Nox2 Mediates Skeletal Muscle Insulin Resistance Induced by a High Fat Diet*§

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Background: The role and source of ROS in insulin resistance induced by a high fat diet remain uncertain.
Results: Insulin resistance induced by a high fat diet, palmitate, or a high concentration of glucose is mitigated in the absence of Nox2.
Conclusion: Nox2 mediates insulin resistance in skeletal muscle.
Significance: Nox2 represents a new target for the treatment of metabolic syndrome and its associated complications.

Inflammation and oxidative stress through the production of reactive oxygen species (ROS) are consistently associated with metabolic syndrome/type 2 diabetes. Although the role of Nox2, a major ROS-generating enzyme, is well described in host defense and inflammation, little is known about its potential role in insulin resistance in skeletal muscle. Insulin resistance induced by a high fat diet was mitigated in Nox2-null mice compared with wild-type mice after 3 or 9 months on the diet. High fat feeding increased Nox2 expression, superoxide production, and impaired insulin signaling in skeletal muscle tissue of wild-type mice but not in Nox2-null mice. Exposure of C2C12 cultured myotubes to either high glucose concentration, palmitate, or H2O2 decreases insulin-induced Akt phosphorylation and glucose uptake. Pretreatment with catalase abrogated these effects, indicating a key role for H2O2 in mediating insulin resistance. Down-regulation of Nox2 in C2C12 cells by shRNA prevented insulin resistance induced by high glucose or palmitate but not H2O2. These data indicate that increased production of ROS in insulin resistance induced by high glucose in skeletal muscle cells is a consequence of Nox2 activation. This is the first report to show that Nox2 is a key mediator of insulin resistance in skeletal muscle.

Insulin resistance and high blood glucose levels, due in part to impaired glucose uptake and utilization by skeletal muscle, are characteristic features of metabolic syndrome/type 2 diabetes. Current evidence suggests that elevated levels of ROS contribute to the alterations in insulin signaling (1). It has been proposed that oxidative stress plays a key role in causing insulin resistance, as the administration of free-radical scavengers or transgenic overexpression of antioxidant enzymes results in decreased insulin resistance (1). However, the relevant sources of ROS and the mechanism by which oxidative stress contributes to insulin resistance remain poorly understood.

NADPH oxidases are an important source of ROS and have been implicated in numerous pathophysiological processes (2–4). Skeletal muscle expresses transcripts for Nox2, protein components of the phagocyte NADPH oxidase complex (p22phox, Nox2, p47phox, and p67phox) (5, 6), and Nox4 (6). Nox2 activity is dependent on the formation of a cytochrome b by its association with p22phox and the recruitment of activating cytosolic cofactors (p67phox, p47phox) to the membrane. Nox4 is unique among Nox enzymes in being constitutively active (7, 8).

Skeletal muscle generates ROS both under resting conditions and during exercise (9). Although the contribution of mitochondria to ROS generation is well established, a potential role for Nox2 remains uncertain (10, 11). Nox2-derived ROS are proposed to play a role in Ca2+ release from the sarcoplasmic reticulum, a key signaling step in muscle contraction (5), and are required for myoblast differentiation (12). Furthermore, both Nox2 and Nox4 are involved in the proliferation of skeletal muscle precursor cells (13).

We investigated whether Nox2 plays a role in the development of insulin resistance. Our data show that Nox2 contributes to whole-body insulin resistance induced by a high fat diet. This phenotype is due, at least in part, to Nox2-dependent alteration of insulin signaling in skeletal muscle. Furthermore, using C2C12 myotubes, we demonstrate that down-regulation...
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of Nox2 protects against insulin resistance induced by palmitate or a high concentration of glucose.

Experimental Procedures

Mice, Diet, and Treatment—Mouse genotyping was carried out with The Jackson Laboratory primers. Wild-type and Nox2 knock-out (Nox2-KO) male mice (n = 80) were maintained in a temperature-controlled room (22–25 °C, 45% humidity) on a 12:12-h dark-light cycle. Beginning at 6 weeks of age, mice were fed ad libitum either a standard chow diet (SD: 57% carbohydrate, 5% fat, and 18% protein; Harlan) or a pelleted high fat diet (HFD: 45% fat Harlan Research diet; D12451) for either 3 or 9 months before testing. At the end of the feeding protocol, mice were fasted overnight then injected or not with 0.75 IU/kg of insulin intraperitoneally 15 min after insulin injection, gastrocnemius muscles were excised, and portions were either snap-frozen in liquid nitrogen or fixed in formalin. National Institutes of Health guidelines for research with vertebrate animals were strictly followed, and all the studies were approved by the Institutional Animal Care and Use Committee.

Measurement of Whole-body Fat and Lean Mass—Whole-body fat mass was measured by dual-energy x-ray absorptiometry (GE Lunar, Madison, WI) as described previously (14).

Serum Measurements—On overnight fasted mice (14 h), serum levels of insulin were determined by ELISA and glucose levels by colorimetric assay kits (Cayman Chemical Co., Ann Arbor, MI). The HOMA index, an estimation of insulin resistance, was calculated as (fasting serum insulin (ng/ml)/22.5) × (fasting serum glucose (mM))/22.5 (15).

Insulin Tolerance Test (ITT) and Glucose Tolerance Test (GTT)—ITT and GTT tests were done on the same set of mice. Briefly, overnight fasted mice (14 h) were injected with 0.75 IU insulin (intrapitoneal) or on a separate day with 1.5 g of glucose (intravenous) per kg of body weight for the ITT or for the GTT, respectively. Blood glucose levels were measured at 0, 15, 30, 60, and 90 min using a OneTouch Ultra glucometer.

Muscle Histology—Gastrocnemius muscles were stained with hematoxylin and eosin (H&E) and toluidine stain (Sigma, St. Louis, MO) using a light microscope (16).

