Nox2 Mediates Skeletal Muscle Insulin Resistance Induced by a High-Fat Diet.

Running title: Nox2-Dependent Insulin Resistance

Alvaro Souto Padron de Figueiredo¹, Adam B. Salmon²,³, Francesca Bruno¹, Fabio Jimenez¹, Herman G. Martinez¹, Ganesh V. Halade¹, Seema S. Ahuja¹,³, Robert A. Clark¹,³, Ralph A DeFronzo¹,³, Hanna E. Abboud¹,³ and Amina El Jamali¹

1- Department of Medicine, University of Texas Health Science Center, San Antonio, Texas 78229-3900 USA
2- The Sam and Ann Barshop Institute for Longevity and Aging Studies 15355 Lambda Drive San Antonio, Texas 78245
3- Audie L. Murphy Hospital, South Texas Veterans Health Care System, San Antonio, TX 78229-3900, USA

To whom correspondence should be addressed: Amina El Jamali, Department of Medicine, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900 USA. Phone: 210-567-1992; Fax: 210-567-4654; E-mail: AkoulouzeBik@uthscsa.edu

Keywords: Insulin resistance, Reactive oxygen species (ROS), NADPH oxidase, Nox2, Skeletal muscle, high-fat diet, mice, Nox2 knockout, palmitate, high concentration of glucose, C2C12 cells.
**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.

**SUMMARY**

Inflammation and oxidative stress, through the production of reactive oxygen species (ROS), are consistently associated with metabolic syndrome/type 2 diabetes. While 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 (HFD) 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 H$_2$O$_2$ decreases insulin-induced Akt phosphorylation and glucose uptake. Pre-treatment with catalase abrogated these effects indicating a key role for H$_2$O$_2$ in mediating insulin resistance. Down-regulation of Nox2 in C2C12 cells by shRNA prevented insulin resistance induced by high glucose or palmitate but not H$_2$O$_2$. 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, since the administration of free-radical scavengers or transgenic over-expression 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 (p22$^{phox}$, Nox2, p47$^{phox}$, and p67$^{phox}$) (5,6) and Nox4 (6). Nox2 activity is dependent on the formation of a cytochrome b by its association with p22$^{phox}$ and the recruitment of activating cytosolic cofactors (p67$^{phox}$, p47$^{phox}$) 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). While 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 Ca$^{2+}$ 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 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 Jackson Lab primers. Wild-type and Nox2 knockout (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 six 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 insulin intraperitoneally [i.p.], 15 min after insulin injection, gastrocnemius muscles were excised and portions 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, USA) as described previously (14).

Serum measurements
On overnight fasted mice (14h), serum levels of insulin were determined by ELISA and glucose levels by colorimetric assay kits (Cayman Chemical Company, Ann Arbor, MI). The HOMA index, an estimation of insulin resistance, was calculated as: [fasting serum insulin (ng/ml) x 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 (i.p.) or on a separate day with 1.5 g of glucose (i.v.) per kg of body weight for the insulin tolerance test (ITT) or for the glucose tolerance test (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 (mATPase staining) to reveal the overall general structure and detect oxidative versus glycolytic fibers. Muscle fiber typing is based on myosin ATPase activity. Frozen muscle sections (10-12 µM) were affixed to slides and were pre-incubated for 8 minutes in a buffer containing 50 mM K acetate; pH 4.4, 17mM CaCl$_2$. The sections were then washed three times in Tris buffer (300 mM Tris-HCl; pH 7.8, 53 mM CaCl$_2$), and then incubated for 25 minutes in a buffer containing 50 mM glycine; pH 9.4, 28 mM CaCl$_2$, 65 mM NaCl, 47.5 mM NaOH, 4 mM ATP. Sections were then rinsed with 1% w/v CaCl$_2$ and stained with 0.1% toluidine blue. Sections were rinsed in H$_2$O, dehydrated in ethanol and pictures were taken using a light microscope (Gollnick, 1984 #1063).

