin-responsive and insulin-resistant myocytes and myotubes to determine their effects on insulin and JNK signaling. Our findings suggest that differential subcellular distributions of JNK-specific phosphatase MKP7 and activated JNK determine the opposite effects of acute and chronic oxidative stress on insulin sensitivity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Myoblast Differentiation, Transient Transfection, and JNK Inhibition**—C2C12 and L6 myoblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37 °C under 5% CO₂. To differentiate into myotubes, C2C12 or L6 myocytes were grown until 95% confluency in normal growth medium, followed by a medium switch into differentiation medium (DMEM containing 2% horse serum). Myocytes were differentiated for 4–5 days with frequent medium changes, and assays were performed on days 5–7 post-differentiation. siRNA transfections were performed with Lipofectamine 2000 according to the manufacturer’s instructions. MKP7 siRNA pool and scrambled siRNA control were purchased from Santa Cruz. To inhibit JNK signaling, we used the cell-permeable peptide JNK inhibitor from Biosource overnight at 10 μM.

**Insulin Resistance and Oxidative Stress Induction**—Insulin pathway activation was induced by a 3-h starvation in serum-free DMEM followed by 100 nM insulin treatment for 15 min at 37 °C. The cells were then placed on ice, washed with ice-cold phosphate-buffered saline and lysed to use in immunoblotting experiments with anti-phospho-AKT and other antibodies (see below). To induce acute oxidative stress myocytes were treated with 200–500 μM hydrogen peroxide in DMEM for 3 h prior to insulin treatment. To induce chronic oxidative stress, myocytes were treated with 1 μM hydrogen peroxide in culture medium for 48 h prior to assay. Medium was changed once at 24 h to account for hydrogen peroxide hydrolysis. To induce insulin resistance, C2C12 cells were cultured in DMEM containing 15 g/liter of glucose, 10% fetal bovine serum, sodium pyruvate, 1 μg/ml of insulin, 100 mM dexamethasone, and 1% penicillin/streptomycin for 1 week. This medium was used in normal passaging procedures. For L6 myotubes, insulin resistance was induced by culturing for 4 days in DMEM containing 2% horse serum, 15 g/liter of glucose, and 1% penicillin/streptomycin.

**Antibodies, Cell Fractionation, Immunoblots, and Immunoprecipitations**—For protein analysis, cells were lysed using a modified RIPA buffer consisting of 50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors mixture (Thermo Scientific, 78430), and phosphatase inhibitors mixture (Thermo Scientific, 78420). For Western blotting, 20–30 μg of protein per lane was loaded. To control for total protein content in loading we used the anti-α-tubulin mouse monoclonal antibody. Immunoprecipitations were performed by first binding the antibody to Amersham Biosciences Fast-Flow Protein A beads for 2 h at 4 °C, followed by washes with immunoprecipitation buffer, and incubation with cell lysates (1 mg of protein/lane) at 4 °C. Precipitated proteins were eluted by boiling in SDS protein sample buffer. The samples were then centrifuged 5 min at 13,000 × g to separate Protein A beads, and the supernatants were loaded onto 4–15% gradient polyacrylamide gels (Invitrogen). For Western blot analysis, proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% milk for 30 min at room temperature, and then incubated with the indicated primary antibodies in phosphate-buffered saline/Tween overnight at 4 °C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies from Pierce, and the signal was detected using Western Lightning Chemiluminescence reagent. The tubulin DM1A antibody was purchased from Abcam. The pAKT Ser473, pAKT Thr308, p-glycogen synthase kinase 3α/β, total AKT, and total JNK antibodies were purchased from Cell Signaling. The p-JNK Thr183 was purchased from Calbiochem. The pIRS-1 Ser307 and total IRS-1 antibodies were purchased from Upstate. The MKP7 G14 goat polyclonal antibody was from Santa Cruz. Cell fractionations to nuclear and cytoplasmic extracts were performed using the NE-PER kit (Pierce Biotechnology). We used the HSP90 antibody (Stressgen) and Histone 3 antibody (Cell Signaling) as controls for the cytoplasmic and nuclear fractions, respectively.

