Angiotensin II-induced NADPH Oxidase Activation Impairs Insulin Signaling in Skeletal Muscle Cells

From the Departments of Internal Medicine and Physiology, University of Arizona, Diabetes Research Center, 1656 E. Mabel St., P.O. 245218, Tucson, AZ 85724-5218. E-mail: stumpc@email.arizona.edu.

Insulin resistance and hypertension often coexist and frequently progress to diabetes and cardiovascular disease (1, 2). A reduced response by target tissues to insulin is the hallmark of the metabolic syndrome (1–5), which is defined by a cluster of abnormalities including impaired glucose tolerance, hypertension, abdominal obesity, and dyslipidemia (6). Insulin signaling is a complex process involving multiple pathways and phosphorylation events. Phosphorylation of protein kinase B (Akt) is a key step leading to the translocation of glucose transporter 4 (GLUT4) from intracellular compartments to the plasma membrane. GLUT4 helps mediate the increase in glucose uptake in skeletal muscle and adipose tissues in the presence of insulin (7, 8). Skeletal muscle insulin receptor signaling is defective in metabolic syndrome and type 2 diabetes mellitus (T2DM) both in experimental models and humans (1–6, 9). Many factors have been reported to induce insulin resistance in vitro and in vivo, including angiotensin II (Ang II), tumor necrosis factor (TNF)-α, interleukin 6 (IL-6), and free fatty acids (10–13).

Ang II is an important physiological regulator of blood pressure, cardiac function, and salt and fluid homeostasis. Its hypertensive, growth, and remodeling effects are mediated through the Ang II receptor 1 (AT1R) (1, 5, 11). In addition, Ang II appears to be antagonistic to insulin action and contributes to insulin resistance. Several prospective clinical studies have shown that treating hypertensive patients with angiotensin converting enzyme (ACE) inhibitors or AT1R blockers results in a lesser incidence of newly diagnosed T2DM when compared with other anti-hypertensive agents (14–16). Furthermore, Ang II acting through the AT1R is known to stimulate NADPH oxidase, which generates reactive oxygen species (ROS) in a variety of cells (5). Indeed, ROS production by NADPH oxidase activation has been strongly associated with hypertension and insulin resistance in vascular tissue (17).

NADPH oxidase is a highly regulated membrane-bound enzyme complex that catalyzes the one-electron reduction of oxygen to superoxide anion with the simultaneous oxidation of cytosolic NADPH (17–19). Components of NADPH oxidase complex of phagocytes include the membrane-bound cytochrome b558, composed of 2 subunits, p22phox and gp91phox, and 4 cytosolic subunits, p47phox, p67phox, p40phox, and the small GTP-binding protein, Rac1/Rac2 (18, 19). Moreover, expression of gp91phox, p22phox, p47phox, and p67phox have been documented in skeletal muscle (20). Activation of NADPH oxidase in phagocytes, which have been studied most extensively,
involves the translocation of cytosolic subunit proteins p47\textsuperscript{phox} and p67\textsuperscript{phox} to the plasma membrane (18, 19). This is regulated in part by the interaction between p47\textsuperscript{phox} and p67\textsuperscript{phox} each harboring two SH3 domains for which deletion or substitution of residues disrupt interaction and diminish enzyme activation (21). Key to activating NADPH oxidase is p47\textsuperscript{phox}, which forms a stable complex with the cytoplasmic region of the membrane-bound cytochrome b\textsubscript{558} (22) and promotes p67\textsuperscript{phox} recruitment to the membrane via SH3-mediated tail-tail interaction (21, 23, 24). It has also been shown that p47\textsuperscript{phox} may regulate gp91\textsuperscript{phox}, Nox1, and other Nox family members (25). Although effects of Ang II via ROS on vascular smooth muscle cells, endothelial cells, and adipocytes have been intensively studied, Ang II effects on skeletal muscle remain largely unknown. Increased superoxide content has been noted in skeletal muscle with local elevated Ang II levels (10). However, whether Ang II-induced ROS production in skeletal muscle is mainly through NADPH oxidase and consequently contributes to insulin resistance in skeletal muscle remains unclear.

In the present investigation, we demonstrated that Ang II increased NADPH oxidase activity, thereby increasing ROS generation and impairing insulin-induced insulin receptor substrate (IRS1) tyrosine phosphorylation, Akt activation, and GLUT4 translocation to plasma membranes. In addition, blocking AT\textsubscript{1}, R by losartan or inhibiting NADPH oxidase by apocynin suppressed ROS production and restored insulin stimulated IRS1 and Akt activation and GLUT4 translocation. Furthermore, knockdown of p47\textsuperscript{phox} utilizing p47 siRNA-reduced NADPH oxidase activity and improved insulin-mediated Akt phosphorylation and GLUT4 translocation in the presence of Ang II.

