Abrogation of oxidative stress improves insulin sensitivity in the Ren-2 rat model of tissue angiotensin II overexpression

Mihaela C. Blendea, David Jacobs, Craig S. Stump, Samy I. McFarlane, Cristina Ogrin, Gul Bahtiyor, Samir Stas, Pawan Kumar, Quan Sha, Carlos M. Ferrario, and James R. Sowers.

Abrogation of oxidative stress improves insulin sensitivity in the Ren-2 rat model of tissue angiotensin II overexpression. Am J Physiol Endocrinol Metab 288: E353–E359, 2005. First published October 19, 2004; doi:10.1152/ajpendo.00402.2004.—To evaluate the role of renin-angiotensin system (RAS)-mediated oxidative stress in insulin resistance (IR), we compared the effects of the angiotensin II (ANG II) receptor blocker (ARB) valsartan and a superoxide dismutase (SOD) mimetic, tempol, on whole body glucose tolerance and soleus muscle insulin-stimulated glucose uptake in transgenic hypertensive TG(mREN-2)27 (Ren-2) rats. Ren-2 rats and Sprague-Dawley (SD) controls were given valsartan (30 mg/kg) or tempol (1 mmol/l) in their drinking water for 21 days. IR was measured by glucose tolerance testing (1 g/kg glucose ip). IR index (AUCglucose/AUCinsulin) was significantly higher in the Ren-2 animals compared with SD controls (30.5 ± 7.0 × 10⁶ arbitrary units in Ren-2 vs. 10.2 ± 2.4 × 10⁶ in SD, P < 0.01). Both valsartan and tempol treatment normalized Ren-2 IR index. Compared with SD controls (100%), there was a significant increase in superoxide anion production (measured by lucigenin-enhanced chemiluminescence) in soleus muscles of Ren-2 rats (133 ± 15%). However, superoxide production was reduced in both valsartan- and tempol-treated (85 ± 22% and 59 ± 12%, respectively) Ren-2 rats. Insulin (INS)-mediated 2-deoxyglucose (2-DG) uptake (%SD basal levels) was substantially lower in Ren-2 rat soleus muscle compared with SD (Ren-2 + INS = 110 ± 3% vs. SD + INS = 206 ± 12%, P < 0.05). However, Ren-2 rats treated with valsartan or tempol exhibited a significant increase in insulin-mediated 2-DG uptake compared with untreated transgenic animals. Improvements in skeletal muscle insulin-dependent glucose uptake and whole body IR in rats overexpressing ANG II by ARB or SOD mimetic indicate that oxidative stress plays an important role in ANG II-mediated insulin resistance.

INSULIN RESISTANCE; SUPEROXIDE; VALSARTAN

ANGIOTENSIN II (ANG II) is a potent vasoconstrictor and can contribute to the pathogenesis of hypertension (8, 46). Furthermore, many tissues possess intrinsic angiotensinogen and angiotensin-converting enzyme activity, and thus are able to express a tissue renin-angiotensin system (RAS) (8). A rodent model for tissue overexpression of RAS is the TG(mREN-2)27 (Ren-2) rat, a transgenic animal that harbors the mouse Ren-2 renin gene (27, 31, 37). This rodent model develops severe hypertension as a consequence of increases in the local RAS that culminates in elevated tissue ANG II levels (31, 37). Elevations in ANG II have been associated with the development of insulin resistance (IR) for skeletal muscle glucose uptake (27, 43). Indeed, the Ren-2 rat has been reported to manifest in vivo IR (20, 27) and to have diminished in vitro skeletal muscle insulin-mediated glucose transport (27). The data collectively indicate that tissue overexpression of ANG II is associated with IR at the skeletal muscle level.