Oxidative Fluorescent Microtopography of Skeletal Muscle—Sections (10 μm) of embedded frozen gastrocnemius muscles were stained using a light microscope. To measure superoxide, dihydroethidium (DHE, 300 nM), a cell-permeant superoxide-sensitive dye was applied to the skeletal muscle sections. In some experiments, muscle sections were preincubated with either 400 units/ml superoxide dismutase or 10 μM diphenylene iodonium. In situ fluorescence was assessed using a Zeiss confocal microscope.

C2C12 Cell Cultures and Treatment—C2C12 cells (17) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Differentiation into myotubes was induced by switching to DMEM containing 1% FBS for 4 days. Myotubes were treated for 48 h with or without H2O2 (100 μM) or in the presence of a standard concentration of glucose (control, 5 mM) or a high concentration of glucose (HG; 25 mM) or palmitate (200 μM). Where indicated, myotubes were pretreated with catalase 900 units/ml.

shRNA Nox2 Myotubes—Knockdown of Nox2 expression was performed using recombinant lentivirus containing shRNA to murine Nox2 or the GAPDH shRNA (Open Biosystems, Waltham, MA; catalog nos. RHS4372 and RHS4372, respectively). Stable expressing clones were selected in the appropriate antibiotic and tested for Nox2 and GAPDH transcript expression (Life Technologies, catalog nos. Mm00627011-M1 and catalog 4352932E, respectively) using the Taqman gene expression assay. Clones that showed a decrease of the Nox2 mRNA level were then tested by Western blot. Because the Nox2 antibody used in this study cross-reacts with GRP58/ERp57 (18), reactive sites were blocked by preincubating the polyvinylidene difluoride (PVDF) membrane with ERp57 antibody (Cell Signaling, Danvers, MA, catalog no. 2881).

Gene Expression Assays—cDNAs were synthesized from 2 μg of DNA-free RNA using the Go script reverse transcription system (Promega, Madison, WI; catalog no. A5000). Gene expression was determined by RT-PCR using commercially available Taqman probe/primer sets (Nox2 catalog no. Mm01287743_m1; Nox4 catalog no. Mm00479239_g1; 18S catalog no. Mm03928990_g1). Characterization of Nox enzymes expressed in skeletal muscle tissue and in C2C12 cells was also done using classical PCR and the following primers for Nox2 (sense, GGCACACATTCACACTGACC; antisense, GCATGTCTCCCTTCTCTGCAT), Nox4 (sense, TCTCAGGTGTGCCATGTAGCC; antisense, AAAAACCTCGAGGCAAAGAT), p47phox (sense, AACTGAAAATGCTCCAGATT; antisense, AGCCATCAGGAGTATAG), p67phox (sense, TGGCCTACTTCCAGAGAGGA; antisense, TCTTGTGAACCA- CAGATGC); p22phox (sense, AAAGAGAAAAAGGGGTCCA; antisense, CTCTCTTTCACCGTCACTCG).

Homogenate Preparation—For tissue, approximately 1 g of skeletal muscle was homogenized in a lysis buffer (20 mM HEPE, pH 7.9, 350 mM NaCl, 500 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM MgCl2, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 0.1 mM Na3V, 8 mM β-glycerolphosphate, phosphatase inhibitor mixture I and II (Sigma), and 1 table protease inhibitor mixture/50 ml of buffer (Roche Applied Science)) using the polytom (DIAX 900, Heidelberg-Instruments, Schwabach, Germany; 4 × 5 s at 1800 rpm). For cells, after treatment, the medium was removed, and the cells (~106 cells per dish) were scraped into ice-cold PBS, centrifuged, and homogenized in lysis buffer.

Preparation of Enriched Plasma Membrane Fraction—An enriched plasma membrane fraction of the muscle cells was prepared as previously described (19). Briefly, the cells were homogenized in Buffer A (10 mM Tris–HCl, pH 7.8, 10 mM KCl, 1.5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM...
dithiothreitol, 5 μg/ml aprotinin, and 10 μg/ml leupeptin containing 0.1% Nonidet P-40), passed through a 22-gauge needle three times, and spun at 1000 × g for 10 min at 4 °C two times. The plasma membrane fraction was obtained by resuspending the resulting pellet in Buffer A containing 1% Nonidet P-40 and centrifugation at 10,000 × g for 20 min at 4 °C. The validity of this preparation was tested by immunoblot using protein markers of subcellular compartments (supplemental Fig. 1).

Western Blot Analysis—Proteins were separated on 4–15% SDS-polyacrylamide gradient gels and transferred to PVDF membranes. The filters were incubated with antibodies directed against phospho-Akt and Akt (Cell Signaling, catalog no. 9271 and #9272, respectively), Glut4 (Santa Cruz Biotechnology, catalog no. sc-53566), myosin heavy chain type I (Developmental Studies Hybridoma Bank catalog no. MF20), Nox2 (kindly provided by Mark Quinn, Montana State University), p67phox (William Nauseef, University of Iowa), Nox4 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-301141), p22phox (Santa Cruz Biotechnology, catalog no. sc-20781). Antibodies to protein disulfide isomerase (EMD Millipore, Darmstadt, Germany; catalog no. 539229 EMD Biosciences), actin, or Pan cadherin (Sigma, catalog nos. A5441 and C1821, respectively) were used as loading controls. The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences).

Glucose Uptake Measurement—The assay was performed as described previously (20). Serum-starved C2C12 myotubes washed with Krebs-Ringer phosphate HEPES buffer were incubated without or with 100 nM insulin for 45 min. The cells were then incubated for 5 min with 2-deoxy-[14C]glucose (0.4 mM, 0.1 μCi/ml; PerkinElmer Life Sciences and Analytical Science). The reaction was stopped with PBS containing 10 μM cytochalasin B (Sigma). The cells were washed with ice-cold PBS and lysed with 0.2M NaOH. The radioactivity taken up by the cells was determined using a scintillation counter (Beckman Instruments, Fullerton, CA) and normalized to protein content.

Statistical Analysis—Data are presented as the mean ± S.E. of the values and are normalized to controls. Statistical analysis was performed using the Newman-Keuls multiple comparisons test to adjust for multiple testing when comparing several means against the mean for a common control sample. A value for p < 0.05 was accepted as significant.