**EXPERIMENTAL PROCEDURES**

**Materials**—siRNA targeted against p47\textsuperscript{phox}, scrambled control siRNA, fluorescein conjugate siRNA, and siRNA transfection reagent were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p67\textsuperscript{phox} and p47\textsuperscript{phox} were purchased from Santa Cruz Biotechnology. Antibodies for Akt and phospho-Akt (Ser473), IRS1, phospho-IRS1 were purchased from Upstate Signaling Technology (Beverly, MA) and Abcam Inc (Cambridge, MA). Angiotensin II, insulin, and NADPH oxidase and Insulin Signaling in Skeletal Muscle

Primary skeletal muscle cells were prepared from Sprague Dawley rats according to the methods as previously described (27), with some modifications. Animals were anesthetized with a single intraperitoneal injection of pentobarbital. The gastrocnemius and EDL muscles were removed and washed in sterile PBS, pH 7.4 to remove blood cells, trimmed of excess connective tissue and fat, and homogenized with scissors in sterile PBS, and subjected to enzymatic digestion by incubation at 37 °C in DMEM with 0.2% collagenase for 1 h and centrifuged at 1,500 × g. Pellets were then resuspended in sterile PBS, mixed with a vortex mixer for 20 s, and centrifuged for 10 min at 500 × g. The supernatant containing the cell fraction was removed, followed by centrifugation at 1,500 × g for 5 min. Cell pellets were pooled and resuspended in DMEM with 10% horse serum and 1% penicillin/streptomycin antibiotic solution and passed through sterile 40 μm cell strainer (BD Biosciences). The filtered suspension was centrifuged again at 1,500 × g, and the pellet was then resuspended in 10 volumes of DMEM containing 10% horse serum and 1% penicillin/streptomycin antibiotic solution and passed through sterile 40 μm cell strainer (BD Biosciences). The filtered suspension was centrifuged again at 1,500 × g, and the pellet was then resuspended in 10 volumes of DMEM containing 10% horse serum and 1% penicillin/streptomycin and plated onto uncoated 100-mm dishes for 3 h in a 37 °C incubator humidified with 5% CO\textsubscript{2}. The unattached cells floating in the medium after the 3 h of incubation were then transferred onto a fresh dish, and this procedure was repeated twice. The cells were further purified using magnetic beads coated with anti-desmin antibody (Hybrudima, ATCC). The isolated satellite cells were cultured on rat-tail collagen-coated plates in high serum medium (Ham’s F-10 nutrient mixture containing 20% fetal bovine serum, 1% chicken embryo extract, 1% penicillin/streptomycin, and 1% glucose). The cells were counted using a hemacytometer and seeded at 1 × 10\textsuperscript{5} cells per well on a 6-well plate in 2 ml of F-10 with 10% FBS until ~80% confluency. To induce differentiation, cells were further cultured in DMEM containing 2% FBS for 6–8 days.

**Transfection of siRNA**—L6 and primary myotubes were transfected with p47siRNA (sc-45918), control siRNA (sc-37007), control siRNA fluorescein conjugate (sc-36869, sc-44239) as per the manufacturer’s instruction with minor modifications (28). The differentiation medium was changed to antibiotic-free medium on day 4, and 0.6 μg of siRNA was transfected using transfection reagent in 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 additional 24 h. In some experiments, the cells were further stimulated with insulin for 30 min, as detailed in figure legends.

**Cell Culture**—L6 rat skeletal muscle cells (American Type Culture Collection) were grown in DMEM with 10% (v/v) FBS and 1% v/v antibiotic-antimycotic solution (10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B) were purchased from Invitrogen.

**Culture**—L6 rat skeletal muscle cells (American Type Culture Collection) were grown in DMEM with 10% (v/v) FBS and 1% v/v antibiotic-antimycotic solution (10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B) at 5% CO\textsubscript{2} and 37 °C until ~80% confluency. 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 expression is highest (26). Myogenic differentiation to myotubes was confirmed by light microscopy with morphological alignment, elongation, and fusion.

Primary skeletal muscle cells were prepared from Sprague Dawley rats according to the methods as previously described (27), with some modifications. Animals were anesthetized with a single intraperitoneal injection of pentobarbital. The gastrocnemius and EDL muscles were removed and washed in sterile PBS, pH 7.4 to remove blood cells, trimmed of excess connective tissue and fat, and homogenized with scissors in sterile PBS, and subjected to enzymatic digestion by incubation at 37 °C in DMEM with 0.2% collagenase for 1 h and centrifuged at 1,500 × g. Pellets were then resuspended in sterile PBS, mixed with a vortex mixer for 20 s, and centrifuged for 10 min at 500 × g. The supernatant containing the cell fraction was removed, followed by centrifugation at 1,500 × g for 5 min. Cell pellets were pooled and resuspended in DMEM with 10% horse serum and 1% penicillin/streptomycin antibiotic solution and passed through sterile 40 μm cell strainer (BD Biosciences). The filtered suspension was centrifuged again at 1,500 × g, and the pellet was then resuspended in 10 volumes of DMEM containing 10% horse serum and 1% penicillin/streptomycin and plated onto uncoated 100-mm dishes for 3 h in a 37 °C incubator humidified with 5% CO\textsubscript{2}. The unattached cells floating in the medium after the 3 h of incubation were then transferred onto a fresh dish, and this procedure was repeated twice. The cells were further purified using magnetic beads coated with anti-desmin antibody (Hybrudima, ATCC). The isolated satellite cells were cultured on rat-tail collagen-coated plates in high serum medium (Ham’s F-10 nutrient mixture containing 20% fetal bovine serum, 1% chicken embryo extract, 1% penicillin/streptomycin, and 1% glucose). The cells were counted using a hemacytometer and seeded at 1 × 10\textsuperscript{5} cells per well on a 6-well plate in 2 ml of F-10 with 10% FBS until ~80% confluency. To induce differentiation, cells were further cultured in DMEM containing 2% FBS for 6–8 days.

