Angiotensin II induced skeletal muscle insulin resistance mediated by NF-κB activation via NADPH oxidase

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ABSTRACT

Reduced insulin sensitivity is a key factor in the pathogenesis of type 2 diabetes and hypertension. Skeletal muscle insulin resistance is particularly important as the major site for insulin-mediated glucose disposal. Angiotensin II (Ang II) is integral in regulating blood pressure and plays a role in the pathogenesis of hypertension. In addition, we have documented that Ang II-induced skeletal muscle insulin resistance is associated with reactive oxygen species (ROS) generation. However, the linkage between ROS and insulin resistance in skeletal muscle remains unclear. To explore potential mechanisms, we employed the transgenic TG(mRen2)27 (Ren2) hypertensive rat that harbors the mouse renin transgene exhibits elevated tissue Ang II levels, and skeletal muscle cell culture. Compared with Sprague Dawley (SD) normtensive control rats, Ren2 skeletal muscle exhibited significantly increased oxidative stress, NF-κB activation and TNF-α expression, which were attenuated by in vivo treatment with either an angiotensin type 1 receptor (AT₁R) blocker (valsartan) or superoxide dismutase (SOD)/catalase mimetic (tempol). Moreover, Ang II treatment of L6 myotubes induced NF-κB activation and TNF-α production, and decreased insulin-stimulated Akt activation and GLUT4 glucose transporter translocation to plasma membranes. These effects were markedly diminished when myotubes were pre-treated with valsartan, antioxidant N-acetylcysteine (NAC), NADPH oxidase inhibiting peptide (gp91ds-tat), or NF-κB inhibitor (MG132). Similarly, NF-κB p65 siRNA reduced NF-κB p65 subunit expression and nuclear translocation, TNF-α production, while improving insulin-stimulated Akt Ser⁴⁷³ phosphorylation and GLUT4 translocation. These findings suggest that NF-κB plays an important role in Ang II/ROS-induced skeletal muscle insulin resistance.
**Key words:** renin-angiotensin system, reactive oxygen species, TG(mRen2)27 rat, tumor necrosis factor – alpha, angiotensin receptor blocker

**INTRODUCTION**

The metabolic syndrome which is closely associated with obesity, dyslipidemia, hypertension and type 2 diabetes mellitus (T2DM) affects 47 million people or 24% of the U.S. adult population (8; 23; 26). Whereas, insulin resistance is a key factor in the pathogenesis of the metabolic syndrome, skeletal muscle is particularly important in the development of insulin resistance since it is responsible for approximately 75%-95% of insulin-mediated glucose disposal (26). Skeletal muscle insulin signaling involves insulin binding to its receptor followed by a series of post-receptor phosphorylation events including the activation of Akt (protein kinase B)(24). Therefore, factors such as angiotensin II (Ang II) that impair this signaling processes cause insulin resistance and contribute to systemic glucose intolerance, (2; 16; 28).

The renin-angiotensin system (RAS) plays a vital role in regulating blood pressure and the pathogenesis of hypertension and cardiovascular disease (CVD). Recent evidence indicates that inhibiting RAS not only improves cardiovascular outcomes but may have metabolic benefits as well, e.g. angiotensin converting enzyme (ACE) inhibitors or angiotensin II type 1 (AT₁R) blockers increase insulin sensitivity (3; 9; 29; 31) and reduce new onset of T2DM compared with other anti-hypertensive agents (25). Adverse effects of the RAS appear to act directly upon skeletal muscle since interstitial infusion of Ang II has been shown to induce insulin resistance that is independent of vascular effects, while Ang II also impairs insulin signaling in cultured myotubes (19; 30). Ang II has pro-oxidant effects by inducing reactive oxygen species (ROS) generation. We have shown that ROS mediate Ang II-induced skeletal muscle insulin resistance and glucose intolerance in the transgenic TG(mRen2)27 (Ren2) hypertensive rat that harbors the
mouse renin gene and exhibits elevated tissue Ang II levels (3). Yet, the intermediate steps linking ROS to insulin resistance in skeletal muscle remain unknown. It is known that ROS activates multiple transcription factors including NF-κB (10; 13). NF-κB activation is involved in high fat diet-induced liver insulin resistance in mice (5). However, it has not been determined whether Ang II activates skeletal muscle NF-κB thereby providing a potential mechanism for Ang II-induced insulin resistance. In the present study we employed Ren2 rats and Ang II treated myotubes to determine the role of ROS mediated NF-κB activation in blunting insulin effects upon skeletal muscle. Our results suggest that Ang II induced ROS generation via NADPH oxidase activates NF-κB which in turn contributes to skeletal muscle insulin resistance.
MATERIALS AND METHODS