Results

Body Weight composition of Wild-type and Nox2-null Mice Fed a High Fat Diet—When fed either a standard diet or a high fat diet, Nox2-KO mice achieved lower body weights than similarly fed wild-type mice (Fig. 1A). These differences, observed only after 3 months of diet, reached significance only for the
high fat diet group. The weight gain induced by the high fat diet was proportionately similar in wild-type and Nox2-KO mice (Fig. 1A). At 3 and 9 months, analysis of body composition revealed no difference in fat and lean mass between wild-type and Nox2-KO mice when fed the same diet. They exhibit 15–20% fat mass on the standard diet versus 40–45% on the high fat diet (Fig. 1, B and C). At 9 months, although Nox2-KO mice achieved lower body weights than similarly fed wild-type mice, no significant variations were observed in the distribution of fat mass versus lean mass between Nox2-KO and wild-type mice under the same diet (Fig. 1, C, D, and E).

Insulin Resistance Induced by a High Fat Diet Is Mitigated in Nox2-KO Mice—After 3 months, wild-type mice fed a high fat diet (WT-HFD mice) exhibited significant increases in levels of fasting blood glucose, insulin, and HOMA-IR insulin resistance index compared with wild-type mice fed a standard diet (WT-SD mice; Fig. 2, A–C). In contrast, Nox2-KO mice fed a high fat diet (Nox2-KO HFD mice) exhibited very modest and mostly non-significant changes in these same indices, such that all parameters were significantly lower in Nox2-KO-HFD (Nox2-KO mice fed a high fat diet) than in WT-HFD animals. After 9 months mice fed a high fat diet were glucose-intolerant (GTT) and insulin-resistant (ITT) (Fig. 2, D–G). The absence of Nox2 was significantly protective against the development of glucose intolerance and insulin resistance. Our data show that Nox2-KO mice fed a high fat diet responded similarly to wild-type mice for both intraperitoneally injected GTT and ITT (Fig. 2, D–G). Interestingly, on a standard diet the decrease in glucose level after insulin injection tends to occur faster in Nox2-KO mice than in wild-type mice. This kinetic difference indicates that Nox2-KO mice are more sensitive to insulin than wild-type mice despite the lack of significant differences of the overall area under the curve GTT. Collectively, these data suggest that the absence of Nox2 has a protective effect against insulin resistance induced by a high fat diet.

**FIGURE 2.** Nox2 deletion reduces insulin resistance induced by a high fat diet. In wild-type and Nox2-KO mice fed for 3 months with either a standard or high fat diet, fasting glucose (A), insulin (B), and HOMA-insulin resistance index (C) were determined. Intraperitoneally injected glucose tolerance test (GTT) and insulin tolerance test (ITT) (E) were performed in wild-type and Nox2-KO mice fed for 9 months with either a standard or a high fat diet. Overall insulin tolerance and glucose tolerance are expressed as the area under curve (F and G). Data are the mean ± S.E. of results obtained from 6–10 mice/group. Statistical significance versus WT-SD mice is *p* < 0.05 (*), *p* < 0.01 (**), and *p* < 0.001 (***) and versus WT-HFD is *p* < 0.05 (#) and *p* < 0.01 (##).
Effect of the High Fat Diet on Skeletal Muscle Histology—We observed that muscle fiber size tends to be smaller in Nox2-KO mice than in wild-type mice when fed a standard diet. This difference was no longer observed in mice fed a high fat diet. The high fat diet led to an accumulation of adipocytes around the muscle and between the fibers and increased fat mass in skeletal muscle to a similar degree in wild-type and Nox2-KO mice (Fig. 3, A and B). Interestingly and in accord with the improvement of insulin sensitivity in Nox2-KO mice, we found a greater increase in lean mass in Nox2-KO mice than in wild-type mice fed a high fat diet (Fig. 3B). We used the staining of mATPase and the quantification of myosin heavy chain I (MHC I) expression levels to determine the proportion of oxidative (slow) versus glycolytic (fast) muscle fibers, as it was reported that slow fibers are both more insulin-sensitive and more insulin-responsive compared with fast-twitch fibers (21–23). We observed that a large portion of the muscle contained intermediate fibers. Some areas presented a mosaic pattern containing both slow and fast fibers, but there was no significant change in the proportion of oxidative versus glycolytic fibers due to either the genotype (Fig. 3C) or the diet (not shown). Furthermore we observed no significant difference in the expression levels of MHC I in muscle of both wild-type and Nox2-KO mice fed a standard or a high fat diet (Fig. 3D). These data suggest that there is no significant change in the proportion of oxidative versus glycolytic fibers due either to the diet or the genotype.

Effect of the High Fat Diet on Nox Gene Expression in Skeletal Muscle Tissue—The genotypes of wild-type and Nox2-KO mice were confirmed by PCR (Fig. 4A). We found that Nox4, Nox2, p22phox, p67phox, and p47phox proteins were all expressed in skeletal muscle tissue (Fig. 4B). The residual bands observed in the immunoblot using Nox2 antibody using Nox2 knock-out mouse skeletal muscle tissue protein extract represents the cross reactivity of the antibody with the GRP58/ERP57 (18). Also, to further assess Nox2 deletion, we phenotypically confirmed the absence of a functional Nox2 protein in Nox2-KO mice by measuring superoxide production by purified blood neutrophils (data not shown).

We observed that the gene expression of Nox2 was consistently increased by the high fat diet at 3 and 9 months (Fig. 4C), whereas Nox4 expression was significantly increased only after 9 months of diet. Despite the increased Nox2 and Nox4 mRNA
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expression (Fig. 4C), only Nox2 and p22<sub>phox</sub> protein expression levels were consistently increased after 3 (data not shown) and 9 months of high fat diet (Fig. 4D).