The role of tissue overexpression of ANG II in promoting IR in animal models (19, 40, 44) and humans (13, 25, 33) is further supported by the observation that treatment with specific ANG II receptor (AT1 subtype) antagonists can improve insulin sensitivity and decrease the development of diabetes. Although improvements in blood flow to skeletal muscles may mediate some of the improvements in glucose uptake with AT1 receptor (AT1R) blockade, there is mounting evidence that ANG II, acting via the AT1R, inhibits insulin signaling and glucose transport (5, 8, 24). ANG II is synthesized by skeletal muscle tissue (24, 54), and AT1R are plentiful in skeletal muscle (32, 36). Indeed, skeletal muscle comprises >40% of mammalian body mass (21) and is the major site of insulin-stimulated glucose disposal (6). Furthermore, there is increasing evidence that increased generation of reactive oxygen species (ROS) by ANG II contributes significantly to the development of skeletal muscle insulin resistance (46, 47). AT1 receptor blockade greatly attenuates superoxide levels in skeletal muscle under conditions associated with high ANG II levels (56) and in skeletal muscle from diabetic rats (45).

To further assess the role of RAS-mediated oxidant stress in IR, we have investigated the effects of AT1R blockade and the administration of a superoxide dismutase (SOD) mimetic in both in vivo and skeletal muscle ex vivo insulin-stimulated glucose uptake in the Ren-2 rat. We have utilized this rodent model because of its known insulin resistance (27) and enhanced tissue IR (20, 27, 37).

MATERIAL AND METHODS

Chemicals. t-Glucose, insulin, mannitol, bovine serum albumin (BSA), tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), bis-N-methyl-acridinium nitrate (lucigenin), diphenyleneiodonium (DPI), and pyruvic acid were purchased from Sigma Chemical (St. Louis, MO). 2-Deoxy-d-[1,2-3H]glucose and d-[14C]mannitol were purchased from Perkin-Elmer, and valsartan was provided by Novartis (East Hanover, NJ).

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Animals and treatments. Male transgenic Ren-2 rats and male Sprague-Dawley (SD) controls were received at 6 wk of age from Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC. The animals were randomly distributed into untreated (SD and Ren-2) or treated groups. The treated animals received either valsartan at 30 mg·kg⁻¹·day⁻¹ (Ren-2-V) or 1 mmol/l tempol (Ren-2-T) in their drinking water for 21 days.

Systolic blood pressure (SBP) was measured in triplicate using the tail cuff method (Harvard Systems, Student Oscillometric Recorder) initially and on day 19 or 20. All animal procedures were approved by the SUNY Downstate Institutional Animal Care And Use Committee.

**Intraperitoneal glucose tolerance test.** Animals were food restricted overnight before the experiment. On day 21, the rats were weighed and anesthetized with Nembutal (35 mg/kg ip). The femoral artery was carefully dissected and cannulated with a 27-gauge angiocath. A 200-μl blood sample was drawn for insulin measurement, and blood glucose (BG) was immediately measured with a glucometer (Dex, Bayer). A dose of dextrose (50% solution, 1 g/kg body wt) was administered intraperitoneally, and blood was drawn at 15, 30, and 60 min for insulin and glucose determination. Serum was separated and frozen at −80°C until analyzed for insulin (RIA kit; Linco, St. Charles, MO). IR index was calculated as the product of areas under the glucose and insulin curves (AUCglucose × AUCinsulin) as previously described (18).

**Soleus muscle glucose transport.** After an overnight fast, rats were deeply anesthetized as above, and both soleus muscles were dissected, separated into ~25-mg intact strips, and placed in ice-cold oxygenated Krebs-Henseleit bicarbonate (KHB) buffer. Glucose transport activity, in the presence or absence of 100 nM insulin, was assessed as 2-deoxyglucose (2-DG) uptake, as described previously (18). Briefly, muscles were incubated for 20 min at 37°C in 2 ml of KHB supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% BSA in the absence or presence of insulin (100 nM) and shaken continuously while oxygenated (95% O₂-5% CO₂). After this initial treatment, the tissue was rinsed for 10 min at 37°C in 2 ml of oxygenated KHB supplemented with 40 mM mannitol, 0.1% BSA, and insulin, if present before. Strips were then transferred and incubated for 20 min in flasks containing 1.5 ml of KHB, 0.1% BSA, 1 mM 2-deoxy-D-[¹⁴C]glucose (300 μCi/mmol), 39 mM [¹⁴C]mannitol (0.8 μCi/mmole), and insulin, if present before. The muscles were snap frozen in liquid nitrogen, weighed, and dissolved in 0.5 N NaOH, and radioactivity was measured in a scintillation counter (Beckman) set for dual-channel detection (¹⁴C and ¹⁴N).