Materials

Antibodies against 4-Hydroxy-2-Nonenal (4-HNE), TNF-α, NF-κB p65, IκB-α, Akt, phospho-Akt (Ser\(^{473}\)), glucose transporter (GLUT4 and 1) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Upstate Signaling Technology (Beverly, MA), and Abcam Inc. (Cambridge, MA), Cell Signaling (Beverly, MA), respectively. The antibody against the Na-K ATPase alpha-1 subunit was obtained from Upstate Biotechnology (Lake Placid, NY). The rat TNF-α ELISA detection kit was obtained from R&D Systems Inc. (Minneapolis, MN). NF-κB NoShift Assay Kit was purchased from Novagen, EMD Biosciences Inc. (San Diego, CA). Ang II, human insulin and NADPH were purchased from Sigma (St. Louis, MO). Dulbecco’s modified Eagle’s Medium (DMEM), fetal bovine serum (FBS) and antibiotic-antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B) were purchased from GIBCO (Invitrogen). Gp91ds-tat is a generous gift from Dr. Patrick Pagano (Henry Ford Research Institute and Hospital, Detroit, MI) and the scrambled siRNA as controls was purchased from Bio-Synthesis (Lewisville, TX) with or without a fluorescent tag (carboxytetramethylrhodamine, TAMRA).

Animals and Treatments

Male Ren-2 and Sprague-Dawley (SD) rats were received at 5-6 wk of age from Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC. Animal protocols were reviewed and approved by the Harry S. Truman VA Medical Center Animal Care Committee. After a short period of adaptation, Ren2 rats were randomly assigned to treatment with the AT\(_1\)R blocker valsartan (RV; 30 mg/kg/day) or the superoxide dismutase/catalase mimetic tempol (RT; 1mM) in their drinking water for 21 days, or remained untreated (RC). These were compared to
age-matched, untreated SD rats. The rats were weighed and anesthetized with Nembutal (35 mg/kg ip) and soleus muscles dissected and trimmed, and either frozen in liquid nitrogen and stored at -80°C, fixed or homogenized for further analysis as described below.

**Cell Culture**

L6 rat skeletal muscle cells (American Type Culture Collection) were grown in DMEM with 10% (v/v) fetal bovine serum (FBS) and 1% vol/vol antibiotic-antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B) at 5% CO₂ 37°C until ~80% confluence. To induce differentiation, cells were further cultured in DMEM containing 2% FBS for 6-8 days. Cells were fed fresh medium every 48 h and used at the stage of myotubes (60-70%) when GLUT4 glucose transporter expression is highest (11). Myogenic differentiation to myotubes was confirmed by light microscopy with morphological alignment, elongation, and fusion.

**Gp91ds-tat Transfection**

In selected experiments, the NAD(P)H oxidase inhibitor gp91ds-tat (gift from P. Pagano) and the scrambled gp91ds-tat as a control (Bio-Synthesis, Lewisville, TX) were used. The peptide gp91ds-tat is linked to a 9-amino acid peptide contained in human immunodeficiency virus (HIV) viral coats (HIV-tat) that is internalized by all cells. The peptide interferes with the binding of the cytosolic subunit p47phox with the membrane subunit gp91phox (Nox2) and inhibits NADPH oxidase activation (18; 32). A scrambled 9-amino acid Nox2 sequence (scramb-tat) was used as a control. In some experiments, Gp91ds-tat and scramb-tat were dissolved in 0.01 mmol/L acetic acid in saline. Myotubes were incubated with gp91ds-tat (20µmol/L) or scramb-tat (20µmol/L) for 1 hour before Ang II incubation.