Deletion of Nox2 Protects against HFD-induced Oxidative Stress and Insulin Resistance in Skeletal Muscle Tissue—We measured superoxide production in longitudinal frozen sections (10 μm) of gastrocnemius muscle using DHE, a fluorescent probe that selectively detects superoxide under the conditions used. The DHE signal was significantly higher in muscle isolated from wild-type mice fed a high fat diet than in those fed a standard diet for 3 months (Fig. 5A). This effect was totally abrogated when muscle tissues were preincubated with either superoxide dismutase or diphenylene iodonium, indicating that superoxide is produced by a flavoprotein oxidase such as Nox2 (Fig. 5A). We found that muscle of Nox2-KO mice fed a standard diet produced slightly more superoxide than the wild-type mice fed a standard diet (Fig. 5B). This unexpected effect, which might be due to compensatory mechanisms linked to the absence of Nox2, does not account for the absence of a high fat diet effect on superoxide production in Nox2-KO mice (Fig. 5B). Consistent with these results, we found that only wild-type mice fed a high fat diet showed an increase in membrane-associated p67<sub>phox</sub> compatible with the activation of Nox2, which requires the translocation of the cytosolic co-factors (24) (Fig. 5C). Collectively these data suggest that superoxide production induced by the high fat diet in skeletal muscle is due to increased Nox2 expression and activity. In parallel, we found that Nox2 knock-out mice expressed more Glut4 than wild-type mice. We observed that the high fat diet significantly reduced Glut4 expression in wild-type mice, whereas its expression was further increased in Nox2-KO mice. This finding not only suggests that the effect of the high fat diet on Glut4 expression is Nox2-dependent but that under physiologic conditions Nox2 represses Glut4 expression. Furthermore we
found that insulin-induced Akt phosphorylation and Glut4 translocation to the plasma membrane were decreased in skeletal muscle of wild-type mice fed for 3 months with a standard or a high fat diet were taken after 10 min of incubation with DHE. Where indicated, gastrocnemius muscle before imaging was preincubated with diphenylene iodonium or superoxide dismutase. Each pair of photomicrograph panels corresponds to superoxide production (red fluorescence) and the differential interference contrast image (gray picture). The scale bar indicates a length of 10 μm. A.U., arbitrary units. B, confocal images of superoxide production in longitudinal sections of optimal cutting temperature compound (OCT)-embedded frozen gastrocnemius muscle isolated from wild-type and Nox2-KO mice fed for 3 months with either a standard diet or a high fat diet. Fluorescence intensity levels were quantified using Image J. Data are representative of results obtained from 6–10 mice/group. Statistical significance versus WT-SD mice is p < 0.001 (**). C, immunoblots were performed using either membrane fractions or homogenates of skeletal muscle tissue isolated from wild-type and Nox2-KO mice fed either a standard or a high fat diet for 9 months. Immunoblots with antibodies against Akt or Glut4 proteins were performed on the membrane fraction of skeletal muscle isolated from mice injected with insulin 15 min before sacrifice. The levels of phospho-Akt and membrane-associated p67\(^{phox}\) and Glut4 were calculated from their individual autoradiographic densities and normalized to the corresponding level of Akt, protein disulfide isomerase (PDI), cadherin, or GAPDH. Data are representative of results obtained from 6–10 mice/group. Statistical significance versus WT-SD mice is p < 0.05 (*) and p < 0.01 (**). Statistical significance versus WT-HFD is p < 0.05 (#) and/or p < 0.001 (###).

**FIGURE 5.** Nox2 deletion reduces high fat diet-induced oxidative stress and improves insulin signaling in skeletal muscle tissue. A, confocal images of superoxide production in longitudinal sections of optimal cutting temperature compound (OCT)-embedded frozen gastrocnemius muscle isolated from wild-type mice fed for 3 months with a standard or a high fat diet were taken after 10 min of incubation with DHE. Where indicated, gastrocnemius muscle before imaging was preincubated with diphenylene iodonium or superoxide dismutase. Each pair of photomicrograph panels corresponds to superoxide production (red fluorescence) and the differential interference contrast image (gray picture). The scale bar indicates a length of 10 μm. A.U., arbitrary units. B, confocal images of superoxide production in longitudinal sections of optimal cutting temperature compound (OCT)-embedded frozen gastrocnemius muscle isolated from wild-type and Nox2-KO mice fed for 3 months with either a standard diet or a high fat diet. Fluorescence intensity levels were quantified using Image J. Data are representative of results obtained from 6–10 mice/group. Statistical significance versus WT-SD mice is p < 0.001 (**). C, immunoblots were performed using either membrane fractions or homogenates of skeletal muscle tissue isolated from wild-type and Nox2-KO mice fed either a standard or a high fat diet for 9 months. Immunoblots with antibodies against Akt or Glut4 proteins were performed on the membrane fraction of skeletal muscle isolated from mice injected with insulin 15 min before sacrifice. The levels of phospho-Akt and membrane-associated p67\(^{phox}\) and Glut4 were calculated from their individual autoradiographic densities and normalized to the corresponding level of Akt, protein disulfide isomerase (PDI), cadherin, or GAPDH. Data are representative of results obtained from 6–10 mice/group. Statistical significance versus WT-SD mice is p < 0.05 (*) and p < 0.01 (**). Statistical significance versus WT-HFD is p < 0.05 (#) and/or p < 0.001 (###).

Chronic H\(_2\)O\(_2\)-dependent Effect of HG Levels on Insulin Signaling in Myotubes—We next assessed the potential role of Nox2 in ROS-induced insulin resistance in C2C12 cells, a murine myoblastic cell line. The differentiation of C2C12 cells leads to the formation of multinucleated cells that form tubes (Fig. 6A) and specific expression of myogenin (Fig. 6B). Both undifferentiated and differentiated C2C12 cells expressed Nox4, Nox2, p67\(^{phox}\), p47\(^{phox}\), and p22\(^{phox}\) transcripts and proteins, although levels of Nox system proteins tended to be higher after differentiation (Fig. 6C).

