Oxidative stress-induced insulin resistance in rat skeletal muscle: role of glycogen synthase kinase-3

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Dokken BB, Saengsirisuwan V, Kim JS, Teachey MK, Henriksen EJ. Oxidative stress-induced insulin resistance in rat skeletal muscle: role of glycogen synthase kinase-3. Am J Physiol Endocrinol Metab 294: E615–E621, 2008. First published December 18, 2007; doi:10.1152/ajpendo.00578.2007.—Oxidative stress can contribute to the multifactorial etiology of whole body and skeletal muscle insulin resistance. No investigation has directly assessed the effect of an in vitro oxidant stress on insulin action in intact mammalian skeletal muscle. Therefore, the purpose of the present study was to characterize the molecular actions of a low-grade oxidant stress (H2O2) on insulin signaling and glucose transport in isolated skeletal muscle of lean Zucker rats. Soleus strips were incubated in 8 mM glucose for 2 h in the presence of 100 μM H2O2. By itself, H2O2 significantly reduced basal glucose transport activity, net glycogen synthesis, and glycogen synthase activity and increased phosphorylation of insulin receptor (Tyr), Akt (Ser473), and GSK-3β (Serα). In contrast, this oxidant stress significantly inhibited the expected insulin-mediated enhancements in glucose transport, glycogen synthesis, and these signaling factors and allowed GSK-3β to retain a more active form. In the presence of CT-98014, a selective GSK-3 inhibitor, the ability of insulin to stimulate glucose transport and glycogen synthesis during exposure to this oxidant stress was enhanced by 20% and 39% (P < 0.05), respectively, and insulin stimulation of the phosphorylation of insulin receptor, Akt, and GSK-3β was significantly increased by 36–58% (P < 0.05). These results indicate that an oxidant stress can directly and rapidly induce substantial insulin resistance of skeletal muscle insulin signaling, glucose transport, and glycogen synthesis. Moreover, a small, but significant, portion of this oxidative stress-induced insulin resistance is associated with a reduced insulin-mediated suppression of the active form of GSK-3β.

Insulin resistance of skeletal muscle glucose transport and glycogen metabolism are primary defects associated with the development of type 2 diabetes. Specific defects in the ability of insulin to normally activate insulin signaling factors, including the insulin receptor (IR), IR substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI 3-kinase), and Akt are known to contribute to this insulin resistance in skeletal muscle (9, 11, 35).

Oxidative stress, the imbalance between the cellular production of oxidants and the antioxidant defenses within cells and in the plasma, can contribute to the multifactorial etiology of whole body and skeletal muscle insulin resistance (for review see Refs. 2, 8, 11). Evidence from cell culture studies has shown that exposure to low levels of oxidants, such as H2O2, will induce a reduction in insulin stimulation of glucose transport due to impaired insulin signaling at the level of PI 3-kinase in 3T3-L1 adipocytes (27) and L6 myocytes (20). Blair et al. (1) and Maddux et al. (20), using L6 myocytes, and Kim et al. (17), using isolated rat skeletal muscle, showed that exposure to a low level of the oxidant H2O2 activates the stress-activated serine/threonine kinase p38 MAPK, and although a portion of the impairment of insulin signaling due to an oxidant stress may be associated with p38 MAPK activation, other mechanisms certainly exist.

For example, overactivity of another serine/threonine kinase, glycogen synthase kinase-3 (GSK-3), is also associated with the insulin-resistant state (for review see Ref. 12), and acute selective inhibition of GSK-3 improves insulin signaling and glucose transport activity in skeletal muscle from prediabetic and type 2 diabetic rats (6, 14, 15). However, no information is available regarding the potential role of GSK-3 in the oxidant stress-induced insulin resistance in mammalian skeletal muscle.