**2-Deoxyglucose Uptake**—Differentiated L6 myotubes were washed twice with 1X phosphate-buffered saline and serum starved in KRH (4.5% NaCl, 5.75% KCl, 7.85% CaCl₂, 19.1% MgSO₄, 0.25X Heps, pH 7.5) supplemented with 0.5% bovine serum albumin, 2 mM sodium pyruvate for 2 h. The cells were then stimulated with or without 20 and 200 nM insulin for 20 min. To control for nonspecific uptake, cytochalasin B (Sigma) was added at a final concentration of 5 μg/ml. [3H]-2-deoxyglucose was diluted in 5.1 mM cold 2-deoxyglucose (Sigma)/KRH at a ratio of 1:20 as substrate solution. At the end of insulin stimulation, 10 μl of substrate solution were added to each well and incubated for 5 min at 37 °C. The cells were washed with ice-cold KRH and 0.4 ml of 1% SDS were added to each well for lysis. Solubilized cells were transferred to scintillation vials and the amount of 2-deoxyglucose uptake into the cells was measured by liquid scintillation counter.

**Immunofluorescence and Confocal Microscopy**—For detection of p-JNK, total JNK, and MKP7 immunofluorescence, cells were cultured in 35-mm coverslip-bottomed dishes and subjected to oxidative stress treatments and/or insulin resistance protocol as described above. At the end of the treatments, cells were fixed in 4% paraformaldehyde for 10 min, washed, and treated with primary antibodies overnight at 4 °C, followed by a 1-h incubation with Alexa Fluor 488- or 573-conjugated goat anti-rabbit, mouse, or donkey anti-goat IgG (1:500 dilution). The p-JNK antibody (1:100) and MKP7 G14 goat polyclonal antibody (1:50) were from Santa Cruz. Fluorescence imaging was assessed with a Zeiss LSM 510 confocal microscope.
RESULTS

Acute and Chronic Oxidative Stress Treatments Have Opposite Effects on Insulin Sensitivity in Muscle Cells—Oxidative stress has been reported to either induce or impair insulin signaling in the muscle, depending on the model and treatments tested (20–22). We hypothesized that this could be at least in part due to differences in stress treatments, some of which could be more short-term and acute than others. To try and resolve the controversy of the effects of oxidative stress on muscle insulin signaling, we used experimental conditions in which we can administer either acute or chronic oxidative stress and assess effects on insulin signaling and glucose uptake.

To test the effects of acute and chronic oxidative stress on muscle cells we treated C2C12 myoblasts acutely for 3 h with 500 μM hydrogen peroxide, or chronically (48 h) with 1 μM hydrogen peroxide. We chose the above peroxide concentrations because these were the highest concentrations that did not reduce cell viability and ATP levels after the relevant time periods, 3 and 48 h, respectively (data not shown). We then tested basal and insulin-induced AKT activation. We found that chronic peroxide stress resulted in a decrease in insulin-induced AKT activation (Fig. 1A), consistent with the development of insulin resistance in individuals with hyperglycemia and hyperlipidemia that have chronically elevated plasma ROS levels. Acute peroxide treatment resulted in a significant increase in AKT phosphorylation elicited by insulin (Fig. 1A). Acute oxidative stress also induced AKT phosphorylation at Thr308 and phosphorylation of the AKT downstream target glycogen synthase kinase 3 (GSK3) (Fig. 1A), suggesting that signaling downstream of AKT is also up-regulated by acute oxidative stress. We obtained similar results with acute and chronic oxidative stress induced by paraquat (supplemental Fig. S1A). Moreover, acute and chronic oxidative stress had opposite effects on insulin signaling in differentiated C2C12 myotubes (supplemental Fig. S1B), as well as in L6 myotubes (supplemental Fig. S2), suggesting that myocytes and myotubes respond similarly to oxidative stress.

To test whether insulin-stimulated AKT activation observed with acute stress translates into a functional measure of muscle insulin sensitivity, we performed insulin-stimulated glucose uptake experiments. C2C12 myotubes do not respond well to insulin due to the lack of an insulin-responsive vesicle compartment (26). We therefore performed 2-deoxyglucose uptake in L6 myotubes, which have been previously used for this assay (20). AKT activation and oxidative stress response were similar in C2C12 and L6 myocytes (supplemental Fig. S1). We differentiated L6 myocytes into myotubes and then treated L6 myo-
tubes with H$_2$O$_2$ to cause acute and chronic oxidative stress, similar to the treatments in C2C12 myoblasts. Without stress, insulin-stimulated 2-deoxyglucose uptake in L6 myotubes resulted in a 50–75% increase relative to basal glucose uptake (Fig. 1C). Acute peroxide treatment further increased insulin-stimulated 2-deoxyglucose uptake, up to 2.5-fold (250%). By contrast, chronic oxidative stress impaired insulin-stimulated 2-deoxyglucose uptake, rendering the cells insulin-resistant (Fig. 1C).