Because hyperglycemia and hyperlipidemia are major factors in the pathogenesis of insulin resistance induced by a high fat diet, we investigated the role of Nox2 in cells treated with high concentration of glucose (25 mM) or palmitate (200 μM). In agreement with previous reports (25), pretreatment with HG or palmitate induced a state of insulin resistance, as reflected by impaired insulin-induced Akt phosphorylation and glucose uptake (Fig. 7, A–D). These effects were abrogated by catalase pretreatment, suggesting that H\(_2\)O\(_2\) mediates HG and palmitate-induced insulin resistance (Fig. 7). In fact, treatment with 100 μM H\(_2\)O\(_2\) mimicked effects of HG and palmitate (Fig. 7, E–H).
and F) and catalase pretreatment abrogated the effects of H₂O₂ treatment. These observations altogether demonstrate that insulin resistance induced by HG or palmitate is dependent on H₂O₂ production.

**Down-regulation of Nox2 Counteracts the Insulin Resistance Induced by HG but Not by H₂O₂**—Because Nox2 is a possible source of H₂O₂ in myotubes, we explored its role in insulin resistance using shRNA Nox2-expressing C2C12 myotubes. C2C12 expressing Nox2 or control shRNA cultured in 1% FBS morphologically changed into elongated tubular forms and expressed myogenin and MHC, two important markers of myoblast differentiation into myotubes (Fig. 8A). HG or palmitate treatment of shRNA control cells did not significantly alter the expression level of Nox2 or Nox4 but reduced Akt phosphorylation (Fig. 8, B, C, E, and F) and glucose uptake induced by insulin (Fig. 8, D and G). To the contrary, these treatments of shRNA Nox2 myotubes did not induce insulin resistance as measured by the same indices (Fig. 8, B–G). H₂O₂ treatment of control shRNA myotubes mimicked HG and palmitate treatments and induced insulin resistance (Fig. 8, H–J). Interestingly, the down-regulation of Nox2 did not prevent the induction of insulin resistance by H₂O₂ (Fig. 8, H–J), suggesting that H₂O₂ induction of insulin resistance bypasses Nox2. Altogether, these data demonstrate that Nox2 in skeletal muscle cells is implicated in insulin resistance induced by high concentration of glucose or palmitate. Moreover, our data suggest that Nox2 is a major source of H₂O₂ production that mediates insulin resistance induced by HG or palmitate.

**Discussion**

Skeletal muscle is responsible for 70–90% of total body glucose uptake. Understanding the factors that contribute to insulin resistance in this tissue and specifically Nox2 as a source of ROS is highly relevant to the pathogenesis of metabolic syndrome. Our data provide the first evidence that Nox2 plays an important role in insulin resistance induced by a high fat diet. Furthermore, we demonstrate that deficiency of Nox2 protects against high fat diet-induced insulin resistance by improving glucose uptake in skeletal muscle cells.

Body weight is tightly linked to metabolic pathways, and beneficial effects of weight loss on metabolism are well documented (26, 27). We observed no significant difference in fat mass/body weight between WT and Nox2-KO mice on the same diet (Fig. 1, D and E). Also total body weight and composition are unlikely to explain the early (3 months) and late (9 months) beneficial effect of Nox2 deletion on insulin sensitivity. Such improvement of glucose metabolism without reduction of fat mass has been observed (28) and is common in metabolically healthy obese individuals.

Although ectopic accumulation of fat can be deleterious, the impact of intramyocellular fat in skeletal muscle remains controversial (29, 30). In our high fat diet model, we did not detect intramyocellular fat using oil red O staining (data not shown), but we did observe adipocyte accumulation in skeletal muscle tissue of both high fat-fed wild-type and Nox2-KO mice. Also, improvement of insulin resistance does not correlate with a reduction of fat in skeletal muscle of Nox2-KO mice (Fig. 3B). A recent report showed that Nox2-KO mice fed high fat diet had smaller visceral adipose deposits, attenuated visceral adipocyte hypertrophy, and diminished visceral adipocyte macrophage infiltration (31). We are currently assessing fat cells to determine further the impact of Nox2 on fat metabolism and oxidative stress.
We observed that a high fat diet increased superoxide production in skeletal muscle fibers of wild-type mice, evidenced by DHE fluorescence within the muscle fibers and in spots surrounding the fibers. These spots of red fluorescence may correspond to nuclei of the skeletal muscle fibers as well as cells of the endomysium. Because we observed that the red fluorescence surrounding muscle fibers is present in Nox2-KO mice fed either a standard or a high fat diet, it appears to arise from a source other than Nox2. Nox4 is expressed in many cells present in skeletal muscle tissue (13, 32, 33), including myoprogenitor cells (13), endothelial cells (33), and adipocytes (34), any of which could contribute to the superoxide signals detected in skeletal muscle by confocal imaging. However, we found that the expression of Nox4 protein in skeletal muscle tissue is not significantly altered in Nox2-KO mice or by the high fat diet. Although Nox4 activity is mainly regulated by its expression level, the possibility of other mechanisms cannot be excluded. Whether this adaptive mechanism compensating for the absence of Nox2 occurs through up-regulation of Nox4 or another superoxide-generating enzyme, it is not responsible for the effect of the high fat diet on superoxide production in skeletal muscle, as the diet did not increase superoxide production in skeletal muscle tissue of Nox2-KO mice. This supports our main conclusion that Nox2 is the primary source of muscle oxidative stress induced by a high fat diet.