Oxidative fluorescent microtopography of skeletal muscle
Sections (10 µm) of embedded frozen gastrocnemius muscles were maintained in Krebs-ringer buffer and incubated in a light-protected and humidified chamber at 37°C. To measure superoxide, dihydroethidine (DHE, 300 nM), a cell-permeant superoxide-sensitive dye, was applied to the skeletal muscle sections. In some experiments, muscle sections were pre-incubated with either superoxide dismutase (SOD), 400 U/ml or diphenylene iodonium (DPI) 10 µM. In situ fluorescence was assessed using a Zeiss confocal microscope.

C2C12 cell cultures and treatment.
C2C12 cells (16) were grown in Dulbeco’s modified Eagle 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 H$_2$O$_2$ (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 pre-treated with catalase 900 U/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, cat# RMM4534-NM 007807 and # RHS3472 respectively). Stable expressing clones were selected in the appropriate antibiotic and tested for Nox2 and GAPDH transcript expression (Life Technologies, Grand Island, NY, cat# Mm00627011-M1 and cat#
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 (17), reactive sites were blocked by pre-incubating the PVDF membrane with ERp57 antibody (Cell Signaling, Danvers, MA, cat# 2881).

Gene expression assays
cDNAs were synthesized from 2μg of DNA-free RNA using the Go script reverse transcription system (Promega, Madison, WI, cat#A5000). Gene expression was determined by RT-PCR using commercially available Taqman probe/primer sets (Nox2 cat# Mm01287743_m1; Nox4 cat# Mm00479239_g1; 18S cat # 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: GCCACACATTCACTGACC, antisense: GCATTGTTCCCTTCTCTGAT), Nox4 (sense: TCTCGGTGTGCAATGAGGCC, antisense: AAAACCCTCAGGGAAGAT), p47phox (sense: AACTGAAACTGCCCACTGAC, antisense: AGCCATCCAGGCTATAG); p67phox (sense: TGGCCTACTTCCAGAGAGGA, antisense: TCTTGGTGAACCACAGATGC); and p22phox (sense: AAAGAGGAAAAAGGGGTCCA, antisense: CTCTTCCACCTCACTCG).

Homogenate preparation. Tissue: ~1 g of muscle was homogenized in a lysis buffer (20 mM HEPES, 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 NaV, 8 mM β-glycerophosphate, phosphatase inhibitor cocktail I and II (Sigma) and 1 tablet protease inhibitor cocktail/50 ml buffer [Roche, Mannheim, Germany]) using the polytron (DIAX 900, Heidolph-Instruments, Schwabach, Germany, 4 X 5 sec at 1800 rpm). Cells: After treatment, the medium was removed, and the cells (~10⁶ cells per dish) 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 (18). Briefly, the cells were homogenized in Buffer A (10 mM Tris–HCl, pH 7.8, 10 mM KCl, 1.5 mM MgCl2, 1 mM phenylmethylsulfonylfuoride, 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 1,000g 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 10,000g for 20 min at 4°C. The validity of this preparation was tested by immunoblot using protein markers of subcellular compartments (Suppl. 1).

Western blot analysis
Proteins were separated on 4-15% SDS polyacrylamide gradient gels and transferred to polyvinylidene difluoride (PVDF) membranes. The filters were incubated with antibodies directed against phospho-Akt and Akt (Cell Signaling, cat# 9271 and 9272, respectively), Glut4 (Santa Cruz Biotechnology, cat# sc-53566), Myosin heavy chain type I (Developmental studies hybridoma bank cat# MF20), Nox2 (kindly provided by Mark Quinn, Montana State University), p67phox (William Nauseef, University of Iowa), Nox4 (Santa Cruz Biotechnology, Santa Cruz, CA, cat# sc-301141), p22phox (Santa Cruz Biotechnology, cat# sc-20781). Antibodies to protein disulfide isomerase (PDI, EMD Millipore, Darmstadt, Germany, cat #539229 EMD Biosciences), actin or Pan cadherin (SIGMA, St. Louis, MO, cat # A5441 and C1821, respectively) were used as loading controls. The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham, Pittsburgh, PA).