**NF-κB p65 siRNA Transfection**
L6 myotubes were transfected with NF-κB p65siRNA or control siRNA (Santa Cruz Biotechnology) as described before (30). The differentiation medium was changed to antibiotic-free medium on day 4, and 0.6 μg of siRNA was added to the transfection medium. 6 h after transfection, DMEM containing 4% FBS was added to each well to a final concentration of 2% FBS (v/v). Cells were transfected again on day 6 with 1 μg of siRNA. 6 h after transfection, the cells were further incubated with Ang II for an additional 24 h. In some experiments, the cells were stimulated with insulin for 30 min, as detailed in figure legends.

**NF-κB Activation Measurement**

Skeletal muscles were dissected and weighed, homogenized in 6 volumes of ice-cold Buffer (10 mmol/HEPES, pH 7.9 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 0.1 mmol/l dithiothreitol, and 1 mmol/l PMSF) using a Duall homogenizer. The homogenates were centrifuged at 500g for 30 min at 4°C. The supernatant was resuspended in NucBuster Reagent and vortex twice during incubation on ice for 5 min and centrifuged at 16,000g for 5 min at 4°C and washed with ice-cold PBS (7). L6 myotubes were rinsed with PBS and lysed using lysis buffer as above. Nuclear fragments were extracted as manufacture’s introduction. 5μl of nuclear extract was mixed with 4x NoShift Bind buffer (Poly(dI-dC)·Poly(dI-dC), Salmon Sperm DNA, WT DNA) and incubated for 30 min on ice. The Streptavidin Plate was washed 3 times for 5 min with 200ul 1x NoShift Wash Buffer. NoShift Bind Buffer (80 μl) was added to each NoShift reaction prepared in step 1. Reaction solution (100µl) was then dispensed into wells of the freshly washed streptavidin plate. The plate was sealed with aluminum and incubated for 60 min at 37°C. After washing steps, primary antibody (100 μl) was added to each well and incubated for 60 min at 37°C, HPR-conjugated secondary antibody (1:1000) was incubated for 30 min at 37°C, and substrate TMB was incubated for 15 min in the dark at room temperature,
respectively. Thereafter, 100 µl of 1N HCl was mixed into each well. The absorbance at 450 nm was measured using a plate reader spectrophotometer (Bio-Tek EL808).

**TNF-α Immunofluorescence**

Soleus muscle paraffin section (5µ, cross section) were cut for TNF-α staining. After dewax, the slides were blocked with 5% rabbit serum containing 1% BSA and 0.1% saponin. Thereafter, slides were incubated with anti-TNF-α at 1:200 dilutions overnight at 4°C. After 3 washes in PBS, rabbit anti-goat IgG antibody conjugated with Alex 468 (Molecular Probes) was added for 1 hour at room temperature. The sections were extensively washed in PBS, mounted on glass slides with mounting media (Vector), and images were acquired with the fluorescence microscope (Nikon, Eclipse 50i) using Meta Imaging Software (Molecular Devices Corporation).

**Western blot**

L6 myotubes were treated with Ang II (10⁻⁷M) for 24 h in the absence or presence of losartan (10⁻⁶M), N-acetylcysteine (NAC; 30mM) or gp91ds-tat (20µM). Thereafter, in some experiments, myotubes were stimulated with insulin (100 nM) for 15 min. Subcellular fractionation of myotubes was performed as previously described (30). Briefly, the myotubes were homogenized in homogenization buffer. The homogenate was centrifuged at 1000g for 30 min at 4°C. An aliquot of the supernatant (S1, as total homogenate) was frozen and saved at -80°C and remaining S1 was centrifuged at 12,000g for 20 min at 4°C resulting in pellet (mitochondria) and supernatant (S2). The S2 was centrifuged at 30,000g for 1 hour at 4°C. The pellet containing the Golgi and sarcoplasmic reticulum were discarded, and the supernatant (S3) was centrifuged at 100,000 x g to enrich the plasma membrane (PM) fraction in the pellet and separate the cytosolic fraction (supernatant). Protein concentrations were determined by the method of Bradford (Bio-Rad reagent). 40 µg of proteins from S2 was loaded in 10% SDS-
PAGE gel and probed with rabbit anti-Akt or phospho-Akt ser^{473} (1:1000) antibodies. 40 µg of PM enriched fraction and total homogenate (S2) protein was subjected to 10% SDS-PAGE and immunoblotted using rabbit anti-GLUT4 (1:1000 dilution in 5% milk-TBS). PM enrichment was confirmed by Na-K ATPase alpha-1 subunit immunoblotting. A horseradish peroxidase HRP-coupled secondary anti-rabbit IgG antibody was applied and reacted with enhanced chemiluminescence (ECL) reagent (Amersham Life Science). The membranes were read and quantified with the Bio-Rad Molecular Imager FX Pro Plus MultiImager System. β-actin was used as a loading control.