In this context, one purpose of the present investigation was to assess the potential influence of a low-grade oxidant stress will directly induce insulin resistance of glucose transport and glycogen synthesis in isolated mammalian skeletal muscle. An additional purpose was to assess the potential influence of a low-grade oxidant stress on distal and proximal insulin signaling factors involved in activation of glucose transport and glycogen synthesis in mammalian skeletal muscle. Finally, this investigation addressed the specific role of GSK-3 in oxidant stress-associated insulin resistance in mammalian skeletal muscle.

METHODS

Animals. Female lean (fa/−) Zucker rats were received from Harlan (Indianapolis, IN) and used at 9–10 wk of age at 150–170 g body wt. Animals were housed in a temperature-controlled (20–22°C) room with a 12:12-h light-dark cycle (lights on from 7 AM to 7 PM) at the Central Animal Facility of the University of Arizona. The animals had free access to chow (diet no. 7001; Teklad, Madison, WI) and water. Before each experiment, animals were restricted overnight starting at 5 PM to 4 g of chow. Experiments began at 8 AM on the following morning. All the procedures used in the present study were approved by the Institutional Animal Care and Use Committee at the University of Arizona.

Oxidant exposure. After an overnight food restriction (chow was restricted to 4 g at 5 PM and was consumed immediately), animals were deeply anesthetized at 8 AM with an injection of pentobarbital sodium (50 mg/kg ip), and strips of soleus muscles (~25–30 mg) were prepared for in vitro incubation in the unmounted state. Muscles were initially incubated for 2 h at 37°C in oxygenated Krebs-Henseleit...
buffer containing 8 mM glucose, 32 mM mannitol, and 0.1% BSA (RIA grade; Sigma, St. Louis, MO), in the absence or presence of 100 nM insulin (Humulin R, Lilly, Indianapolis, IN) in the absence or presence of glucose oxidase. Muscles were transferred to fresh medium after the initial 60 min of incubation. The level of H$_2$O$_2$ in the medium at the end of the second 60-min incubation was assessed spectrophotometrically (20) and reached ~90 μM (17).

In some experiments, incubations also included 1 μM CT-98014 (kindly provided by Dr. Stephen D. Harrison; Chiron, Emeryville, CA), a selective inhibitor of GSK-3β (4, 6, 14, 15, 24, 23, 32). CT-98014 inhibits GSK-3α and GSK-3β with inhibitor constant <10 nM in an ATP-competitive manner (12, 32). The compound was >95% pure by HPLC and was in free-base form diluted from a DMSO stock solution. The final DMSO concentration did not exceed 0.5%.

Glucose transport and glycogen synthesis in skeletal muscle. Glucose transport in isolated soleus strips was assessed in vitro by the filter paper assay of Thomas et al. (31) was used to determine the determination of insulin (5 mU/ml) (13). In addition, in separate experiments, the intracellular accumulation of 2-deoxyglucose (1 mM) in the absence or presence of a maximally effective concentration (5 mM) of glucose transport activity in isolated soleus strips was assessed in vitro by spectrophotometrically (20) and reached

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Measurement of signaling factors. For measurement of tyrosine-phosphorylated IR-β, immunoprecipitations and subsequent immunoblotting were performed. Muscles were homogenized in 1 ml of ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na$_2$VO$_4$, 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 mmol/l phenylmethylsulfonyl fluoride). 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 bicinchoninic acid method (Sigma Chemical). For assessment of tyrosine-phosphorylated IR-β, 0.5 ml of diluted homogenate was immunoprecipitated with 15 μl of recombinant agarose-conjugated anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Lake Placid, NY). After an overnight incubation at 4°C, samples were pulse centrifuged, and the supernatant was removed. The agarose beads were washed three times with ice-cold PBS, mixed with SDS sample buffer, and boiled for 5 min. Equal amounts of the protein of interest were separated by SDS-PAGE on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with the appropriate dilution of an antibody against insulin receptor β-subunit (Upstate Biotechnology). Thereafter, the membranes were incubated with secondary goat anti-mouse antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were visualized on Kodak X-Omat AR film (Kodak, Rochester, NY) using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ) and quantified with a imaging densitometer (model GS-800; Bio-Rad Laboratories, Hercules, CA) using Quantity One software (Bio-Rad Laboratories). The level of the phosphorylated signaling element was expressed relative to the total amount of that protein from the same sample.