**Figure 7.** Chronic H$_2$O$_2$-dependent insulin resistance. Akt phosphorylation and glucose uptake were determined in myotubes cultured for 48 h with 5 mM (control) or 25 mM (HG) glucose (A and B), 200 µM palmitate (C and D), or with 100 µM H$_2$O$_2$ (E and F) and then stimulated for 20 min with 100 nM insulin. Where indicated, cells were pretreated with catalase (900 units/ml, 10 min) before HG, palmitate, or H$_2$O$_2$ exposure. Phosphorylation of Akt was calculated from individual autoradiographic densities and normalized to the corresponding total Akt levels (A, C, and E). These ratios were determined only in cells stimulated with insulin to accurately determine the effect of HG, palmitate, or H$_2$O$_2$ pretreatment. Glucose uptake was determined as the amount of deoxy-$[^{14}$C$]$glucose in cells and expressed as fold increase compared with values obtained in untreated cells (B, D, and F). Data are representative of results obtained from a total of 4–6 experiments. Statistical significance versus WT-SD mice is $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) and versus cells stimulated with insulin is $p < 0.05$ (†), $p < 0.001$ (‡‡), and $p < 0.01$ (‡).
The confocal imaging with DHE did not show the type of pattern that would be expected with a mitochondrial source. Because superoxide is a short-lived ROS that is restricted to its site of production, the data argue against mitochondrial superoxide generation in this model. It is, however, possible that mitochondria are a secondary source of ROS that might amplify or sustain the oxidative stress. Further study is needed to determine whether Nox2 affects mitochondrial function as an additional mechanism contributing to insulin resistance.

We observed that insulin signaling was altered in skeletal muscle of mice fed a high-fat diet. This effect correlated with an increase in Nox2 and p22phox proteins as well as membrane-associated p67phox, an index of Nox2 activation. Furthermore, we observed that Nox2 repressed Glut4 expression and that the high-fat diet reduced Glut4 expression in a Nox2-dependent manner. Interestingly, it has been shown that Glut4 is reduced in slow muscle fibers of type 2 diabetic patients. Also, it is possible that the Glut4 expression level is an early indicator of fiber metabolic alteration that precedes the disruption of the relative proportion of oxidative versus glycolytic fibers in skeletal muscle (37, 38). In addition to down-regulating Glut4 expression, we also observed that Nox2 decreased Glut4 translocation and Akt phosphorylation induced by insulin. These results collectively reinforce the prominent role of Nox2 in insulin resistance in skeletal muscle tissue through the regulation of essential intermediates (Fig. 5).

Although the present study implicates Nox2 in insulin resistance, other work shows that Nox2 is required to induce insulin-stimulated calcium release (39). This bimodal pattern has been observed in many systems where ROS, although essential for physiologic function, are deleterious when a certain threshold is attained. Also, our findings suggest that the increase of Nox2 expression and activity (Fig. 5) might increase ROS production beyond that threshold, thereby accounting for the apparent discrepancy in Nox2 effect on insulin signaling. These observations implicate Nox2 as an impor-
tant contributor of both physiologic and pathophysiologic insulin signaling in skeletal muscle.

To provide in vitro support for the importance of Nox2 expressed in skeletal muscle cells in the induction of insulin resistance, we used C2C12, a murine myoblastic cell line. We observed that HG- or palmitate-induced insulin resistance was alleviated by treatment with catalase and that H$_{2}$O$_{2}$ mimicked their effects. These data demonstrate that HG and palmitate exert their action in a H$_{2}$O$_{2}$-dependent manner. Because H$_{2}$O$_{2}$ can be produced through the dismutation of superoxide generated by Nox2 expressed in myotubes, we generated myotubes with stably down-regulated Nox2. The level of proliferation of these cells was reduced, but their ability to form myotubes remained intact (13). These results contrast with a previous report showing that Nox2 is required for both myoblast proliferation and differentiation (12). It may be that the extent of Nox2 down-regulation differs between the two studies and that the residual Nox2 in our shRNA-treated cells is sufficient to support proliferation and differentiation. Alternatively, the shRNA construct used by Piao et al. (12) might have altered Nox4 levels, which according to Mofarrah et al. (13), is down-regulated during differentiation.

Our data showed that down-regulation of Nox2 expression in myotubes mitigated the effects of HG and palmitate on insulin signaling and glucose uptake. However, shRNA Nox2 cells treated with H$_{2}$O$_{2}$ remained insulin-resistant, indicating that exogenous H$_{2}$O$_{2}$ bypasses Nox2 and is sufficient by itself to induce the deleterious redox signaling. Moreover, these findings suggest that in cells exposed to HG or palmitate, the production of H$_{2}$O$_{2}$ is subsequent to Nox2 activation or that Nox2 is required to amplify the amount of H$_{2}$O$_{2}$ produced to reach the necessary threshold to induce the insulin-resistant phenotype. Feedback regulation of ROS-generating enzymes by ROS themselves, as previously reported, may contribute to the generation of high levels of ROS (40–42). In vitro, we were unable to see any significant increase of Nox2 expression in cells treated with either HG or palmitate. This might be due to the fact that the duration of treatment is not comparable to months of exposure of the tissue to hyperglycemia and hyperlipidemia or that additional stimuli are present in vivo that act in concert to up-regulate Nox2 expression in skeletal muscle. Another possibility is that other cell types present in skeletal muscle tissue contribute to the up-regulation of Nox2. Leukocytes are good candidates as they abundantly express Nox2; however, we did not observe any significant increase of leukocytes in mouse skeletal muscle tissue (data not shown). An important aspect of Nox2 is that it requires the translocation of cytosolic factors to be activated. Also, the deleterious superoxide production by Nox2 probably involves the activation of PKC by high glucose or free fatty acids (43, 44). Also, we do not expect the up-regulation of Nox2 by itself to induce insulin resistance in the in vitro model. Nevertheless, our data demonstrate that Nox2 expressed in skeletal muscle cells is a mediator of insulin resistance induced by HG and palmitate.

In summary, this study provides novel and specific evidence that Nox2-generated ROS contribute to insulin resistance in mice fed a high fat diet and that deletion of Nox2 restores skeletal muscle insulin sensitivity. Moreover, we provide evidence that down-regulation of Nox2 expressed in skeletal muscle cells improves insulin signaling. Because skeletal muscle insulin resistance is the earliest step in the pathogenesis of metabolic syndrome/type 2 diabetes (45–47), our data indicate that Nox2 may become a complementary therapeutic target for the treatment of metabolic syndrome and its associated complications. The potential beneficial impact of such therapeutics is further supported by studies showing that Nox2 inhibition is protective against tissue injury in metabolic diseases (31, 48–50).