**Measurement of 4-Hydroxy-2-Nonenal**

4-Hydroxy-2-Nonenal (4-HNE), a by-product of lipid peroxidation and marker of oxidative stress, was measured by immunofluorescence staining and Western blot using anti 4-HNE antibody. Paraffin sections (5µm) of the soleus muscle were microwaved for 10 minutes for antigen retrieval. The sections were incubated with monoclonal mouse antibody against 4-HNE (OXIS) overnight at 4°C. Goat anti-mouse IgG antibody conjugated with Alex568 (Molecular Probe) was used as secondary antibody. The images were acquired with the Laser-Scanning confocal microscope (Olympus IX70). Soleus muscle were also homogenized on ice in homogenization buffer (50 mM phosphate buffer, 0.01 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, 2 µM pepstatin A, pH 7.4) using a Duall homogenizer. The homogenate was centrifuged at 1000g for 30 min at 4°C and the supernatant (S1) further centrifuged at 13,000g for 20 min, 4°C, with the resulting supernatant (S2) and pellet containing mitochondria. 40 µg of protein (S2) was loaded in SDS-PAGE gel and probed with primary antibodies (1:1000) against 4-HNE modified proteins. After washing, the membrane was incubated with HRP-conjugated secondary antibodies (1:10,000). The intensities of the
immunoblot lanes were quantified using Quantity One software (Bio-RAD). β-actin was used as a loading control

**RT-PCR Amplification**

TNF-α mRNA expression was measured by RT-PCR using primers, forward, TGGCCCAGACCTCACA CTC, reversed, CTCCTGGTATGAAATGGCAAATC. To determine the relative initial amounts of TNF-α cDNA, the cDNA sample was serially diluted 1:5 and 1:25. GAPDH was used as a housekeeping gene to verify that the same amount of RNA was amplified. The PCR products were analyzed using a digital imaging system (Kodak).

**TNF-α ELISA**

L6 myotubes were stimulated with Ang II at different concentrations from $10^{-9}$ to $10^{-5}$ mM, with and without inhibitors (Fig. 4) for 24 hours. Media samples were collected and centrifuged at 1000g for 30 min. TNF-α was determined in supernatants using ELISA as the manufacture’s instruction (R&D Systems, Inc., Minneapolis, MN).

**Statistical analysis**

Values are reported as the means ± SE from at least three different experiments. One way ANOVA or Student’s t-test was used to determine the significance between groups. When an ANOVA F-test indicated significance a protected Fisher LSD post hoc analysis was performed. A value of P < 0.05 was considered to be statistically significant.

**RESULTS**

**Increased oxidative stress in soleus muscle from Ren2 rats**

We have shown that Ang II increases ROS formation in soleus muscle from Ren2 rats (3) and L6 myotubes (30) using a lucigenin assay. ROS are short-lived molecules that exert local effects...
such as increased formation of aldehyde by-products including 4-hydroxy-2-nonenal (4-HNE), which have longer-longer half lives than ROS (4; 6). Therefore, 4-HNE is considered to be a reliable index of the deleterious effects of ROS on various cellular components including membranes, proteins and DNA (4). Western blot analysis and immunhistochemistry have been used to detect modified proteins by 4-HNE (15). In the present study, 4-HNE staining on western blot was increased in soleus muscles from Ren2 rat (RC) compared with SD (194%) (Figure 1A). When Ren2 rats were treated with valsartan (RV) or tempol (RT), 4-HNE was significantly reduced compared with untreated RC rats (Figure 1A). Likewise, immunofluorecent staining of soleus muscle sections showed increased 4-HNE accumulation Ren2 rats than in the SD controls, and that valsartan or tempol treatment reduced 4-HNE staining intensity (Figure 1B).