Our results indicate that acute and chronic oxidative stress lead to opposite effects on insulin sensitivity in muscle cells and suggest that different cellular pathways are activated by acute and chronic stress in the muscle.

**Acute Oxidative Stress Restores Insulin Sensitivity to Insulin-resistant Muscle Cells, Despite JNK Activation**—Hyperglycemia and hyperlipidemia impair insulin signaling, primarily due to activation of JNK resulting in inhibition of IRS1 (8). We asked how acute and chronic stress will affect insulin signaling in the muscle under insulin-resistant conditions. Myoblasts in culture can be made insulin-resistant by long term incubation in high glucose, high insulin medium, which is intended to mimic the *in vivo* hyperglycemia state (see “Experimental Procedures” for details). Culturing C2C12 myoblasts for 1–2 weeks in high glucose, high insulin medium leads to a significant decrease in AKT activation (Fig. 2A) and reduced IRS-1 levels (Fig. 2C). Chronic oxidative stress did not significantly change the AKT activation in insulin-resistant C2C12 myoblasts (data not shown), whereas acute stress significantly increased insulin responsiveness in these cells (Fig. 2A).

We then tested whether glucose uptake in myotubes will be similarly affected. Culturing differentiated L6 myotubes in high glucose medium for 72 h prevented insulin-induced increase in glucose uptake, to a similar extent as observed with low grade chronic peroxide stress (Fig. 2B). Treating insulin-resistant L6 myotubes chronically with peroxide did not alter insulin resistance. Acute oxidative stress, however, increased insulin-dependent 2-deoxyglucose uptake in insulin-resistant myotubes more than 2-fold, restoring it to a level similar to that in normal insulin-sensitive cells (Fig. 2B). These data suggest that acute oxidative stress can fully restore insulin responsiveness in hyperglycemia-induced insulin-resistant myocytes.

Because many of the effects of hyperglycemia and obesity on insulin signaling are mediated through JNK activation, we looked at the activation of JNK (i.e. phosphorylation at Thr$^{183}$/
Tyr185) in regular and insulin-resistant myoblasts before and after stress. As expected, in insulin-resistant cells we observed elevated JNK phosphorylation/activation relative to normal cells. However, acute oxidative stress also resulted in a similar JNK activation (Fig. 2A). The primary effect of activated JNK on insulin signaling is serine phosphorylation of IRS1 that targets IRS1 to degradation, impairing the downstream pathway activation (including AKT phosphorylation). Therefore, we tested the effects of acute oxidative stress on IRS1 levels and activation. We immunoprecipitated IRS1 with a specific antibody from extracts from regular and insulin-resistant C2C12 myoblasts with or without oxidative stress, and detected total IRS1 levels or IRS1 p-Ser307 levels in the pulldowns. As expected, IRS1 levels were low in insulin-resistant cells, and serine 307 phosphorylation of IRS1 was increased (Fig. 2C). Despite high levels of activated JNK upon acute stress, we did not see further reduction in total IRS1 levels with acute stress. On the contrary, total IRS1 levels were increased following acute stress in these insulin-resistant cells with acute stress. These results indicate that acute oxidative stress prevents activated JNK from phosphorylating IRS1 and targeting it for degradation.

**Acute and Chronic Oxidative Stress Differentially Affect JNK Activation in Muscle Cells**—How does acute oxidative stress prevent phosphorylated JNK from inhibiting IRS1 in insulin-resistant cells? We hypothesized that JNK could be localized to different subcellular compartments following acute and chronic oxidative stress. To test this idea, we determined the subcellular localization of phosphorylated JNK by immunofluorescence in normal and insulin-resistant C2C12 myoblasts. Under normal conditions, phospho-JNK levels are low and it is ubiquitously distributed in insulin-sensitive cells. Upon acute stress, as activation of JNK is increased, levels of nuclear phospho-JNK are increased in insulin-sensitive cells (Fig. 3A). Insulin-resistant cells in the absence of any stress showed increased JNK phosphorylation in the cytoplasmic fraction of insulin-resistant myoblasts, compared with normal myoblasts. Note that after acute oxidative stress, there was significantly less phospho-JNK present in the cytoplasmic fractions of both insulin-resistant and control myoblasts. Three independent experiments were performed with similar results.