For determination of Akt, GSK-3, and p38 MAPK phosphorylation, samples containing equal amounts of total protein were separated by SDS-PAGE on 7.5% or 12% polyacrylamide gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes. Membranes were incubated overnight with antibodies against phosphorylated (Ser$^{273}$) Akt, phosphorylated (Ser$^{216}$) GSK-3α/β, or phosphorylated (Thr$^{179}$/Tyr$^{182}$) p38 MAPK (Cell Signaling, Beverly, MA) or antibodies against total Akt1/2 (Cell Signaling), GSK-3α/β (Cell Signaling), or p38 MAPK (Santa Cruz Biotechnology). In our hands, 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 of GSK-3β. Subsequently, membranes were incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (Chemicon, Temecula, CA). The proteins were visualized, and autoradiographs were quantified as described above.

Statistical analysis. Values are means ± SE. Paired t-tests were used to determine statistical differences between group means derived from paired muscles (strips originating from the same intact muscle), the treatment of which differed only due to a stimulus (H$_2$O$_2$) or an inhibitor (CT-98014). P ≤ 0.05 was set for statistical significance.

RESULTS

Effects of an oxidant stress on insulin action. Treatment with glucose oxidase alone, which produced H$_2$O$_2$ at ~90 μM, induced 26–30% increases (P < 0.05) in glucose transport activity (Fig. 1), glycogen synthase activity (Fig. 2, left), and net glycogen synthesis (Fig. 2, right) in the soleus muscle of lean Zucker rats. Insulin alone caused substantial increases in glucose transport activity (~3-fold), glycogen synthase activity (38%), and net glycogen synthesis (~4-fold). However, the oxidant stress significantly antagonized insulin-stimulated glucose transport activity by 40%, with the rate being only 45% of the expected additive value. Insulin-stimulated glycogen synthase activity and net glycogen synthesis (14% and 31%, respectively) were similarly antagonized by H$_2$O$_2$, with values only 70% and 65% of the respective theoretical additive values.

To gain insight into the potential cellular mechanisms for this oxidant stress-induced insulin resistance, several signaling factors associated with insulin action were assessed in muscles treated with glucose oxidase alone and glucose oxidase in the presence of insulin. The oxidant stress caused a large (~4-fold) increase in tyrosine phosphorylation of the IR-β (Fig. 3, left), as we reported previously (17). Insulin alone caused a large increase in this variable, and this insulin-stimulated increase in IR-β tyrosine phosphorylation was significantly antagonized (44%) by the oxidant stress, with the measured value being only ~50% of the theoretical additive value. IR-β protein

![Fig. 1. Effect of an oxidant stress on basal and insulin-stimulated glucose transport activity in skeletal muscle of lean Zucker rats. Dashed line, theoretical additive value of individual effects of oxidant stress and insulin. Values are ± SE for 5–6 muscles per group. *P < 0.05 vs. paired muscles in the absence of H$_2$O$_2$ and insulin. §P < 0.05 vs. paired muscles in the presence of insulin without H$_2$O$_2.$](http://ajpendo.physiology.org/)
expression was not changed by the oxidant stress (data not shown).

More distal insulin signaling elements were examined next. 

H$_2$O$_2$ alone caused a twofold increase in phosphorylation (Ser$^{473}$) of Akt (Fig. 3, middle), in agreement with our previous observation in the soleus (17). However, this oxidant stress antagonized insulin-stimulated phosphorylation (Ser$^{473}$) of Akt by 62%, with the observed value being only 50% of the expected additive values. The oxidant exposure stimulated basal phosphorylation (Ser$^{9}$) of GSK-3 by 60% (Fig. 3, right) but reduced (by 49%) the ability of insulin to induce this variable, allowing GSK-3 to retain a more active form. The measured value for GSK-3 phosphorylation in the presence of the oxidant stress and insulin was less than half of the theoretical additive value. Akt and GSK-3 protein expression was not changed by the oxidant stress (data not shown).