**Increased NF-κB activation in soleus muscle from Ren2 rats**

Oxidative stress activates multiple redox signaling pathways including NF-κB (13). To test whether increased oxidative stress induced by Ang II was associated with NF-κB activation in Ren2 skeletal muscle, nuclear fractions were isolated and NF-κB p65 nuclear translocation was determined by NoShift assay. As shown in Figure 2A, NF-κB p65 nuclear translocation was significantly increased by 189% in soleus muscles from Ren2 compared with SD rats. However, NF-κB p65 nuclear translocation was greatly attenuated in muscle from Ren2 rats treated with valsartan or tempol. Under these conditions NF-κB activation was significantly correlated (r=0.786, p<0.01) to 4-HNE levels in rat skeletal muscle (Figure 2B). Moreover, decreased levels of cytosolic IκB-α were detected in Ren2 skeletal muscle, whereas treatment with valsartan or tempol treatment reversed this effect (Figure 2C).

**Increased TNF-α expression in soleus muscle from Ren2 rats**
NF-κB activation is known to upregulate inflammatory cytokines such as TNF-α (12), whereas TNF-α is a multifunctional cytokine that has been linked to insulin resistance (26). To determine whether NF-κB activation in skeletal muscle is associated with an increase in the inflammatory cytokine TNF-α, RT-PCR and immunostaining were performed. Increased TNF-α mRNA and protein levels were observed in Ren2 skeletal muscle compared to SD (Figure 2D and 2E). Again, this effect was attenuated in Ren2 soleus muscle treated with the AT1R blocker valsartan or the superoxide scavenger tempol (Figure 2D and 2E).

**Ang II induced ROS activates NF-κB in L6 myotubes**

The above *in vivo* results utilizing Ren2 rats suggest that increased ROS might mediate the Ang II-induced NF-κB activation expression in skeletal muscle. To further evaluate this possibility, L6 myoblasts were differentiated into myotubes and then treated with Ang II alone or Ang II plus each of the followings: gp91 ds-tat (a specific NADPH inhibitor peptide which blocks p47phox binding to gp91phox); valsartan (AT1R blocker); NAC (antioxidant); or MG132 (NF-κB inhibitor). NF-κB p65 nuclear translocation and cytosolic IκB-α were determined by NoShift assay and immunoblot, respectively. As shown in Figure 3, Ang II significantly increased NF-κB p65 nuclear translocation and concomitant treatment with gp91 ds-tat, valsartan, NAC or MG132 inhibited Ang II-induced NF-κB p65 nuclear binding activity in L6 myotubes, (Figure 3A). Furthermore, when L6 myotubes were transfected with NF-κB p65 siRNA not only was p65 subunit expression reduced (Figure 3B), but NF-κB nuclear translocation in the presence of Ang II was significantly lower than when L6 myotubes were transfected with scrambled siRNA, or treated with Ang II alone (Figure 3C). NF-κB translocation with and without inhibitors was inversely associated with cytosolic IκB protein levels measured by Western blotting (data not shown).
**Ang II-induced NF-κB activation mediated TNF-α production in L6 myotubes**

Ang II induced TNF-α production in a dose-dependent manner which reached statistical significance at $10^{-7}$ M of Ang II when compared to untreated L6 myotubes (Figure 4A). Alternatively, co-administration of valsartan, NAC, gp91 ds-tat or MG132 reversed Ang II induced TNF-α production in L6 myotubes. Likewise, co-administration of NF-κB inhibitor MG132 (Figure 4B) or transfection of NF-κB p65 siRNA (Figure 4C) reversed Ang II-induced TNF-α production in the presence of Ang II. These findings suggest that increased TNF-α production by skeletal muscle in the presence of Ang II is mediated by ROS induced NF-κB activation.