**FIGURE 3.** Myoblasts show differential phospho-JNK localization upon acute and chronic oxidative stress. **A**, C2C12 myoblasts were subjected to insulin resistance protocol and/or acute or chronic oxidative stress. Activated JNK was visualized by immunofluorescence with an anti-phospho-JNK antibody (green staining), and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Chronic oxidative stress caused p-JNK distribution similar to that in insulin-resistant myoblasts, i.e. primarily in the cytoplasm, whereas acute oxidative stress caused increased nuclear p-JNK and decreased cytoplasmic p-JNK. B, C2C12 myoblasts were subjected to a insulin-resistance protocol and/or acute oxidative stress. Cell fractionation experiments in regular and insulin-resistant C2C12 myoblasts showed increased JNK phosphorylation in the nucleus following acute oxidative stress. Active (phosphorylated) JNK was present in the cytoplasm and the amount of phospho-JNK was increased in the cytoplasmic fraction of insulin-resistant myoblasts, compared with normal myoblasts. Note that after acute oxidative stress, there was significantly less phospho-JNK present in the cytoplasmic fractions of both insulin-resistant and control myoblasts. Three independent experiments were performed with similar results.
firmed these observations by subcellular fractionation followed by Western blotting for phospho-JNK in nuclear and cytoplasmic cellular fractions (Fig. 3B). There was a striking decrease in phospho-JNK but not total JNK in the cytoplasm upon acute stress in both control and insulin-resistant cells. In the nucleus, levels of phospho-JNK increased upon acute stress (Fig. 3B). These results suggest that the subcellular localization of phosphorylated JNK correlates with insulin sensitivity of these cells. In both normal and insulin-resistant cells acute stress leads to accumulation of nuclear phospho-JNK, which correlates with insulin sensitivity. By contrast, chronic stress, induced by either peroxide or hyperglycemia, results in accumulation of phospho-JNK in the cytoplasm and insulin resistance.

We then asked whether acute oxidative stress activated insulin signaling by accumulating phospho-JNK in the nucleus, or by reducing phospho-JNK in the cytoplasm, the latter of which could reduce phosphorylation of IRS1 at the inhibitory serine residues. To address this question we treated insulin-resistant C2C12 myoblasts with a specific peptide JNK inhibitor, with or without acute oxidative stress.

**FIGURE 4.** JNK inhibition restores insulin sensitivity to insulin-resistant myocytes. C2C12 myoblasts were subjected to insulin-resistance protocol and acute oxidative stress (H2O2), JNK inhibitor peptide, or both. The total IRS1 level was detected as described in the legend to Fig. 2 and AKT activation was detected by anti-p-AKT473 as described in the legend to Fig. 1. Note that JNK inhibitor alone increased the IRS1 level and promoted insulin-dependent AKT activation in insulin-resistant myocytes, but did not further activate AKT upon acute oxidative stress conditions. Bottom panel shows quantitation of AKT activation by densitometry and normalization to tubulin. #, p < 0.05 versus normal myocytes; *, p < 0.05 versus insulin-resistant myocytes. Error bars are standard deviations from the mean. This experiment was repeated three times with similar results. A representative experiment is shown.

Control experiments indicated that this inhibitor, which prevents association between JNK and its specific scaffold protein JIP (required for tethering JNK with its upstream kinases MEKK4 and MEKK7), reduced basal levels of active JNK and impaired JNK activation following acute and chronic stress (data not shown). Administration of the JNK inhibitor increased the IRS1 level and restored AKT activation in insulin-resistant myotubes (Fig. 4), indicating that inhibiting JNK can prevent hyperglycemia-induced insulin resistance in this system. Moreover, the JNK inhibitor did not cause further activation of AKT phosphorylation after acute peroxide stress (Fig. 4), suggesting that acute oxidative stress and JNK inhibition activate insulin signaling by the same mechanism, i.e. inhibition of cytoplasmic JNK.