**Transfection of siRNA**—L6 and primary myotubes were transfected with p47siRNA (sc-45918), control siRNA (sc-37007), control siRNA fluorescein conjugate (sc-36869, sc-44239) as per the manufacturer’s instruction with minor modifications (28). The differentiation medium was changed to antibiotic-free medium on day 4, and 0.6 μg of siRNA was transfected using transfection reagent in 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 additional 24 h. In some experiments, the cells were further stimulated with insulin for 30 min, as detailed in figure legends.

**Cell Culture**—L6 rat skeletal muscle cells (American Type Culture Collection) were grown in DMEM with 10% (v/v) FBS and 1% v/v antibiotic-antimycotic solution (10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B) at 5% CO\textsubscript{2} and 37 °C until ~80% confluency. 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 expression is highest (26). Myogenic differentiation to myotubes was confirmed by light microscopy with morphological alignment, elongation, and fusion.
NADPH Oxidase Activity—After 6 h of incubation in serum-free DMEM supplemented with 0.1% bovine serum albumin, L6 myotubes were treated with various concentrations (10⁻⁹ to 10⁻⁶ M) of Ang II for 24 h. In other experiments, L6 myotubes were stimulated with Ang II for 24 h in the absence or presence of apocynin (200 μM), which blocks association of p47phox with membrane-associated subunits, p22phox and gp91phox (29), or with losartan (10⁻⁶ M), a blocker of AT₁R. Subcellular fractionation of myotubes was performed as previously described (20), with minor modifications. Myotubes were washed with ice-cold PBS, scraped gently from 10-cm plates, centrifuged at 1000 g for 10 min, resuspended in 300 μl of homogenization buffer (50 mM phosphate buffer, 0.01 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 2 mM pepstatin A, pH 7.4) and placed on ice. The myotubes were then homogenized using a Duni homogenizer and sonication. An aliquot of the total homogenate was saved, and the remaining homogenate was then centrifuged at 13,000 g for 20 min, 4 °C, the supernatant was collected and was further centrifuged at 100,000 g for 90 min at 4 °C with the resulting supernatant yielding the plasma membrane-enriched fractions (PM). Homogenate (50 μg of protein) was incubated with NADPH (100 μM) at 37 °C, and the rate of NADPH consumption monitored by measuring the decline in absorbance (340 nm) every 10 min, using a plate reader spectrophotometer (Bio-Tek EL808). To verify NADPH oxidase contribution and exclusion of other molecular sources of NADPH consumption, the procedure was repeated in the presence of DPI (2 μM), l-NAME (1 mM), apocynin (200 μM), oxyurinol (100 μM), or rotenone (250 μM) (10, 11, 29) (Table 1).

Lucigenin-enhanced Chemiluminescence Detection of ROS in Myotubes—ROS production was measured using a lucigenin method (30) with minor modifications, as previously described (31). Briefly, 1 × 10⁵ of L6 myoblasts/well was plated in 6-well plates and cultured with DMEM with low glucose and 2% FBS for 6–8 days. The resulting L6 myotubes were treated with L6 myotubes was evaluated by use of the oxidative fluorescent dye dihydroethidium (DHE). L6 myotubes were cultured in chamber slides and treated as above. After washing with ice-cold PBS, L6 myotubes were incubated with DHE 1 μM/l in a light-protected humidified chamber at 37 °C for 10 min. DHE specifically reacts with intracellular superoxide and is converted to the red fluorescent compound ethidium, which then binds irreversibly to double-stranded DNA and appears as punctuate nuclear staining (32). The specificity of DHE signals for O₂⁻ detection was confirmed by preincubation with polyethylene glycol-superoxide dismutase (PEG-SOD, 500 units/ml, Sigma).

mRNA Transcripts by Real Time PCR—L6 cells were grown under the conditions specified above. They were treated in the same way as for protein extraction. The cells were scraped off in ice-cold PBS and separated (1000 × g). The supernatant was removed and RNA. Later was added for preservation. Total mRNA was extracted using RNeasy midi-kits from Qiagen (Germany). The RNA was then subjected to an additional round of DNase1 digestion (Inviitrogen). 1 μg of RNA was reverse-transcribed using RT kit from ABI Systems (Roche Applied Science). 100 ng of starting cDNA was used to run the real time reactions in 50 μl of reaction mixture under universal cycling parameters for Rac1 and p67phox (20, 33, 34). The reactions were run on an ABI Prizm 7000 machine, and the results were analyzed using guidelines from ABI Prizm. PCR primers for Rac1, Forward: GTAAAACCTGCCTGCTCATC, Reverse: GCTTCGTCAAAACTGCTTTG; p67phox, Forward: CGAG-GGAACAGCCTGATAGA, Reverse: CATAGGCACGCTGA-GCTTCA (34).