We previously reported a role of p38 MAPK in the stimulation of glucose transport by an oxidant stress (17), and we therefore compared the effect of this oxidant exposure on p38 MAPK phosphorylation in the absence and presence of insulin (Fig. 4). H$_2$O$_2$ alone elicited a 45% increase in p38 MAPK phosphorylation, whereas insulin under these conditions had no effect on this variable. The oxidant-induced enhancement of p38 MAPK phosphorylation was the same in the presence and absence of insulin. The protein expression of p38 MAPK was not changed by the oxidant stress (data not shown).

Role of GSK-3 in oxidant stress-associated insulin resistance. Inasmuch as the oxidant stress exposure caused GSK-3 to assume a more active form (Fig. 3, right), we used a selective GSK-3 inhibitor, CT-98014 (4, 6, 14, 15, 21, 24, 32), to determine the role of this GSK-3 overactivity in the etiology of oxidant-induced insulin resistance. By itself, the GSK-3 inhibitor had no effect on basal or insulin-stimulated glucose transport activity or phosphorylation of IR-β, Akt, or GSK-3 (data not shown). However, CT-98014 did elicit increases in basal glycogen synthase activity (0.27 ± 0.01 vs. 0.36 ± 0.01) and net glycogen synthesis (2.1 ± 0.2 vs. 2.7 ± 0.1 nmol·mg$^{-1}$·min$^{-1}$, both $P < 0.05$). Moreover, insulin-stimulated glycogen synthase activity (Fig. 5, middle) and net glycogen synthesis (Fig. 5, right) were also increased by CT-98014. This effect of GSK-3 inhibition on glycogen synthase activity in insulin-sensitive muscle has been reported by our group previously (14, 24).
In the presence of insulin and the oxidant stress, glucose transport activity was enhanced 20% ($P < 0.05$) by the selective inhibition of GSK-3 (Fig. 5, left). Similarly, in the presence of the oxidant stress, CT-98014 increased insulin-stimulated glycogen synthase activity by 42% (Fig. 5, middle) and net glycogen synthesis by 35% (Fig. 5, right). The enhanced phosphorylation of p38 MAPK by the oxidant stress was not affected by the GSK-3 inhibitor (data not shown).

These increases in insulin-stimulated glucose transport and glycogen synthesis in the presence of the oxidative stress in muscle treated simultaneously with the selective GSK-3 inhibitor were associated with enhancements ($P < 0.05$) of tyrosine phosphorylation of IR ($36\%$; Fig. 6, left), phosphorylation (Ser473) of Akt ($58\%$; Fig. 6, middle), and phosphorylation (Ser9) of GSK-3 ($49\%$; Fig. 6, right).

**DISCUSSION**

In the present investigation, we have demonstrated for the first time in isolated mammalian skeletal muscle that a low-grade oxidant stress ($\sim 90 \mu M H_2O_2$) can directly and rapidly (within 2 h) induce an impairment of insulin action on glucose transport activity (Fig. 1) and glycogen synthesis (Fig. 2). Moreover, the oxidant-induced reductions in these physiological processes are associated with diminished insulin action on proximal (tyrosine phosphorylation of IR) and distal (serine phosphorylation of Akt and GSK-3β) insulin-signaling events (Fig. 3). The decrease in insulin-stimulated glycogen synthesis in the face of the oxidant stress is also linked to an impaired ability of insulin to stimulate the activity of glycogen synthase (Fig. 2).

We observed small, but significant, increases in basal glucose transport (Fig. 1), glycogen synthesis (Fig. 2), and insulin signaling (Fig. 3) due to the oxidant stress, similar to our previously reported findings (17). These increases in the skeletal muscle glucose transport system are not normally observed in conditions of oxidative stress-associated insulin resistance in vivo (8). However, the oxidant-induced increases in these variables have been accounted for in the analysis of the deleterious effects of the in vitro oxidant stress on insulin action, and this isolated skeletal muscle model is useful for the investigation of the molecular mechanisms underlying oxidant-associated insulin resistance in the skeletal muscle glucose transport system.