**Measurement of superoxide production in soleus muscle.** Superoxide production in soleus muscle strips (~40 mg wet wt each) was determined by the lucigenin-enhanced chemiluminescence method (23, 39). A lucigenin solution (5 μM) in Krebs buffer was prepared and dark-adapted for 30 min. Muscle strips were added to 2 ml of lucigenin solution in scintillation vials at room temperature. Photon emission was measured every minute calculated from minute 15 to minute 20 in a scintillation counter (Beckman) switched to off-coincidence mode and averaged. The superoxide production was calculated as counts per milligram of fresh tissue for each sample, and, after subtraction of the background activity, was expressed relative to activity of SD controls.

**Statistical analysis.** All results are presented as means ± SE. Unpaired Student’s t-tests were used to compare independent means, and analysis of variance (ANOVA) was used to compare multiple mean values of groups and/or time points. A P value of <0.05 was considered statistically significant.

**Results**

**Characteristics of animals studied.** The initial weights of SD and Ren-2 animals were similar (Table 1). Weight gain was expressed as percent increase compared with the initial weight of each animal. Untreated Ren-2 had significantly less weight gain (24 ± 3%) compared with SD control (31 ± 2%, P < 0.05), Ren-2-V (33 ± 4%, P < 0.05) or Ren-2-T animals (33 ± 1%; Table 1).

At 6 wk, before initiation of treatments, Ren-2 rats had higher SBP (230 ± 7 mmHg) compared with SD animals (156 ± 7 mmHg, P < 0.001). A similar difference was found at 9 wk (227 ± 5 mmHg in Ren-2 vs. 146 ± 7 mmHg in SD, P < 0.0001). In contrast, after 21 days of treatment, Ren-2-V or Ren-2-T animals had significantly lower SBP (165 ± 8 and 209 ± 6 mmHg, respectively) compared with untreated Ren-2 (227 ± 5 mmHg), both P < 0.05 (Table 1).

Untreated Ren-2 animals also had higher heart-to-body weight ratio, an index of cardiac hypertrophy, compared with SD controls (4.4 ± 0.1 mg/g in Ren-2 compared with 3.6 ± 0.1 mg/g in SD, P < 0.05). Compared with untreated Ren-2 animals, valsartan (3.9 ± 0.2 mg/g)- and tempol (3.8 ± 0.1 mg/g)-treated rats exhibited a significantly lower heart-to-body weight ratio after 21 days of treatment (Fig. 1).

**Soleus muscle ROS.** ROS production was measured as counts per milligram tissue with the lucigenin-enhanced chemiluminescence method (23) and expressed as percent increase compared with SD controls. Lucigenin chemiluminescence is specific for the detection of the superoxide anion (39). Superoxide anion production in the soleus muscle was significantly increased in Ren-2 rats (133 ± 15%, P < 0.05 vs. SD) and was reduced after 21 days of treatment with valsartan.

### Table 1. Weight gain and SBP in experimental groups

<table>
<thead>
<tr>
<th></th>
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<th>Ren-2</th>
<th>Ren-2-V</th>
<th>Ren-2-T</th>
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<tr>
<td>Initial SBP, mmHg</td>
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<td>230±7</td>
<td>219±6</td>
<td>228±8</td>
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<tr>
<td>Final SBP, mmHg</td>
<td>146±7*</td>
<td>227±5</td>
<td>165±8*</td>
<td>209±6*</td>
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Values are means ± SE. Weight gains and systolic blood pressures (SBP) of untreated (Ren-2), valsartan-treated (Ren-2-V), and tempol-treated (Ren-2-T) transgenic hypertensive TG(mRen-2-27) (Ren-2) rats compared with Sprague-Dawley (SD) controls. *Significantly different from untreated Ren-2 animals (P < 0.05); †significantly different from untreated Ren-2 animals (P < 0.01).
shown). 