Immunofluorescence—L6 myotubes grown on chamber slides were treated as indicated above, washed with PBS and fixed in methanol/acetone (50:50, v/v) for 10 min, and blocked with 5% goat serum containing 1% bovine serum albumin, and 0.1% saponin. Thereafter, myotubes were incubated overnight at 4 °C with goat anti-p67phox antibody at 1:200 dilutions. After three washes in PBS, rabbit anti-goat IgG antibody conju-
gated with Alex 568 (Molecular Probes) was added for 1 h at room temperature. Cells 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).

p47phox and p67phox Translocation—L6 myotubes were treated with Ang II for 5 min, 15 min, 30 min, 60 min, and 24 h and the plasma membrane-enriched fractions isolated by differential centrifugation see above and further centrifuged at 100,000 × g. The intensities of the immunoblot bands were quantified by using Quantity One software (Bio-Rad). We have verified PM enriched fractions by noting robust Na+/K+ ATPase α-subunit expression by Western blot and by confirming negligible citrate synthase (mitochondria activities) (data not shown). β-Actin was used as a loading control.

Immunoprecipitation of IRS1—L6 myotubes or primary myotubes were treated with Ang II (10−7 M) for 24 h in the absence or presence of losartan (10−6 M) or apocynin (200 μM) or transfected with p47siRNA and then were incubated with Ang II (10−7 M) for 24 h. The cells were then stimulated with insulin for 30 min. IRS1 was immunoprecipitated from L6 myotubes or primary myotubes as described previously (35). Briefly, 1 mg of protein of whole cell lysates in lysis buffer (protease inhibitor mixture, Pierce) were incubated with IRS1-specific antibodies at 4 °C overnight under constant rotation, the antibody-antigen complex was pulled-down by protein A/G-Sepharose beads. The pelleted protein was resolved by 7.5% SDS-PAGE, and tyrosine phosphorylation of IRS1 was detected by immunoblotting with monoclonal anti-phosphotyrosine antibody.

Immunoblotting of Akt and Phospho-Akt—L6 myotubes or primary myotubes were treated with Ang II (10−7 M) for 24 h in the absence or presence of losartan (10−6 M) or apocynin (200 μM). In some experiments, the cells were transfected with p47siRNA or control siRNA as described above and treated with Ang II (10−7 M). Thereafter, myotubes were stimulated with insulin (100 nM) for 30 min, scraped in ice-cold PBS, and homogenized with lysis buffer. Samples were centrifuged at 1000 × g for 30 min and the subsequent supernatant (S1) centrifuged at 12,000 × g for 20 min. 40 μg of proteins from this supernatant (S2) was loaded in SDS-PAGE gel and probed with rabbit anti-Akt and phospho-Akt Ser473 (1:1000) antibodies. The remaining S2 was further centrifuged at 100,000 × g for 90 min at 4 °C to pellet the plasma membrane fraction, which was resuspended in lysis buffer.

GLUT4 Membrane Translocation—L6 myotubes or primary myotubes were treated as described above (for detection phospho-Akt and total Akt). Plasma membrane proteins (40 μg) or total cell lysate were resolved by SDS-PAGE and probed with rabbit anti-GLUT4 (1:2000) antibody. The intensities of the immunoblot bands were quantified by using Quantity One software (Bio-Rad).

Western blot analysis using antibodies to p47phox were resuspended in homogenization buffer and analyzed by Western blot analysis using antibodies to p47phox or p67phox (1:1000). The intensities of the immunoblot bands were quantified by using Quantity One software (Bio-Rad). We have verified PM enriched fractions by noting robust Na+/K+ ATPase α-subunit expression by Western blot and by confirming negligible citrate synthase (mitochondria activities) (data not shown). β-Actin was used as a loading control.

Effect of various inhibitors on NADPH oxidase activity in L6 myotubes—L6 myotubes were stimulated with Ang II (10−7 M) for 24 h or incubated without Ang II (Control). Membrane protein (50 μg) was used for measurement of NADPH consumption (340 nm). The results are mean ± S.E of three experiments produced in triplicate. *, p < 0.05 versus control. **, p < 0.05 versus Ang II-mediated NADPH oxidase activity in L6 myotubes. L6 myotubes were treated with Ang II, Ang II plus AT1R blocker losartan (10−6 M), or NADPH oxidase inhibitor apocynin (200 μM). Results are mean ± S.E of three experiments produced in triplicate. *, p < 0.05 versus control; **, p < 0.05 versus Ang II-treated myotubes.

### TABLE 1

Effect of various inhibitors on NADPH oxidase activity in L6 myotubes

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>NADPH consumption (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>17.5 ± 2.5</td>
</tr>
<tr>
<td>Apocynin (200 μM)</td>
<td>3.8 ± 0.6*</td>
</tr>
<tr>
<td>DPI (2 μM)</td>
<td>8.6 ± 1.3*</td>
</tr>
<tr>
<td>Oxyurinol (100 μM)</td>
<td>15.9 ± 2.2</td>
</tr>
<tr>
<td>Rotenone (250 μM)</td>
<td>14.5 ± 2.8</td>
</tr>
<tr>
<td>t-NAME (1 mM)</td>
<td>16.3 ± 3.6*</td>
</tr>
</tbody>
</table>

* p < 0.05, compared to no inhibitor.