This oxidant stress-induced insulin resistance of glucose transport and glycogen synthase activation in normal mammalian skeletal muscle is similar to that reported in studies utilizing insulin-sensitive cell lines. Rudich and colleagues (26, 27) demonstrated in 3T3-L1 adipocytes and L6 myocytes that prolonged exposure to a low-grade H_2O_2 stress markedly decreases insulin-stimulated glucose metabolism. This decreased insulin responsiveness was associated with increased GLUT1 protein and mRNA and decreased GLUT4 protein and mRNA (19, 26) and impaired insulin signaling at the level of PI 3-kinase (27), a finding confirmed by Maddux et al. (20).
The findings of the present investigation indicate that the results from these cell culture studies are relevant to the etiology of insulin resistance that develops with exposure to oxidative stress in mammalian tissues, including skeletal muscle. Every experimental cell line and animal model employed in scientific investigations has inherent advantages and disadvantages regarding how well it reflects the condition(s) of human insulin resistance. Although it is clear that the duration of exposure (2 h) to the oxidant stress is relatively short, the present study represents the first comprehensive investigation of the direct effects of an oxidant stress on insulin signaling and glucose transport activity in a mammalian skeletal muscle preparation using the experimental design of previous investigations in cell lines (2, 3, 23). The proof of principle of this deleterious effect of an oxidant stress in mammalian skeletal muscle has been clearly demonstrated, with the added novel observation that part of this effect is mediated by GSK-3 overactivity. Future investigations using this isolated muscle approach should employ longer-term incubations with lower levels of the oxidant stress to further elucidate the signaling defects induced in oxidant stress-associated insulin resistance.

The reduced insulin-dependent activation of glycogen synthase in the presence of the oxidant stress (Fig. 2) is likely secondary to the impairment of insulin-stimulated Akt activation (Fig. 3), inasmuch as Akt can phosphorylate GSK-3 on serine residues and inhibit its activity (4). The decreased insulin-dependent activation of Akt observed under these conditions would therefore allow GSK-3 to attain an overactive state and inhibit glycogen synthase activity via serine phosphorylation (22, 25, 34). In support of this concept is our observation of a significant correlation between phosphorylation (Ser473) of Akt and the glycogen synthase activity ratio in the presence of insulin without or with an oxidant stress. Representative bands are shown. Values are means ± SE for 4–6 muscles per group. *P < 0.05 vs. muscles in the presence of insulin and oxidant stress. §P < 0.05 vs. muscles in the presence of insulin without H2O2.

Fig. 6. Effects of selective GSK-3 inhibition on IR-β tyrosine phosphorylation, Akt (Ser473) phosphorylation, and GSK-3β (Ser9) phosphorylation in skeletal muscle in the presence of insulin without or with an oxidant stress. Representative bands are shown. Values are means ± SE for 4–6 muscles per group. *P < 0.05 vs. muscles in the presence of insulin and oxidant stress. §P < 0.05 vs. muscles in the presence of insulin without H2O2.