**MKP7 Is Required for Activation of Insulin Signaling by Acute Oxidative Stress**—The observation that levels of cytoplasmic phospho-JNK but not total JNK are reduced following acute stress suggests that JNK can be inactivated in the cytoplasm following acute stress by dephosphorylation. MKP7 is a JNK-specific phosphatase, which can shuttle between the cytoplasm and the nucleus (17). To determine whether MKP7 could play a role in restoring insulin sensitivity by acute oxidative stress, we assessed MKP7 levels and subcellular localization in insulin-resistant C2C12 myoblasts. Without stress, we detected endogenous MKP7 throughout the cell (Fig. 5A). Strikingly, acute oxidative stress resulted in exclusion of MKP7 from the nucleus and accumulation in the cytoplasm (Fig. 5A). Similar results were obtained by subcellular fractionation experiments (Fig. 5B). These data suggest that decreased levels of phosphorylated JNK in the cytoplasm following acute oxidative stress may be due to the redistribution of MKP7.

To obtain functional evidence that MKP7 plays a role in activation of insulin signaling by acute oxidative stress, we used siRNA to reduce MKP7 levels in insulin-resistant C2C12 myoblasts by over 80% (Fig. 5C). Without stress, MKP7 knockdown did not alter AKT activation in normal or insulin-resistant myoblasts (Fig. 5D, data not shown). However, upon acute oxidative stress MKP7 knockdown prevented restoration of IRS1 levels and insulin-dependent AKT phosphorylation in insulin-resistant myoblasts (Fig. 5D). This result indicates that MKP7 is required for the positive effect of acute oxidative stress on insulin signaling in muscle cells, consistent with the hypothesis that
MKP7 mediates dephosphorylation of cytoplasmic JNK following acute oxidative stress.

**DISCUSSION**

In this study, we evaluated the effects of acute and chronic oxidative stress on insulin and MAPK signaling in regular and insulin-resistant muscle cells. It has been widely established that chronic oxidative stress is detrimental to glucose homeostasis, leading to insulin resistance in muscle and adipose tissue, hyperglycemia, and metabolic disease. Recent work demonstrated that reactive oxygen species play a causative role in the development of insulin resistance and glucose intolerance (8). Oxidative stress leads to the activation of MAPK pathways that have been shown by numerous studies to mediate inhibitory serine phosphorylation of IRS1, thereby decreasing the downstream insulin signaling, GLUT4 translocation to the plasma membrane, and glucose uptake (6, 7, 23). Indeed, animals fed a high-fat diet show increased ROS levels in plasma, increased activation of JNK, and are insulin-resistant. The causality of JNK activation in the development of insulin resistance is demonstrated by the fact that deletion of JNK1 significantly improves insulin sensitivity and glucose homeostasis in high-fat fed mice (14, 23–25). Consistent with the current dogma, we found that chronic oxidative stress caused by either low grade peroxide administration or the hyperglycemia/hyperinsulinemia regimen impairs insulin signaling in two different muscle cell lines. This was due to the reduction in the IRS1 protein level mediated by inhibitory phosphorylation by JNK. JNK activation was essential for the development of insulin resistance in our system, because a specific inhibitor of JNK activation restored insulin-dependent signaling, consistent with the results in db/db and DIO mice, where JNK inhibition improves glucose homeostasis (26).

By contrast, we discovered that acute oxidative stress not only did not impair insulin signaling, but increased insulin-dependent AKT phosphorylation and reversed hyperglycemia-induced insulin resistance, restoring insulin stimulation of glucose uptake. Several reports indicate positive effects of oxidative stress on glucose uptake in adipose cells and in isolated muscles. For example, glucose oxidase and peroxide administration has been shown to induce glucose uptake in L6 myotubes (13). Moreover, oxidative stress induced by either peroxide or xanthine oxidase increases glucose uptake in isolated human muscle, in a phosphatidylinositol 3-kinase-dependent manner (22). Another example of a positive effect of oxidative stress on insulin sensitivity is aerobic exercise. Muscle