**Statistical Analysis**—Values are reported as the means ± S.E. from at least three different experiments. ANOVA and Student’s t test was used to determine the significance among groups. A value of p < 0.05 was considered to be statistically significant.
NADPH Oxidase and Insulin Signaling in Skeletal Muscle

RESULTS

Ang II-induced Activation of NADPH Oxidase and ROS Production in L6 Myotubes—ROS production was significantly increased in Ang II-treated myotubes (192%) compared with untreated L6 myotubes, which was prevented by pretreatment with losartan and apocynin (Fig. 1, A and B). To further determine whether NADPH oxidase contributed to Ang II-induced ROS production, NADPH oxidase activity was measured in plasma membrane fractions. Addition of Ang II (10^{-9} to 10^{-6} M) to L6 myotubes induced a dose-dependent activation of NADPH oxidase (Fig. 2A) with maximal activity at 10^{-7} M (17.5 nmol/mg protein/min compared with 8.2 nmol/mg protein/min untreated cells). NADPH oxidase activity was markedly suppressed by losartan (10^{-6} M) and apocynin (200 μM) (Fig. 2B). To assess the molecular sources of NADPH consumption, the procedure was repeated in the presence of DPI (2 μM, NADPH oxidase inhibitor), L-NAME (1 mM, NOS inhibitor), apocynin (500 μM, a NADPH oxidase inhibitor), allopurinol (100 μM, xanthine oxidase inhibitor), and rotenone (250 μM, mitochondria inhibitor). NADPH consumption was inhibited by DPI and apocynin, but not by L-NAME, allopurinol, or rotenone (Table 1), suggesting that NADPH oxidase contribute to Ang II-induced ROS generation in skeletal muscle cells.

NADPH Oxidase Subunit mRNA Expression—We confirmed mRNA transcripts for p47^{phox}, p67^{phox}, gp91^{phox}, and p22^{phox}, as well as gp91^{phox} homologs Nox-1 and Nox-4, in rat gastrocnemius muscle, using RT-PCR (data not shown) which is consistent with previous reports (20). In untreated L6 myotubes p47^{phox} and p67^{phox} subunits were expressed. However, gp91^{phox} and p22^{phox} mRNA were not detected in untreated L6 myotubes and up-regulated in Ang II-stimulated L6 myotubes (data not shown). In addition, there were strong bands for gp91^{phox} homologs Nox-1 and Nox-4 (data not shown).

AngII-induced p47^{phox} Expression and Translocation—NADPH oxidase activation requires participation of cytosolic factors including, p47^{phox}, p67^{phox}, and Rac1 which associate with gp91^{phox} (or Nox-1, Nox-4) and p22^{phox} at the plasma membrane (36, 37). The L6 myotubes were incubated with Ang II (10^{-7} M) for 5, 15, 30, 60 min, and 24 h or without Ang II. The cells were collected, plasma membrane fractions separated using differential centrifugation, and protein extracts analyzed by Western blotting with anti-p47 antibody. Ang II (10^{-7} M) increased p47^{phox} subunit detected in the plasma membrane by 5 min, reached statistical significance by 15 min and remained elevated at 24-h poststimulation (Fig. 3A). Increased total cellular p47^{phox} levels were also noted at 24 h (Fig. 3C).

FIGURE 3. A, plasma membrane p47^{phox} protein levels after Ang II (10^{-7} M) stimulation for 5 min, 15 min, 30 min, 60 min, and 24 h. 50 μg of plasma membrane proteins were applied per immunoblot lane. The results are mean ± S.E. of three experiments produced in triplicate. *, p < 0.05 versus control. B, uptake of fluorescent-labeled siRNA in L6 myotubes. Cells were transfected with control fluorescent-labeled siRNA (green) and blue-stained nuclei. Shown here are representative areas of aceton-fixed cells. C, L6 myotubes were treated with control (scrambled) siRNA (CsiRNA) or siRNA-targeting NADPH oxidase subunit p47^{phox}, and then the cells were stimulated with Ang II for 24 h. Total p47^{phox} levels were shown by representative immunoblots. The results are mean ± S.E. of three experiments performed in duplicate. *, p < 0.05 Ang II versus control. D, p47^{phox} protein levels after Ang II (10^{-7} M) stimulation for 5 min, 15 min, 30 min, 60 min, and 24 h or without Ang II. The cells were collected, plasma membrane fractions separated using differential centrifugation, and protein extracts analyzed by Western blotting with anti-p47 antibody. Ang II (10^{-7} M) increased p47^{phox} subunit detected in the plasma membrane by 5 min, reached statistical significance by 15 min and remained elevated at 24-h poststimulation (Fig. 3A). Increased total cellular p47^{phox} levels were also noted at 24 h (Fig. 3C).
*p67phox Expression and Translocation*—To evaluate Ang II affects on the p67phox subunit, we analyzed p67phox mRNA and protein levels in myotubes with and without Ang II for 24 h. Real-time PCR results indicated that p67phox mRNA was not significantly changed with Ang II treatment (data not shown). However, Ang II increased total cellular expression of p67phox protein at 24 h (2.3-fold compared with untreated cells), which was blocked by losartan (Fig. 4A). Consistent with Western blot results, immunostaining indicated that Ang II dramatically increased fluorescence intensity of p67phox which was reduced by losartan (Fig. 4B). To examine p67phox translocation, L6 myotubes were treated with Ang II for 5 min, 15 min, 30 min, 1 h, and 24 h or without Ang II. Plasma membrane fractions were separated using differential centrifugation. Plasma membrane p67phox increase was noted by 5 min of Ang II treatment, becoming significant by 15 min and remaining elevated at 24 h (Fig. 4C).