**Inhibition of NF-κB improved insulin-mediated Akt ser473 phosphorylation and GLUT4 translocation in L6 myotubes**

To determine whether activation of NF-κB mediates Ang II-induced insulin resistance in skeletal muscle, L6 myotubes were preincubated with valsartan, NAC, gp91ds-tat, or NF-κB inhibitor MG132 for 1 hour and then co-incubated with Ang II for 24 hours. Thereafter, the myotubes were exposed to insulin (100 nM) for 15 min. Akt Ser$^{473}$ phosphorylation and GLUT4 translocation to PM were measured by Western blot (Figure 5). In these experiments, Ang II reduced insulin-mediated Akt Ser$^{473}$ phosphorylation and GLUT4 translocation. This inhibition was largely prevented with valsartan, NAC, gp91ds-tat, or NF-κB inhibitor MG132 pre-treatment (Figure 5A and 5B), suggesting that ROS-induced NF-κB activation plays an important role in Ang II-induced skeletal muscles insulin resistance.

**NF-κB p65 siRNA knockdown of p65 protein expression prevented Ang II inhibition of insulin signaling**
To further confirm the role of NF-κB in Ang II-induced skeletal muscle insulin resistance, siRNA targeting NF-κB subunit p65 was employed in L6 myotubes. NF-κB p65 siRNA transfection significantly reduced NF-κB p65 levels and inhibited Ang II-induced NF-κB activation (Figure 3B and 3C), while also reversing decreases in insulin-stimulated Akt ser\textsuperscript{473} phosphorylation (Figure 5C) and GLUT4 translocation in L6 myotube PM (Figure 5D). GLUT1 transporter levels while detected in L6 myotube PM were not altered with Ang II treatment (data not shown).

DISCUSSION

The prevalence of the metabolic syndrome is steadily increasing. Hypertension and T2DM, two important manifestations of the metabolic syndrome, often co-exist and frequently progress to cardiovascular disease. Insulin resistance plays a central role in the pathogenesis of the metabolic syndrome. This may be attributable in part to Ang II, which is not only pivotal role in the development of hypertension, but impairs insulin actions upon skeletal muscle by way of AT\textsubscript{1}R (25; 26). As the major tissue for insulin-mediated glucose disposal, skeletal muscle is particularly crucial in the development of insulin resistance. Skeletal muscle expresses many components of RAS including AT\textsubscript{1}R (26), and Ang II has direct effects on skeletal muscle which lead to insulin resistance (3; 22; 30). Ang II appears to induce insulin resistance at the levels of IRS-1 tyrosine phosphorylation (22), Akt activation, and/or distal to Akt activation, perhaps at the level of glucose transporter (GLUT-4) translocation (14). Nevertheless, this insulin resistance appears closely linked to an increase in oxidative stress (3; 14; 30). Remaining uncertain are the intermediate steps linking Ang II induced ROS to impaired insulin signaling in skeletal muscle.
The primary finding in this investigation is that Ang II induced oxidative stress activates NF-κB in skeletal muscle which in turn diminishes insulin signaling and GLUT4 translocation. This interpretation is based on the following: 1) Ang II induced oxidative stress, NF-κB activation and increased TNF-α were documented in skeletal muscle from insulin resistant Ren2 rats and Ang II-treated L6 myotubes; 2) Antioxidant treatment inhibited Ang II-induced NF-κB activation and TNF-α production in both Ren2 skeletal muscle and L6 myotubes; 3) Ang II inhibition of insulin-mediated Akt activation and GLUT4 translocation in L6 myotubes were significantly attenuated by the AT₁R blockade (valsartan), antioxidant treatment (NAC), NF-κB inhibition (MG132) or NF-κB p65 knockdown (siRNA; Figures 3 and 5). Multiple sources of increased ROS are possible, but NADPH oxidase remains a leading candidate since pre-treatment of L6 cells with the specific inhibitor inhibitor gp91 ds-tat peptide reduced NF-κB activation and improved GLUT4 translocation in the presence of insulin. To our knowledge, this is the first study to document that NF-κB activation mediates Ang II-induced insulin resistance in skeletal muscle in a NADPH oxidase dependent manner. NF-κB activation is known to promote increases in inflammatory cytokines, including TNF-α, which have been linked to insulin resistance. Interestingly, in the present study Ang II induced TNF-α generation in L6 myotube cultures was attenuated when the myotubes were pre-incubated with either an antioxidant (NAC), NADPH oxidase inhibitor (gp91 ds-tat), NF-κB inhibitor (MG132) or previously transfected with NF-κB p65 siRNA (Figure 4).