Glucose and insulin concentrations. Fasting BG was higher in Ren-2 compared with SD controls (9.0 ± 1.3 mmol/l in Ren-2 vs. 6.5 ± 0.4 mmol/l in SD, P < 0.05). A significantly higher AUC for glucose was also calculated during intraperitoneal glucose tolerance test (IPGTT) for the Ren-2 rats (743 ± 78 mmol-l⁻¹-min in Ren-2 compared with 642 ± 49 mmol-l⁻¹-min in SD, P < 0.05). Ren-2-V animals had lower 15-, 30-, and 60-min BG values and AUC_{glucose} (516 ± 40 mmol-l⁻¹-min, P < 0.05) compared with untreated Ren-2 rats during IPGTT (Fig. 3). Tempol treatment decreased BG at 15 min, but the decrease in AUC_{glucose} (577 ± 42 mmol-l⁻¹-min) did not reach statistical significance compared with untreated Ren-2 (Fig. 3).

The higher fasting insulin levels in the Ren-2 compared with the SD rats did not reach statistical significance (134 ± 102 pmol/l in Ren-2 vs. 32 ± 6 pmol/l in SD, P = 0.19). However, after intraperitoneal glucose administration, Ren-2 serum insulin levels at 15, 30, and 60 min (Fig. 3) and the AUC_{insulin} (34.5 ± 6.2 × 10⁴ pmol-l⁻¹-min) were higher compared with SD values (14.4 ± 4.4 pmol-l⁻¹-min, P < 0.01). Ren-2-V animals exhibited lower 30- and 60-min insulin levels during IPGTT and AUC_{insulin} values (16.8 ± 6.6 × 10³ pmol-l⁻¹-min, P < 0.05) compared with the untreated Ren-2 rats (Fig. 3). Tempol treatment (Ren-2-T) also decreased the 15-min insulin and AUC_{insulin} (15.7 ± 7.4 × 10³ pmol-l⁻¹-min) values compared with the untreated Ren-2 condition (Fig. 3).

The IR index was calculated for each animal as the product of areas under glucose and insulin curves (AUC_{glucose} × AUC_{insulin}) and is considered to represent an indirect marker of peripheral insulin action (18). As shown in Fig. 4, IR index was substantially higher in the untreated Ren-2 compared with SD animals (30.5 ± 7.0 × 10⁴ in Ren-2 vs. 10.2 ± 2.4 × 10⁶, P ≤ 0.01). Furthermore, IR index was significantly lower in Ren-2-T compared with untreated Ren-2 rats (8.3 ± 3.9 × 10⁶, P ≤ 0.05 vs. Ren-2) and Ren-2-V compared with untreated Ren-2 rats (7.6 ± 3.6 × 10⁶, P ≤ 0.05 vs. Ren-2; Fig. 4).

Skeletal muscle glucose transport. Glucose transport activity, measured by 2-DG uptake, in the presence (insulin-stimulated) or absence (basal) of 100 nM insulin was measured in the slow-twitch, highly oxidative soleus muscle, which is more insulin sensitive than other skeletal muscles (17). Basal uptake (%SD/mg tissue) was moderately decreased in the untreated Ren-2 by ~25% (SD = 100%, Ren-2 = 74 ± 6%, P < 0.05 vs. SD). Insulin-stimulated glucose uptake was also significantly decreased in the untreated Ren-2 compared with SD animals (SD + INS = 206 ± 12%, Ren-2 + INS = 110 ± 3%,}
$P < 0.05$). Blocking the AT$_1$R with valsartan (Ren-2-V) normalized insulin-stimulated glucose uptake (Fig. 5). Likewise, tempol treatment improved the significantly diminished insulin-stimulated 2-DG uptake in soleus muscle of Ren-2 compared with the untreated condition (Fig. 6).