**Insulin Receptor Substrate 1 (IRS1)**—To examine whether Ang II affects insulin-mediated IRS1 activation through NADPH oxidase-derived ROS production, L6 myotubes were preincubated with losartan (10^{-6} M) or apocynin (200 μM) for 1 h and then co-treated with Ang II for 24 h. The cells were then stimulated with insulin for 30 min. As shown in Fig. 5, Ang II significantly inhibited insulin-stimulated IRS1 tyrosine phosphorylation. Blockage of AT_{1}R with losartan or inhibiting NADPH oxidase with apocynin prevented Ang II inhibition of insulin-stimulated IRS1 tyrosine phosphorylation (Fig. 5). A similar reduction in IRS1 tyrosine phosphorylation by Ang II treatment was also observed using primary skeletal muscle cells (data not shown).

**Insulin-mediated Akt Serine 473 Phosphorylation**—Akt phosphorylation at serine 473 was markedly increased in L6 myotubes in the presence of insulin (2.8-fold; Fig. 6A). However, insulin-stimulated Akt phosphorylation was impaired by Ang II pretreatment (10^{-7} M). This Ang II-induced inhibition of insulin signaling was restored by coinubcation of either AT_{1}R blocker losartan or NADPH oxidase inhibitor apocynin (Fig. 6A). A similar reduction of Akt phosphorylation by Ang II treatment was also noted in primary muscle cell (data not shown).

**Insulin-induced Translocation of GLUT4**—To determine whether impaired insulin-mediated IRS1 and Akt activation by Ang II would suppress GLUT4 translocation to the plasma membrane, L6 myotubes were preincubated with or without AT_{1}R blocker losartan (10^{-6} M) or NADPH oxidase inhibitor apocynin (200 μM) for 1 h and then cotreated with Ang II for 24 h. Pretreated myotubes were then stimulated with insulin for 30 min. As shown in Fig. 7A, Ang II inhibited insulin-stimulated GLUT4 translocation by 59%. Both losartan and apocynin prevented Ang II inhibition of insulin-stimulated GLUT4 translocation to the plasma membrane (Fig. 7A).
investigation was that Ang II up-regulates NADPH oxidase activity and thereby increases ROS in skeletal muscle myotubes. This was coincident with diminished insulin signaling through the IRS1 and Akt pathway, which was restored by blocking the AT1R (losartan), inhibiting NADPH oxidase (apocynin), or knock-down p47(phox) by p47siRNA. To our knowledge, this is the first study to document NADPH oxidase involvement in Ang II-induced insulin resistance in skeletal muscle.

DISCUSSION

There is emerging evidence that Ang II contributes to insulin resistance in experimental animals and humans (1,3–5;10,11). It has also been shown that Ang II impairs the insulin signaling pathway in vascular smooth muscle cells (1,11). However, the precise nature of insulin and Ang II signaling cross-talk remains unclear. Folli et al. (38) have shown in vascular smooth muscle cells that Ang II inhibits IRS-1 tyrosine phosphorylation and its association to phosphoinositol 3-kinase (PI3-K). This effect was inhibited by the Ang II receptor antagonist saralasin, but not the AT1R specific inhibitor losartan. The primary finding in this study was that p47siRNA dramatically inhibited Ang II-induced GLUT4 translocation to membrane, but did not affect total expression of p67(phox) in both L6 (Fig. 6B) and primary skeletal muscle cells (data not shown) and Akt activation in L6 cells (Fig. 6B). p47siRNA transfection of L6 myotubes also inhibited Ang II-induced p67(phox) translocation to plasma membrane, but did not affect total expression of p67(phox) (Fig. 4D). Knockdown p47phox by p47siRNA also restored insulin-stimulated IRS1 tyrosine phosphorylation in L6 cells (Fig. 5) and primary skeletal muscle cells (data not shown) and Akt activation in L6 cells (Fig. 6B) and primary skeletal muscle (data not shown). Inhibition of Ang II to insulin-mediated GLUT4 translocation to membrane was completely prevented by p47siRNA in both L6 (Fig. 7B) and primary myotubes (Fig. 7C).

Figure 5. Insulin-stimulated tyrosine phosphorylation of IRS1 was impaired with Ang II treatment. Myotubes were treated as described under “Experimental Procedures.” Cells were lysed and IRS1 was immunoprecipitated (IP) from 1 mg of protein. Tyrosine phosphorylation of IRS1 was determined by immunoblotting (WB), results are expressed as a fold changes of control. The data are mean ± S.E. of three independent experiments produced in triplicate. *, p < 0.05 versus control; #, p < 0.05 versus insulin (Ins); **, p < 0.05 versus Ang II + Ins.