Ang II exerts pro-oxidant and pro-inflammatory effects that regulate cell growth, apoptosis, migration, inflammation and fibrosis, and impairs insulin signaling in many tissues including skeletal muscle (3; 17; 30). The Ang II effects on insulin signaling in skeletal muscle appear to be at least in part mediated by NADPH oxidase-derived ROS (30). ROS, in turn, may be an
important intermediary between the Ang II / AT₁R binding event and several downstream cellular outcomes. However, little information as to how ROS diminishes insulin signaling has been garnered. ROS has been shown to activate several signaling pathway including those involving redox-sensitive transcription factors (NF-κB, AP-1, and HIF-1) (1; 27). NF-κB, in turn, stimulates the expression of numerous genes including TNF-α and IL-6. It has been also shown that NF-κB is involved in fatty acid induced insulin resistance in liver and adipocytes (5; 21). Further, chronic inflammation and inflammatory cytokines such as TNF-α, IL-6, and IL-8 may play fundamental role in the pathogenesis of insulin resistance and T2D (20). Some of the questions that have remained unanswered, particularly in skeletal muscle, include 1) does Ang II induce NF-κB activation; 2) if so, does NADPH oxidase-generated ROS mediate Ang II-induced NF-κB activation; and 3) does NF-κB activation participate in Ang II-induced skeletal muscle insulin resistance? In the present study, skeletal muscle from insulin resistant Ren-2 rats exhibited increased NF-κB activation and TNF-α production which was reversed when the animals were treated with the AT₁R receptor blocker valsartan or the superoxide scavenger tempol. Moreover, in vitro experiments confirmed that NF-κB activation was essential for perpetuating Ang II-induced skeletal muscle insulin resistance since either NF-κB inhibition with MG132 or NF-κB p65 knockdown by siRNA techniques prevented Ang II induced reductions in insulin-mediated Akt activation and GLUT4 translocation.

Collectively, several novel findings were demonstrated in the present investigation. First, NF-κB activation and nuclear translocation are required for Ang II-induced insulin resistance in skeletal muscle. Second, Ang II increased NF-κB activation and TNF-α production which was dependent on NADPH oxidase-derived ROS. Third, blocking the AT₁R, inhibiting NADPH oxidase or preventing NF-κB activation attenuated TNF-α increases in cultured myotubes. The
data provide solid evidence indicating that NADPH oxidase generated ROS activates NF-κB, thus linking Ang II, ROS, and insulin resistance in skeletal muscle. These findings may provide important insights into the pathogenesis and potential targets for treatment that might be pivotal role in alleviating Ang II-induced insulin resistance.

Acknowledgments

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References


29. **Torlone E, Britta M, Rambotti AM, Perriello G, Santeusanio F, Brunetti P and Bolli GB.** Improved insulin action and glycemic control after long-term angiotensin-converting


Figure legends

Figure 1. Oxidative stress marker 4-HNE in rat skeletal muscle using immunostaining and Western blot. Soleus muscle results from Sprague-Dawly (SD), and Ren2 rats either control (RC), treated with valsartan (RV) or treated with tempol (RT) are shown. A, Representative immunoblot for 4-HNE (top), bar graph illustrates summary of lane densitometry from multiple experiments (bottom; N=4-5 per group), *P<0.05, vs. SD; #P<0.05, vs. RC. B, Representative immunostaining microphotographs showed increased 4-HNE in Ren2 soleus muscle (red) compared with SD which was attenuated in soleus muscle from valsartan or tempol treated Ren2 rats. Microphotograph magnification for B: 400x; scale bar 50 µm.