Glucose uptake measurement
The assay was performed as described previously (19). 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 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 0.2 M...
NaOH. The radioactivity taken up by the cells was determined using a scintillation counter (Beckman Instruments, Fullerton, CA, USA) and normalized to protein content.

**Statistical analysis:**
Data are presented as the mean ± SEM 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 approximately 15 to 20% fat mass on the standard diet vs. 40 to 45% on the high-fat diet (Fig. 1B and 1C). 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 vs. lean mass between Nox2-KO and wild-type mice under the same diet (Fig. 1C, D, 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. 2A-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 than in WT-HFD animals. After 9 months mice fed a high-fat diet were glucose-intolerant (ipGTT) and insulin-resistant (ipITT) (Fig. 2D-2G). 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 animals fed a standard diet for both ipGTT and ipITT (Fig. 2D-2G). 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 AUC GTT. Collectively, these data suggest that the absence of Nox2 has a protective effect against insulin resistance induced by a high-fat diet.

**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. 3A, 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 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 {Kern, 1990 #1060} {Zierath JR, 1996 #1061} {Kriketos, 1996 #1062}. 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 (Fig. 4B). The residual bands observed in the immunoblot using Nox2 antibody using Nox2 knockout mouse skeletal muscle tissue protein extract represents the cross reactivity of the antibody with the GRP58/ERp57 (17). 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), while Nox4 expression was significantly increased only after 9 months of diet. Despite the increased Nox2 and Nox4 mRNA expression (Fig. 4C), only Nox2 and p22phox 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 dihydroethidine (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 pre-incubated with either SOD or DPI, indicating that superoxide is produced by a flavo-protein 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 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 p67phox, compatible with the activation of Nox2, which requires the translocation of the cytosolic co-factors (20) (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 knockout mice express more Glut4 than wild-type mice. We observed that the high-fat diet reduced significantly Glut4 expression in wild-type mice, whereas its expression is 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 a high-fat diet for 3 months (data not shown) or 9 months (Fig. 5C). This effect was not observed in Nox2-KO mice. Collectively, these findings suggest that Nox2-KO mice are more insulin-sensitive and are protected against the deleterious effects of the high-fat diet through a Nox2-dependent regulation of Akt phosphorylation and Glut4 expression and translocation.

**Chronic H2O2-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, p67phox, p47phox, and p22phox transcripts and proteins, although levels of Nox system proteins tended to be higher following 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 (HG; 25 mM) or palmitate (200 µM). In agreement with previous
reports (21), pretreatment with HG or palmitate induced a state of insulin resistance, as reflected by impaired insulin-induced Akt phosphorylation and glucose uptake (Fig. 7A,B, C, D). These effects were abrogated by catalase pre-treatment, suggesting that H₂O₂ mediates HG and palmitate-induced insulin resistance (Fig. 7). In fact, treatment with 100 µM H₂O₂ mimicked these effects of HG (Fig. 7E, F) and catalase pre-treatment 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**

Since 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 myosin heavy chain (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. 8B, C, E, F) and glucose uptake induced by insulin (Fig. 8D, G). To the contrary, these treatments of shRNA Nox2 myotubes did not induce insulin resistance as measured by the same indices (Fig. 8B, C, D, E, F, G). H₂O₂ treatment of control shRNA myotubes mimicked HG and palmitate treatments and induced insulin resistance (Fig. 8H, I, J). Interestingly, the down-regulation of Nox2 did not prevent the induction of insulin resistance by H₂O₂ (Fig. 8H, I, 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 (22,23). We observed no significant difference in fat mass/body weight between WT and Nox2-KO mice on the same diet (Fig 1D, 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 (24) 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 (25,26). 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 adipose macrophage infiltration (27). 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);(28)-(29), including myoprogenitor cells (13), endothelial cells (29) and adipocytes (30), 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, since the diet did not increase superoxide production in skeletal muscle 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. This finding contrasts with the work of Anderson et al., who showed that scavenging H$_2$O$_2$ produced by mitochondria counteracts the effect of a high-fat diet on insulin signaling (31). However, another study using a high-fat high-sucrose diet demonstrated that mitochondrial dysfunction is a consequence rather than a cause of insulin resistance, rather than a cause (32). The confocal imaging with DHE did not show the type of pattern that would be expected with a mitochondrial source. Since 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 p22$^{phox}$ proteins, as well as membrane-associated p67$^{phox}$, 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 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 {Gaster, 2001 #1058} {Gaster, 2000 #1059}. In addition to down-regulating Glut4 expression, we observed also 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. 5D).