Figure 6. A, total and phosphorylated (Ser1177) Akt. L6 myotubes were preincubated with AT1R blocker losartan (10⁻⁷ M) or NADPH oxidase inhibitor apocynin (200 μM) for 1 h and then co-incubated with Ang II (10⁻⁷ M) 24 h. Insulin-(100 nM) stimulated (30 min) Akt phosphorylation in lysates was analyzed by Western blotting. Data are expressed in densitometry units, mean ± S.E. for three experiments performed in duplicate. *, p < 0.05 versus control; #, p < 0.05 versus Ins only; **, p < 0.05 versus Ang II + Ins. B, myotubes were transfected with siRNA targeting specifically against p47phox (p47siRNA) or control siRNA (CsiRNA) as described under “Experimental Procedures” and then incubated with Ang II (10⁻⁷ M) for 24 h and following insulin stimulation for 30 min. Total cell lysates were prepared, and 50 μg of protein was immunoblotted for total and phosphorylated (Ser473) Akt. L6 myotubes were preincubated with Ang II (10⁻⁷ M) 24 h. Insulin-(100 nM) stimulated (30 min) Akt phosphorylation in lysates was analyzed by Western blotting. Data are expressed in densitometry units, mean ± S.E. of three independent experiments performed in triplicate. *, p < 0.05 versus control; #, p < 0.05 versus insulin (Ins); **, p < 0.05 versus Ang II + Ins; ***, p < 0.05 versus Ang II + p47siRNA + Ins.
NADPH Oxidase and Insulin Signaling in Skeletal Muscle

![Graphs and diagrams illustrating GLUT4 plasma and total GLUT4 expression](https://example.com/graphs)

**FIGURE 7.** GLUT4 plasma membrane and total GLUT4 expression were determined by Western blot. A, L6 myotubes were treated with Ang II for 24 h or preincubated with losartan or apocynin for 1 h and co-treated with Ang II for 24 h. Results are expressed as fold change of untreated control and represent three independent experiments performed in duplicate. *, p < 0.01 versus control; #, p < 0.01 versus Ins; **, p < 0.05 versus Ang II + Ins. B (L6 myotubes); and C (primary myotubes), myotubes were transfected with siRNA-targeted specifically against p47<sup>phox</sup> (p47<sup>siRNA</sup>) or control siRNA (C<sup>siRNA</sup>) as described under "Experimental Procedures" and then incubated with Ang II (10<sup>-7</sup> M) for 24 h and following insulin stimulation for 30 min. Total cell lysates were prepared, and plasma membrane fraction were separated using differential centrifugation. 50 μg of protein was immunoblotted for total GLUT4 or plasma membrane (PM) GLUT4. Insulin-stimulated GLUT4 translocation to PM is expressed as fold changes of control, and the results represent the mean ± S.E. of three independent experiments performed in triplicate. *, p < 0.01 versus control; #, p < 0.05 versus insulin (Ins); **, p < 0.05 versus Ang II + Ins; ***. p < 0.01 versus Ang II + p47<sup>siRNA</sup> + Ins. D, plasma membrane fraction enrichment was verified by measuring Na<sup>+</sup>/K<sup>+</sup>-ATPase protein under various treatment conditions (cyt, cytosolic fraction; pm, plasma membrane fraction).

**A**

- Control
- Ang II
- Ang-L-Cln
- Ang + Apocyn

**B**

- Control
- Ang II
- p47<sup>siRNA</sup>
- C<sup>siRNA</sup>
- Ins

**C**

- Control
- Ang II
- p47<sup>siRNA</sup>
- C<sup>siRNA</sup>
- Ins

**D**

- Cyt pm
- Cyt pm
- Cyt pm
- Cyt pm
- Cyt pm

Relative differences in response to the AT<sub>1</sub>R losartan between vascular smooth muscle reported previously (38) and skeletal muscle cells in the present study might be caused by the duration of Ang II and losartan administration (<10 min versus 24 h) or other cell specific signaling mechanisms.

Recently, the role of RAS in the development of insulin resistance and glucose intolerance, in addition to hypertension and vascular dysfunction, has gained attention (39). For example, angiotensin-converting enzyme (ACE) inhibition or AT<sub>1</sub>R blockade in patients with hypertension and/or cardiovascular disease resulted in a lesser incidence of newly diagnosed T2DM (14–16). Further, AT<sub>1</sub>R blockers (10) or antisenes gene therapy (40) have been shown to ameliorate glucose intolerance in insulin-resistant rats.

Although improvements in peripheral blood flow may partially mediate increases in glucose disposal during ACE inhibition or AT<sub>1</sub>R blockade, there is evidence for direct Ang II effects upon skeletal muscle. For example, AT<sub>1</sub>Rs are present in skeletal muscle (41), and Ang II, directly infused into the interstitial space of canine skeletal muscle caused insulin resistance for glucose uptake that could not be attributed to hemodynamic changes (42). Furthermore, data from our laboratory (10) and others (43) have revealed diminished responses to insulin for glucose transport in isolated muscle preparations from a transgenic hypertensive rat model (Ren-2) that overexpresses tissue Ang II (9, 10). This effect was blunted when Ren-2 rats were treated for 3 weeks in vivo with AT<sub>1</sub>R blocking agent valsartan or the superoxide scavenger tempol (10). Similarly, AT<sub>1</sub>R blockade with irbesartan has been shown to improve insulin sensitivity in skeletal muscle from insulin-resistant Zucker obese rats (44).