IRS-1 in skeletal muscle of the prediabetic obese Zucker rat (6) and IR and IRS-1 in skeletal muscle of the type 2 diabetic Zucker diabetic fatty rat (15). Previous investigations have utilized highly selective inhibitors of GSK-3 to determine the role of GSK-3 in the etiology of insulin-resistant states in skeletal muscle and adipose tissue (for review see Refs. 7, 12, 32). For example, in the Zucker diabetic fatty rat, in vitro treatment of soleus muscle with the GSK-3 inhibitor CT-98014 improved insulin-stimulated glucose transport activity (14, 15, 24) and tyrosine phosphorylation of IR and IRS-1 and serine phosphorylation of Akt and GSK-3 (15). In the present investigation, similar enhancements of insulin-stimulated glucose transport activity (Fig. 1) and the functionality of insulin-signaling elements (Fig. 3) were induced by selective GSK-3 inhibition in isolated rat skeletal muscle made insulin resistant with exposure to an oxidant stress. However, the improvements in insulin action on glucose transport activity (Fig. 5) and insulin signaling (Fig. 6) mediated by the GSK-3 inhibitor in skeletal muscle exposed to the oxidant stress were on the order of 20–50%, underscoring the fact that GSK-3 overactivity contributes only partially to this insulin-resistant state and emphasizing the multifactorial etiology of this and other conditions of insulin resistance. In contrast, the GSK-3 inhibition mediated a complete restoration of insulin-stimulated glycogen synthase and glycogen synthesis (Fig. 5). The partial restoration of insulin signaling and insulin-stimulated glucose transport activity could be due to an incomplete inhibition of GSK-3 by CT-98014, inasmuch as GSK-3 activity was not directly assessed in the present study. However, the complete restoration of glycogen synthase activity (a direct substrate of GSK-3) and glycogen synthesis with the GSK-3 inhibitor (Fig. 5) indirectly supports the complete GSK-3 inhibition with CT-98014 in these isolated muscle preparations. Indeed, in a cell-free system, this concentration (1 μM) of CT-98014 completely inhibits GSK-3 kinase activity (24).

We observed that the oxidant stress caused an activation (increased phosphorylation) of the p38 MAPK in the absence and presence of insulin (Fig. 4), confirming in rat skeletal muscle preparations the critical elements of the insulin-signaling cascade, including  

**Fig. 6. Effects of selective GSK-3 inhibition on IR-β tyrosine phosphorylation, Akt (Ser473) phosphorylation, and GSK-3β (Ser9) phosphorylation in skeletal muscle in the presence of insulin without or with an oxidant stress.**

Representative bands are shown. Values are means ± SE for 4–6 muscles per group. *P < 0.05 vs. muscles in the presence of insulin and oxidant stress. §P < 0.05 vs. muscles in the presence of insulin without H2O2.
muscle the previous findings of Blair et al. (1) and Maddux et al. (20) in L6 myocytes. Moreover, we demonstrated in rat skeletal muscle that the same degree of oxidant stress-associated activation of p38 MAPK can be elicited in the presence and absence of insulin (17). Interestingly, we observed no insulin-stimulated increase in p38 MAPK phosphorylation in rat skeletal muscle, in contrast to previous findings in cultured cell lines and isolated skeletal muscle (18, 28–30). Shorter-duration (5–20 min) exposure of skeletal muscle similarly did not activate p38 MAPK (unpublished data). Finally, the GSK-3 inhibitor CT-98014 did not reduce this oxidant-induced enhancement of p38 MAPK phosphorylation (see RESULTS); although we did not measure p38 MAPK kinase activity in this context, we previously showed in a cell-free system that CT-98014 does not affect the related kinase Erk2 (24), and it is highly unlikely that CT-98014 affects p38 MAPK kinase activity at this concentration. Collectively, these findings support a potential role of p38 MAPK activation in the etiology of muscle insulin resistance.

In conclusion, the results of the present investigation demonstrate that exposure of skeletal muscle to an oxidant stress (micromolar levels of H$_2$O$_2$) produced by treatment with glucose oxidase in vitro can directly and rapidly induce insulin resistance of skeletal muscle glucose transport and glycolysis synthesis that is associated with impaired insulin action on the activation of the IR, Akt, GSK-3β, and glycogen synthase. Moreover, a small, but significant, portion of this oxidative stress-induced insulin resistance is associated with a reduced insulin-mediated suppression of the active form of GSK-3β, inasmuch as improvements in insulin action on glucose transport, glycolysis synthesis, and insulin signaling can be elicited with the selective GSK-3 inhibitor CT-98014. The oxidant stress leads to the same activation of p38 MAPK in the absence and presence of insulin. Collectively, these data indicate that GSK-3 overactivity can contribute to the multifactorial etiology of oxidant stress-associated insulin resistance in isolated rat skeletal muscle.

**REFERENCES**