While the present study implicates Nox2 in insulin resistance, other work shows that Nox2 is required to induce insulin-stimulated calcium release (33). This bimodal pattern has been observed in many systems where ROS, though 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 important 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 exerts their action in a H$_2$O$_2$-dependent manner. Since 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
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used by Piao et al. (12), might have altered Nox4 levels, which according to Mofarrhi et al., is down-regulated during differentiation (13).

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 H₂O₂ remained insulin resistant, indicating that exogenous H₂O₂ 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 H₂O₂ is subsequent to Nox2 activation or that Nox2 is required to amplify the amount of H₂O₂ 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 (34-36). 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 didn’t 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 (37,38). 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. Since skeletal muscle insulin resistance is the earliest step in the pathogenesis of metabolic syndrome / type 2 diabetes (39-41), 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 (27,42-44).
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REFERENCES


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Author disclosure statement: Robert Clark is a founder, equity holder, and member of the Scientific Advisory Board of Genkyotex SA, a biotechnology company devoted to the development of small-molecule inhibitors of Nox enzymes. No other conflicts of interest exist.

Abbreviations: AUC: Area under the curve; DPI: diphenylene iodonium; DHE: Dihydroethidine; HFD: High-fat diet; HG: High concentration of glucose; HOMA: Homeostasis Model of Assessment of insulin resistance; i.p.: Intraperitoneal; ipGTT: Glucose tolerance test with glucose injection by i.p. route; ipITT: Insulin tolerance test with insulin injection by i.p. route; Nox: NADPH oxidase; Nox2: NADPH oxidase type 2; Nox4: NADPH oxidase type 4; Nox2-KO: Nox2 knockout; Nox2-KO-HFD: Nox2-KO mice fed a high-fat diet; Nox2-KO-SD: Nox2-KO mice fed a standard diet; ROS: reactive oxygen species; RT mRNA: Reverse-transcribed mRNA; SOD: superoxide dismutase; SD: Standard diet; WT-HFD: wild-type mice fed a high-fat diet; WT-SD: Wild-type fed a standard diet.

Figures Legends

Fig. 1 - Body weight composition and fat mass gain is similar in Nox2-KO mice and wild-type mice fed a high-fat diet. (A) Body weight of wild-type and Nox2-KO mice fed either a standard or a high-fat diet; (B) and (C). Lean mass and fat mass of wild-type and Nox2-KO mice fed either a standard or a high-fat diet for 3 months (B) or 9 months (C); (D) Lean mass and (E) fat mass of mice fed 9 months with either SD or HFD expressed in percent of total body weight. Data are the mean +/- SEM of results obtained from 6-10 mice/group. Statistical significance versus WT-SD mice is ***P<0.001, and vs WT-HFD #P<0.05.

Fig. 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, (A) fasting glucose, (B) insulin and (C) HOMA-insulin resistance index were determined. (D) Glucose tolerance test (ipGTT) and (E) Insulin tolerance test (ipITT) 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 (AUC; F and G). Data are the mean +/- SEM of results obtained from 6-10 mice/group. Statistical significance versus WT-SD mice is *P<0.05, **P<0.01, ***P<0.001, and vs WT-HFD #P<0.05, ##P<0.01.