Oxidative stress resulting from increased ROS and/or reactive nitrogen species (RNS) appears to be important in the progression of insulin resistance (5). The effects of oxidative stress on insulin signaling have been demonstrated in cultured insulin-sensitive cell lines such as 3T3-L1 adipocytes and L6 myocytes with the impairment of PI3K and Akt insulin signaling steps evident (45–48). Furthermore, the addition of anti-oxidants such as α-lipoic acid to the cultures abrogates this effect. In insulin-resistant Zucker obese rats, chronic oxidative stress is evidenced by an increased carbonyl protein levels in skeletal muscle and liver when compared with insulin-sensitive lean rats (49). Likewise, increased superoxide content has been noted in insulin-resistant Ren-2 skeletal muscle (10). Again, treatment of insulin-resistant rats with the anti-oxidants α-lipoic acid or tempol improved whole body glucose tolerance and insulin-stimulated glucose transport in isolated skeletal muscle, respectively. In T2DM patients plasma hydroperoxides, another marker of oxidative stress, are increased especially during poor metabolic control (50).

Ang II, acting through the AT<sub>1</sub>R, increases the generation of ROS in vascular cells primarily through the activation of membrane-bound NADPH oxidase (30, 51). Like phagocytic cells, vascular smooth muscle cell NADPH oxidase consists of two membrane components p<sub>22</sub><sup>phox</sup> and gp91<sup>phox</sup>, cytosolic components p<sub>47</sub><sup>phox</sup> and p<sub>67</sub><sup>phox</sup>, and the small molecular weight G protein Rac1/2 (52, 53). Vascular smooth muscle and endothelial cells also express gp91<sup>phox</sup>, Nox-1 and Nox-4 (non-phagocytic oxidase) (52). The NADPH oxidase enzyme in skeletal muscle also contains multiple subunits (p<sub>22</sub><sup>phox</sup>, gp91<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>) (20). Importantly, this study has also confirmed previous studies (20, 54) showing robust NADPH oxidase activity in skeletal muscle, and suggests that this enzyme...
complex may be a significant contributor to cellular superoxide production (20, 55).

Activation of NADPH oxidase requires the translocation of the cytosolic factors, p47<sub>phox</sub> and p67<sub>phox</sub>, to the plasma membrane where these components interact with cytochrome b<sub>558</sub> (18, 19, 23). Evidence that p47<sub>phox</sub> plays a central role in NADPH oxidase activation comes in part from p47<sup>phox</sup>(<sup>−/−</sup>) knock-out mice, which exhibit significantly lower Ang II-induced superoxide production in endothelial cells (56). It appears that p47<sub>phox</sub> is required for stable complex of cytosolic subunits with membrane-bound cytochrome b<sub>558</sub> (22) and the recruitment of p67<sub>phox</sub> (21, 23, 24). The deletion or substitution for residues in the SH3 domains of p47<sub>phox</sub> disrupt the p47<sub>phox</sub> to p67<sub>phox</sub> interaction and leads to defective activation of the enzyme both in vivo and in vitro (21). Interestingly, in the present study using skeletal muscle cells, not only did siRNA targeting of p47<sub>phox</sub> decrease p47<sub>phox</sub> protein levels (Fig. 3C), and Ang II-induced activation of NADPH oxidase (Fig. 3, D and E), but it also inhibited p67<sub>phox</sub> translocation to the plasma membrane (Fig. 4D) whereas total p67<sub>phox</sub> protein expression in whole cell lysates did not change. These results further confirm that p47<sub>phox</sub> is a critical component for NADPH oxidase activation. However, the exact role and importance of concomitant p67<sub>phox</sub> translocation remains to be determined.

Recently, skeletal muscle superoxide production as assessed by ethidium fluorescence has been shown to be significantly enhanced in rats infused with Ang II for 2 weeks (57) and in KK-Ay diabetic mice compared with non-diabetic mice (57). Evidence that Ang II enhanced in rats infused with Ang II for 2 weeks (57) and in vivo (58). It has been shown that Ang II associated increase in superoxide anion production in soleus muscles from Ren-2 hypertensive rats compared with non-diabetic rats (57). However, the exact role and importance of concomitant p67<sub>phox</sub> translocation remains to be determined.

In summary, we have shown that Ang II impairs insulin-induced activation of IRS1 and Akt, and GLUT4 membrane translocation in skeletal muscle cells. This inhibition may be mediated by NADPH oxidase generation of ROS. Inhibition of NADPH oxidase may provide a new target of therapy for insulin resistance in skeletal muscles.

**REFERENCES**

Angiotensin II-induced NADPH Oxidase Activation Impairs Insulin Signaling in Skeletal Muscle Cells

Yongzhong Wei, James R. Sowers, Ravi Nistala, Heping Gong, Grace M.-E. Uptergrove, Suzanne E. Clark, E. Matthew Morris, Nicholas Szary, Camila Manrique and Craig S. Stump

doi: 10.1074/jbc.M601320200 originally published online September 17, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M601320200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 36 of which can be accessed free at http://www.jbc.org/content/281/46/35137.full.html#ref-list-1