**DISCUSSION**

This investigation is the first to show that AT$_1$R blockade improves skeletal muscle glucose uptake and whole body glucose tolerance in the hypertensive, insulin-resistant TG(mREN-2)27 rat. This finding is in keeping with the hypothesis that locally elevated ANG II in Ren-2 animals (30, 31, 37) contributes directly to the observed IR. Furthermore, this study demonstrates the important link between increased ROS levels and impaired insulin-stimulated glucose uptake in skeletal muscle and that oxidative stress likely mediates the impaired skeletal muscle response to insulin observed when RAS is enhanced at the tissue level.

The findings from this investigation extend the number of animal models in which chronic administration of AT$_1$R antagonists improves insulin sensitivity. Previously, AT$_1$R blockade has been shown to improve whole body insulin sensitivity in fructose-fed (19), spontaneously hypertensive (SHR) (44), and obese Zucker (18) IR rats and KK-Ay diabetic mice (45).

Moreover, AT$_1$R blockade increases skeletal muscle insulin-stimulated glucose transport and soleus muscle GLUT4 protein expression in obese Zucker rats (18) and soleus muscle insulin-stimulated glucose transport in KK-Ay diabetic mice (45).

Unlike Ren-2 animals, enhanced tissue RAS remains to be determined in these models.

The presence and magnitude of IR in Ren-2 animals has been controversial, the present study verifies that male Ren-2 rats exhibit hypertension, whole body glucose intolerance, and insulin resistance in a slow-twitch skeletal muscle (soleus). Moreover, whole body insulin resistance is implied from IR index calculated from glucose and insulin AUC measurements during IPGTT. These findings confirm the work of Kinnick et al. (27), who found that male Ren-2 rats exhibit elevated plasma glucose and insulin levels during an oral glucose tolerance test and reduced insulin-stimulated glucose uptake in both the soleus and the predominantly fast-twitch glycolytic epitrochlearis muscle. Furthermore, Holness and Sugden (20) reported increased fasting plasma insulin concentrations and reduced in vivo glucose uptake into slow-twitch oxidative skeletal muscle in female Ren-2 rats compared with transgene negative, normotensive control rats. However, others found no evidence of insulin resistance at the whole body or skeletal muscle level in female Ren-2 rats (53). It is noteworthy that male Ren-2 rats exhibit higher resting SBP and RAS components compared with female Ren-2 rats (30). These disparate findings may be due to the effects of male sex steroids, which have been shown to contribute to the development of hypertension and hypertensive end-organ damage in Ren-2 rats (2, 3). It remains unknown whether androgen effects explain any sexual dimorphism observed in Ren-2 rats for insulin-stimulated glucose uptake.

ANG II stimulates a number of intracellular signaling pathways (15, 42); among these is NAD(P)H oxidase, which increases production of superoxide anion and H$_2$O$_2$ in a variety of tissues (4, 26, 28). Under normal circumstances, these molecules can act as second messengers in the regulation of signaling pathways, but in pathological conditions they become mediators of inflammation and cellular dysfunction (9). 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 wk (56) and in KK-Ay type 2 diabetic compared with nondiabetic mice (45). Moreover, AT$_1$R blockade with valsartan greatly attenuated the muscle superoxide increase observed in the KK-Ay mice while no effect of AT$_2$R blockade was observed (45). Interestingly, the compensatory hyperinsulinemia associated with insulin-resistant conditions may also lead directly to the generation of oxidative stress (4, 35, 51). Cross talk between insulin and ANG II signaling pathways may occur at multiple levels. ANG II has been shown to increase serine phosphorylation of insulin receptors (12), inhibit basal and insulin-stimulated phosphatidylinositol (PI) 3-kinase activity (52), and inhibit insulin-induced Akt activation (38), all of which could contribute to reduced insulin sensitivity. In contrast, others have
reported that 12 days of ANG II infusion resulted in enhanced PI 3-kinase activation and enhanced Akt phosphorylation (41).