Fig. 3 - Adipocyte accumulation and increased lean mass in skeletal muscle of Nox2-KO mice fed a high-fat diet. (A) Longitudinal and transverse paraffin sections of gastrocnemius muscle of wild-type and Nox2-KO mice fed either a standard or a high-fat diet for 3 months were stained with hematoxylin and eosin (H&E). (B) Hind limb fat and lean mass were measured by Dual-energy X-ray absorptiometry (DXA) before and after 3 months of diet. The gain in fat or lean mass is expressed as a percent of total fat or lean mass in the hind limb. (C) Gastrocnemius muscle fiber typing in wild-type and Nox2-KO mice. (D) MHC I and GAPDH immunblots were performed using muscle homogenates obtained from wild-type and Nox2 knockout mice fed a standard or a high fat diet. The levels of MHC I were calculated from their individual autoradiographic densities and normalized to the corresponding level of GAPDH. Data are the mean +/- SEM of results obtained from 5 mice/group. Statistical significance versus WT-SD mice is *P<0.05, **P<0.01, and vs WT-HFD #P<0.05.

Fig. 4 – Insulin resistance correlates with increased Nox2 expression. (A) RNA extracted from frozen gastrocnemius muscle and from RAW cells (left panel) was reverse transcribed (RTmRNA+) or not (RTmRNA-), then the products and control plasmid containing Nox4 cDNA were subjected to a PCR using primers for mouse p67phox, p47phox, p22phox, Nox2 and Nox4. In addition 50 µg of total protein extract were used to determine p67phox, p47phox, p22phox, Nox2 and Nox4 protein content by immunoblot analysis (right panel). (B) Wild-type and Nox2-KO mice genotyping (Wild type = 240 bp, Mutant = 195 bp). Raw cells were used as a positive control for the PCR. (C) By quantitative RT-PCR, Nox2 and Nox4 mRNA expression levels were determined in skeletal muscle RNA extracts from mice fed the standard diet.
diet (SD) or the high-fat diet (HFD) for 3 and 9 months. The results were quantified by the $\Delta$Ct method using 18S for differences in RNA input and efficiency in cDNA synthesis and expressed as arbitrary units compared with the average expression levels in WT mice. (D) The protein expression levels of Nox2, p22phox and Nox4 were determined by immunoblot in wild-type and Nox2-KO mice fed either a standard diet or a high-fat diet. GAPDH was used as a loading control. Results are expressed as the ratio of the protein of interest and loading control immunoreactivity. All the data are the mean +/- SEM of results obtained from 5-10 mice/group. Statistical significance versus WT-SD mice is *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Fig. 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 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 incubation with dihydroethidine (DHE). Where indicated gastrocnemius muscle prior to imaging was pre-incubated with DPI or SOD. 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. (B) Confocal images of superoxide production in longitudinal sections of 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 membrane fraction of skeletal muscle isolated from mice injected with insulin 15 min prior to sacrifice. The levels of phospho-Akt and membrane-associated p67phox and Glut4 were calculated from their individual autoradiographic densities and normalized to the corresponding level of Akt, PDI, cadherin, or GAPDH. Data are representative of results obtained from 6-10 mice/group. Statistical significance versus WT-SD mice is *$P<0.05$, **$P<0.01$.

Fig. 6: Nox2 and Nox4 are expressed in C2C12 myoblasts and myotubes. (A) Digital images of C2C12 cells grown in either a proliferative (10% FBS) or a differentiating (1% FBS) medium were captured. (B) Differentiated (D) and undifferentiated (U) C2C12 cells were analyzed for myogenin expression by RT-PCR. (C) Expression of Nox2, Nox4, p22phox, p47phox and p67phox in undifferentiated and differentiated C2C12 cells were determined by RT-PCR and immunoblot.

Fig. 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; B), 200 µM Palmitate (C, D) or with 100 µM H$_2$O$_2$ (E, F) and then stimulated for 20 min with 100 nM insulin. Where indicated, cells were pre-treated with catalase (900 U/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, 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 $^{14}$C-deoxy-glucose in cells and expressed as a fold increase compared with values obtained in untreated cells (B, D, 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 versus cells stimulated with insulin #$P<0.05$, ###$P<0.001$, ##$P<0.01$.