Figure 2. NF-κB nuclear translocation and TNF-α expression in soleus muscles from Ren2 rats. A, NoShift assay showed increased NF-κB p65 nuclear translocation in soleus muscle from Ren2 rats compared with SD, while NF-κB p65 nuclear translocation was blunted in soleus muscle from Ren2 rats treated with valsartan or tempol. B, Correlation between skeletal muscle NF-κB activities and 4-HNE staining intensities, r=0.786, p<0.01. Figure includes data from SD, and Ren2 rats treated with valsartan, tempol or untreated. C, Cytosolic IκB-α levels in soleus muscles from Ren2 rats including representative immunoblots with β-actin loading controls (top), and mean band densities compared to SD (bottom). D, RT-PCR for TNF-α mRNA expression normalized to GAPDH. E, Representative microphotographs showed increased TNF-α immunostaining intensity (green) in Ren2 rat soleus muscles. The results are mean ± SE for 4-
5 rats for each group. *P<0.05, vs. SD; #P<0.05, vs. RC. Microphotograph magnification for E: 400x; scale bar 50 μm.

**Figure 3.** NF-κB nuclear translocation in L6 myotubes. A, NF-κB nuclear translocation (activation) was detected by NoShift assay, Ang II significantly induced NF-κB p65 nuclear translocation which was prevented by the AT1R blocker valsartan (Val), antioxidant (NAC), NADPH oxidase inhibitor peptide (gp91 ds-tat), or NF-κB inhibitor (MG132); *P<0.05 vs. untreated control, #P<0.05 vs. Ang II alone. B, NF-κB p65 protein levels were not affected by Ang II alone or Ang II with scrambled RNA. However, siRNA targeting of NF-κB p65 subunit significantly decreased p65 protein levels in L6 myotubes compared to Ang II alone; *P<0.05. C, NF-κB p65 siRNA significantly attenuated Ang II-induced NF-κB p65 nuclear translocation compared with scrambled RNA; *P<0.05 for Ang II alone vs. no treatment, #P<0.05 for NF-κB p65 siRNA vs. Ang II alone. Results are mean ± SE for 3 separate experiments with triplex wells for each group.

**Figure 4.** Ang II induced TNF-α production detected by ELISA in L6 myotubes. A, Ang II induced TNF-α production in dose-dependent manner; *P<0.05 vs no treatment. B, Ang II-induced increase in TNF-α production was inhibited by AT1R blocker valsartan (Val), antioxidant (NAC), NADPH oxidase inhibitor (gp91 ds-tat peptide), or NF-κB inhibitor (MG132); *P<0.05 vs. no treatment (Con), #P<0.05 vs. Ang II alone. C, NF-κB p65 siRNA significantly reduced L6 myotube TNF-α production in the presence of Ang II, whereas scrambled siRNA did not; *P<0.05 vs. no treatment, #P<0.05 vs. Ang II alone. The results are mean ± SE for 3 separate experiments with triplex wells for each group.
Figure 5. Insulin-stimulated Akt Ser\(^{473}\) phosphorylation and GLUT4 translocation to PM in L6 myotubes. Representative immunoblots and bar graphs indicating mean ± SE for insulin-mediated Akt Ser\(^{473}\) phosphorylation (A) and GLUT4 translocation (B) either untreated or in the presence of Ang II (10\(^{-7}\)M), with or without valsartan (Val), NAC, gp91ds-tat or MG132; *P<0.05 vs. no treatment, **P<0.05 vs. insulin (INS) alone treatment, #P<0.05 vs. Ang II+INS. C and D, knockdown of NF-\(κ\)B p65 improved insulin-mediated Akt Ser\(^{473}\) phosphorylation and GLUT4 translocation to the PM; *P<0.05 vs. no treatment, **P<0.05 vs. INS alone, #P<0.05 vs. Ang II+INS. The results are mean ± SE for 3 separate experiments with triplex wells for each group.
Figure 1

A

![4-HNE and Actin Blot](image)

![_bar_graph_image](image)

B

![Immunofluorescence Images](image)
Figure 4

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)