FIGURE 5. JNK phosphatase MKP7 accumulates in the cytoplasm and regulates the activation of insulin signaling following acute oxidative stress. A, insulin-resistant C2C12 myoblasts were subjected to acute oxidative stress, and endogenous MKP7 was visualized by immunofluorescence with an anti-MKP7 antibody. Under normal conditions MKP7 was detected throughout the cell, acute oxidative stress caused translocation of MKP7 from the nucleus and accumulation in the cytoplasm. B, cell fractionation experiments in insulin-resistant C2C12 cells also showed MKP7 accumulation in the cytoplasm following acute oxidative stress. Note that there is less phosphorylated JNK in the cytoplasm following acute oxidative stress. C, C2C12 myocytes transfected with siRNA against MKP7 show ~80% knockdown at the protein level. *, p < 0.01 versus scrambled siRNA control. D, insulin-resistant C2C12 myoblasts were transfected with MKP7 siRNA or negative control siRNA. After 48 h, myoblasts were subjected to acute oxidative stress (H2O2), and IRS1 levels and AKT activation were detected by anti-p-AKT 473 and anti-IRS1 as described in the legends to Figs. 1 and 2. Without stress, MKP7 knockdown did not alter insulin-dependent AKT activation in insulin-resistant myocytes, but upon acute oxidative stress AKT activation was impaired in cells with MKP7 knockdown. Note that IRS1 levels were also not increased following stress in MKP7 knockdown cells. Bottom panel shows quantitation of the above panel bands by densitometry and normalization to tubulin. Error bars are standard deviations from the mean. #, p < 0.01 versus regular cells. *, p < 0.01 versus no stress control. A representative of three independent experiments is shown. DAPI, 4',6-diamidino-2-phenylindole.
Oxidative Stress, JNK Localization, and Insulin Sensitivity

FIGURE 6. A model for the acute and chronic oxidative stress effects on insulin sensitivity in the muscle. See text for details.

contraction, which is accompanied by increased oxidative phosphorylation, was shown to significantly increase oxidative stress in human and rodent muscle, followed by activation of MAPK signaling pathways, including JNK (17, 18, 27). Exercise boosts muscle glucose uptake, in part due to activation of AMP-activated protein kinase signaling, which contributes to GLUT4 translocation in parallel to insulin signaling (27). However, exercise also increases insulin sensitivity in the muscle, by an unknown, AMP-activated protein kinase-independent mechanism, and significantly improves glucose tolerance in diabetic humans and animal models, despite increased levels of ROS and MAPK/JNK activation (16). Notably, mice overexpressing the antioxidant gene glutathione surprisingly develop insulin resistance and other metabolic abnormalities, arguing against a simple negative role for ROS and oxidative stress in glucose homeostasis (28). It has recently been proposed that acute oxidative stress, such as that caused by exercise, and chronic metabolic oxidative stress may elicit different cellular responses resulting in contrasting outcomes on insulin sensitivity and glucose homeostasis in the muscle (22). Understanding the mechanisms underlying signaling changes that occur upon acute and chronic stress can be valuable for optimal design of targeted therapies for prevention of insulin resistance.

We discovered that acute oxidative stress leads to accumulation of activated JNK in the nucleus, whereas chronic oxidative stress, either caused by low grade peroxide administration or hyperglycemia, activates JNK in cytoplasmic pools. This is consistent with the theory that upon chronic metabolic stress JNK phosphorylates IRS1, which is present in the cytoplasm and plasma membrane, to trigger its degradation. Interestingly, acute stress, whereas increasing JNK phosphorylation in the nuclei, significantly decreased activated JNK levels in the cytoplasm of insulin-resistant myoblasts. We found that acute stress led to the exclusion of JNK-specific MKP7 from the nucleus and its accumulation in the cytoplasm. It is plausible that upon acute oxidative stress cytoplasmic JNK is dephosphorylated and inactivated by MKP7. This notion is supported by our finding that MKP7 is required for the increase in insulin sensitivity caused by acute oxidative stress.

Taken together, our results suggest a novel mechanism of stress-mediated regulation of insulin resistance, where chronic and acute oxidative stresses activate JNK in different subcellular compartments, leading to opposite cellular outcomes. We propose that exercise, similarly to acute oxidative stress, can cause redistribution of MKP7 from the nucleus to the cytoplasm, leading to dephosphorylation of JNK in the cytoplasm and plasma membranes (Fig. 6). This reduction in JNK activation in the cytoplasm and at the plasma membrane should result in increased insulin sensitivity due to IRS1 stabilization, activation of the downstream insulin pathway, and increased glucose uptake (Fig. 6). Our model explains the discrepancies between reported effects of oxidative stress on JNK activation and muscle insulin sensitivity and highlights the importance of differential spatial activation of JNK.

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