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References
1. Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2002) Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocr. Rev. 23, 599 – 622
2. Dupuy, C., Ohayon, R., Valent, A., Noël-Hudson, M.-S., Déme, D., and Virion, A. (1999) Purification of a novel flavoprotein involved in the thyroid NADPH oxidase: cloning of the porcine and human cDNAs. J. Biol. Chem. 274, 37265–37269
3. Geiszt, M. (2000) Identification of renox, an NAD(P)H oxidase in kidney. Proc. Natl. Acad. Sci. U.S.A. 97, 8010 – 8014
4. Suh, Y. A. (1999) Cell transformation by the superoxide-generating oxidase Nox1. Nature 401, 79 – 82
5. Hidalgo, C., Sánchez, G., Barrientos, G., and Aracena-Parks, P. (2006) A transverse tubule NADPH oxidase activity stimulates calcium release from isolated triads via ryanodine receptor type 1 S-glutathionylation. J. Biol. Chem. 281, 26473–26482
6. Cheng, G. (2001) Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. Gene 269, 131 – 140
7. Ambasta, R. K., Kumar, P., Griendling, K. K., Schmidt, H. H., Busse, R., and Brandes, R. P. (2004) Direct interaction of the novel nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. J. Biol. Chem. 279, 45935–45941
8. Jordahl, P. L. (2006) Regulation of NADPH oxidases: the role of Rac proteins. Circ. Res. 98, 453 – 462
9. Jackson, M. J. (2008) Free radicals generated by contracting muscle: by-products of metabolism or key regulators of muscle function? Free Radic. Biol. Med. 44, 132–141
10. Lambertucci, R. H., Hirabara, S. M., Silveira Ldos, R., Levada-Pires, A. C., Curi, R., and Pithon-Curi, T. (2008) Palmitate increases superoxide production through mitochondrial electron transport chain and NADPH oxidase activity in skeletal muscle cells. J. Cell. Physiol. 216, 796 – 804
11. Zuo, L., Pasniciuc, S., Wright, V. P., Merola, A. J., and Clanton, T. L. (2003) Expression of human aminopeptidase N (APN) in skeletal muscle enhances insulin signaling in vivo. J. Biol. Chem. 278, 27927–27933
12. Piao, Y. J., Sun, F. H., Hong, F., Kim, J. H., Kim, Y.-J., Kang, M. H., Kim, B. S., Jo, S. A., Jo, I., Jue, D.-M., Kang, I., Ha, J., and Kim, S. S. (2005) Nox 2 stimulates muscle differentiation via NF-[kappa]B/iNOS pathway. Free Radic. Biol. Med. 39, 296 – 303
13. Li, Y., Wu, Y., Zhang, Y., Wang, Y., Wang, G., Li, L., and Li, K. (2008) Enhancement of muscle regeneration by Nox2 knockdown in skeletal muscle of aged mice. J. Biol. Chem. 283, 16763–16768
Goldstein, D. J. (1992) Beneficial health effects of modest weight loss. *Anti-oxidants Redox Signaling* **10**, 559–574

Halade, G. V., El Jamali, A., Williams, P. J., Fajardo, R. J., and Fernandes, G. (2011) Obesity-mediated inflammatory microenvironment stimulates osteoclastogenesis and bone loss in mice. *Exp. Gerontol.* **46**, 43–52

Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., and Turner, R. C. (1985) Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28**, 412–419

Gollnick, P. D., and Matoba, H. (1984) Identification of fiber types in rat skeletal muscle based on the sensitivity of myofibrillar actomyosin ATPase to copper. *Histochemistry* **81**, 379–383

Yaffe, D., and Saxel, O. (1977) A myogenic cell line with altered serum sensitivity. *J. Cell Biol.* **75**, 97–105

Baniulis, D., Nakano, Y., Naseef, W. M., Banfi, B., Cheng, G., Lambeth, D. J., Burritt, J. B., Taylor, R. M., and Jessaitis, A. J. (2005) Evaluation of two anti-gp91(phox) antibodies as immunoprobes for Nox family proteins: mAb 54.1 recognizes recombinant full-length Nox2, Nox3, and the C-terminal domains of Nox1–4 and cross-reacts with GRP 58. *Biochem. Biophys. Acta* **1752**, 186–196

Yap, A., Nishiumi, S., Yoshida, K., and Ashida, H. (2007) Rat L6 myotubes as an *in vitro* model system to study GLUT4-dependent glucose uptake stimulated by insulinotrophic cytokines. *Cytochemistry* **55**, 103–108

Nedachi, T., and Kanzaki, M. (2006) Regulation of glucose transporters by insulin and extracellular glucose in C2C12 myotubes. *Am. J. Physiol. Endocrinol. Metab.* **291**, E817–E828

Kern, M., Wells, J. A., Stephens, J. M., Elton, C. W., Friedman, J. E., Tapp, Scott, T. B., Pekala, P. H., and Dohm, G. L. (1990) Insulin responsiveness in skeletal muscle is determined by glucose transporter (Glut4) protein level. *Biochem. J.* **270**, 397–400

Zierath, J. R., He, L., Gumà A, Odagoord Wahlström E, Klip A, Wallberg-Henriksson H. (1996) Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia* **39**, 1180–1189

Krketos, A. D., Part, D. A., Lilloia, S., Cooney, G. J., Baur, L. A., Milner, M. R., Sutton, J. R., Jenkins, A. B., Bogardus, C., and Storlien, L. H. (1996) Interrelationships between muscle morphology, insulin action, and adiposity. *Am. J. Physiol.* **270**, R1332–R1339

Clark, R. A., Volpp, B. D., Leidal, K. G., and Nauseef, W. M. (1990) Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J. Clin. Invest.* **85**, 714–721

Huang, C., Somwar, R., Patel, N., Niu, W., Török, D., and Klip, A. (2002) Regulation of glucose transporters by fasting plasma glucose and insulin concentrations in man. *Diabetologia* **45**, 1120–1126