Fig. 8: Down-regulation of Nox2 counteracts the insulin resistance induced by high concentration of glucose but not by H$_2$O$_2$: (A) RT-PCR was performed to determine the mRNA levels of myogenin and MHC expressed in control and Nox2 shRNA myoblasts (U) and myotubes (D). Akt phosphorylation (B, C, E, F, H, I) and glucose uptake (D, G, J) stimulated by insulin were determined in control and Nox2 shRNA myotubes treated or not for 48 h with either 5 mM (control) or 25 mM (HG) of glucose (B-D),
200 µM palmitate (E-G) or with 100 µM H₂O₂ (H-J) and then stimulated for 20 min with 100 nM insulin. Immunoblots with Nox2 and Nox4 antibodies were performed to confirm the specific down-regulation of Nox2 by shRNA (B, E, H). Figures C, F, I represent the quantitative analysis of the phosphorylation of Akt stimulated by insulin. Results were quantified and expressed as in Fig. 7. Data are the mean+/−SEM of 4-6 experiments. Statistical analysis is performed versus each untreated shRNA cell type (control (Ctrl) or Nox2 shRNA) *P<0.05, **P<0.01 and versus each shRNA cells type stimulated with insulin †P<0.05, ‡‡‡‡P<0.001, ‡‡P<0.01.
Figure 1

(A) Body weight (g) over days for different groups: Wild type SD, Wild type HFD, Nox2−/− SD, Nox2−/− HFD.

(B) Mass (g) for Lean and Fat groups after 3 months diet: WT SD, WT HFD, Nox2−/− SD, Nox2−/− HFD.

(C) Mass (g) for Lean and Fat groups after 9 months diet: WT SD, WT HFD, Nox2−/− SD, Nox2−/− HFD.

(D) Lean mass expressed in % of body weight for SD and HFD groups: WT SD, Nox2−/− HFD.

(E) Fat mass expressed in % of body weight for SD and HFD groups: WT SD, Nox2−/− HFD.
Nox2-dependent insulin resistance

Figure 2

(A) Glucose (mg/dl) in WT and NOX2−/− mice on SD and HFD diets.

(B) Insulin (μU/ml) in WT and NOX2−/− mice on SD and HFD diets.

(C) HOMA-IR index in WT and NOX2−/− mice on SD and HFD diets.

(D) GTT: Glucose (mg/dl) over time (min) for WT and NOX2−/− mice on SD and HFD diets.

(E) ITT: Glucose (mg/dl) over time (min) for WT and NOX2−/− mice on SD and HFD diets.

(F) Area under the curve of the GTT (arbitrary units) for WT and NOX2−/− mice.

(G) Area under the curve of the ITT (arbitrary units) for WT and NOX2−/− mice.
Nox2-dependent insulin resistance

Figure 3

A

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H&E staining

B

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SD | HFD

C

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mATPase staining

D

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Figure 4

A. Genotyping

B. RT mRNA analysis

C. Relative mRNA expression

D. Western blot analysis of muscle total protein extract
Nox2-dependent insulin resistance

Figure 7

A

HG Ins. - + + + Cat.

p-Akt (Ser 473)

Akt

B

HG Ins. - + + + Cat.

P-Akt/Akt immunoreactivity

Percent vs. insulin stimulated cells

C

Palm. Ins. - + + + Cat.

p-Akt (Ser 473)

Akt

D

Palm. Ins. - + + + Cat.

Fold increase in 14C-deoxyglucose uptake vs. control cells

E

H₂O₂ Ins. - + + + Cat.

p-Akt (Ser 473)

Akt

F

H₂O₂ Ins. - + + + Cat.

Fold increase in 14C-deoxyglucose uptake vs. control cells

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Figure 8

Nox2-dependent insulin resistance
Nox2 Mediates Skeletal Muscle Insulin Resistance Induced by a High-Fat Diet
Alvaro Souto Padron de Figueiredo, Adam B. Salmon, Francesca Bruno, Fabio Jimenez, Hernan G. Martinez, Ganesh V. Halade, Seema S. Ahuja, Robert A. Clark, Ralph A. DeFronzo, Hanna E. Abboud and Amina El Jamali

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