Badin, P. M., Vila, I. K., Louche, K., Mairal, A., Marques, M. A., Bourlier, V., Tavernier, G., Langin, D., and Moro, C. (2013) High fat diet-mediated lipotoxicity and insulin resistance is related to impaired lipase expression in mouse skeletal muscle. *Endocrinology* **154**, 1444–1453

Pepping, J. K., Freeman, L. R., Gupta, S., Keller, J. N., and Bruce-Keller, A. J. (2013) NOX2 deficiency attenuates markers of adiposepathy and brain injury induced by high fat diet. *Am. J. Physiol. Endocrinol. Metab.* **304**, E492–E404

Lee, C. F., Qiao, M., Schröder, K., Zhao, Q., and Asmis, R. (2010) Nox4 is a novel inducible source of reactive oxygen species in monocytes and macrophages and mediates oxidized low density lipoprotein-induced macrophage death. *Circ. Res.* **106**, 1489–1497

Williams, C. R., Lu, X., Sutliff, R. L., and Hart, C. M. (2012) Rosiglitazone attenuates NF-KB-mediated Nox4 upregulation in hyperglycemia-activated endothelial cells. *Am. J. Physiol. Cell Physiol.* **303**, C213–C223

Mahadevan, K., Motoshima, H., Wu, X., Ruddy, J. M., Arnold, R. S., Cheng, G., Lambeth, J. D., and Goldstein, B. J. (2004) The NAD(P)H oxidase homolog nox4 modulates insulin-stimulated generation of H2O2 and plays an integral role in insulin signal transduction. *Mol. Cell Biol.* **24**, 1844–1854

Anderson, E. J., and Lustig, M. E., Boyle, K. E., Woodlief, T. L., Kane, D. A., Lin, C. T., Price, J. W., 3rd, Kang, L., Rabinovitch, P. S., Szeto, H. H., Houmard, J. A., Cortright, R. N., Wasserman, D. H., Neuf, P. D. (2009) Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J. Clin. Invest.* **119**, 573–581

Bonnard, C., Durand, A., Peyrol, S., Chasseuneu, M., Chauvin, M. A., Morio, B., Vidal, H., and Rieux, S. (2008) Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J. Clin. Invest.* **118**, 789–800

Gaster, M., Staehr, P., Beck-Nielsen, H., Schröder, H. D., and Handberg, A. (2001) GLUT4 is reduced in slow muscle fibers of type 2 diabetic patients: is insulin resistance in type 2 diabetes a slow, type 1 diabetes disease? *Diabetes* **50**, 1324–1329

Gaster, M., Poulsen, P., Handberg, A., Schroeder, H. D., and Beck-Nielsen, H. (2000) Direct evidence of fiber type-dependent GLUT4 expression in human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **278**, E910–E926

Espinosa, A., García, A., Härtil, S., Hidalgo, C., and Jainovich, E. (2009) NADPH oxidase and hydrogen peroxide modulate insulin-induced calcium increase in skeletal muscle cells. *J. Biol. Chem.* **284**, 2568–2575

Zorov, D. B., Filburn, C. R., Klotz, L. O., Zweier, J. L., and Sollett, S. J. (2000) Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J. Exp. Med.* **192**, 1001–1014

El Jamali, A., Valente, A. J., Lechleiter, J. D., Gamez, M. J., Pearson, D. W., Nauseef, W. M., and Clark, R. A. (2008) Novel redox-dependent regulation of NOX5 by the tyrosine kinase c-Abl. *Free Radic. Biol. Med.* **44**, 868–881

El Jamali, A., Valente, A. J., and Clark, R. A. (2010) Regulation of phagocyte NADPH oxidase by hydrogen peroxide through a Ca2+/c-Abl signaling pathway. *Free Radic. Biol. Med.* **48**, 798–810

Geraudes, P., and King, G. I. (2010) Activation of protein kinase C isoforms and its impact on diabetic complications. *Circ. Res.* **106**, 1319–1331

Glass, C. K. and Olefsky, J. M. (2012) Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metab.* **15**, 635–645

Martin, B. C., Warram, J. H., Krolewski, A. S., Bergman, R. N., Soeldner, J. S., and Kahn, C. R. (1992) Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. *Lancet* **340**, 925–929

Lilloia, S., Mott, D. M., Spraul, M., Ferraro, R., Foley, I. E., Ravussin, E., Knowler, W. C., Bennett, P. H., and Bogardus, C. (1993) Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus: prospective studies of Pima indians. *N. Engl. J. Med.* **329**, 1988–1992

Song, R., Peng, W., Zhang, Y., Lu, F., Wu, H.-K., Guo, J., Cao, Y., Yi, P., Zhang, X., Jin, L., Zhang, M., Jiang, P., Liu, F., Meng, S., Zhang, X., Jiang, P., Cao, C.-M., and Xiao, R.-P. (2013) Central role of E3 ubiquitin ligase
MG53 in insulin resistance and metabolic disorders. *Nature* 494, 375–379

48. Paik, Y.-H., Iwaisako, K., Seki, E., Inokuchi, S., Schnabl, B., Osterreicher, C. H., Kisseleva, T., and Brenner, D. A. (2011) The nicotinamide adenine dinucleotide phosphate oxidase (NOX) homologues NOX1 and NOX2/gp91phox mediate hepatic fibrosis in mice. *Hepatology* 53, 1730–1741

49. Sukumar, P., Viswambharan, H., Imrie, H., Cubbon, R. M., Yuldasheva, N., Gage, M., Galloway, S., Skromna, A., Kandavelu, P., Santos, C. X., Gatenby, V. K., Smith, J., Beech, D. J., Wheatcroft, S. B., Channon, K. M., Shah, A. M., and Kearney, M. T. (2013) Nox2 NADPH oxidase has a critical role in insulin resistance-related endothelial cell dysfunction. *Diabetes* 62, 2130–2134

50. Xiang, F. L., Lu, X., Strutt, B., Hill, D. J., and Feng, Q. (2010) NOX2 deficiency protects against streptozotocin-induced beta-cell destruction and development of diabetes in mice. *Diabetes* 59, 2603–2611