To our knowledge no previous study had investigated the effect of an AT1R blocker on skeletal muscle ROS or superoxide scavenger on insulin sensitivity and glucose uptake in the Ren-2 model of increased tissue RAS. Therefore, in addition to valsartan, we treated male Ren-2 rats with the SOD mimetic tempol. As expected, superoxide levels measured in ex vivo soleus muscle preparations were significantly reduced in animals treated for 21 days with either valsartan or tempol. Furthermore, tempol treatment was effective in reducing the IR index calculated after intravenous glucose administration (Fig. 4) and soleus glucose uptake after insulin stimulation (Fig. 6), which was similar to the findings with valsartan treatment (Figs. 4 and 5). Tempol has also been shown to reverse IR and normalize PI 3-kinase activation in ANG II-infused rats (41). Although the results from the present study do not prove that ROS generation is directly responsible for the insulin resistance exhibited by Ren-2 animals, a significant correlation ($r = 0.456, P \leq 0.01$) between soleus ROS production and IR index was documented (Fig. 7). Moreover, the results are compelling that ARB and antioxidant strategies may be useful for preventing or combating insulin-resistant conditions.

In the present study, tempol blunted the increase in SBP in the Ren-2 animals only modestly. Partial correction of increased SBP has also been observed in ANG II-infused rats with tempol treatment (41). Interestingly, reductions in SBP are substantially greater when ANG II-infused rats are injected daily with liposomally packaged SOD (29), suggesting improved tissue or cellular availability and/or penetration of antioxidant compared with delivering tempol in the drinking water. Moreover, Ren-2 rats in the present study exhibited more prolonged (≥3 wk) and much higher SBPs (≥225 mmHg) than those achieved by ANG II infusion (5–12 days and ≥180 mmHg, respectively) in other studies (29, 41) and therefore may require more effective superoxide scavenging or interventions at additional blood pressure-regulating sites to achieve comparable results.

ANG II is a powerful stimulator of ROS production (14, 15), and yet the role of oxidative stress in IR related to ANG II remains controversial. Some members of the ROS family such as H2O2 are paradoxically considered either insulinomimetic (11, 50) or contributors to IR (16, 34). The improvement of 2-DG uptake in the soleus muscle with either valsartan or tempol treatment suggests a direct relationship between increased superoxide production under ANG II stimulation and reduced glucose uptake. AT1R blockade with valsartan in diabetic KK-Ay mice appears to increase soleus glucose uptake capacity by enhancing the insulin-signaling cascade and GLUT4 translocation to the plasma membrane. However, although AT1R blocking has been shown to increase total GLUT4 in skeletal muscle from obese Zucker rats (18), it is unknown whether this occurs in muscle from the Ren-2 rats. These animals did not increase GLUT4 expression in the epimyocellular muscle after 6 wk of exercise training despite improvements in insulin-stimulated glucose uptake. Therefore, the possibility exists that increased local levels of ANG II in the Ren-2 rats may prevent enhancements in GLUT4 expression.

As previously reported with AT1R blocker therapy (27), our study showed a dramatic SBP reduction and lower heart-to-body weight ratio in the Ren-2 animals treated for 21 days with valsartan. Remarkably, tempol also reduced cardiac hypertrophy in the Ren-2 animals but reduced the SBP only modestly. This is in accord with previous observations of ROS mediating cardiac hypertrophy through either the mitogen-activated protein kinase (1) or extracellular signal-regulated protein kinase (49) pathways. The role of oxidative stress in the Ren-2 kidney remains largely unknown. However, kidneys from Ren-2 rats have been shown to express significantly higher levels of NAD(P)H oxidase subunit mRNAs (Nox1 and Nox4) compared with normotensive wild-type animals (55). Furthermore, ANG II-mediated oxidative stress at the kidney appears to be important to the development of hypertension in several animal models, including the SHR (10), one-kidney, and one-clip rats (7) and ANG II-infused rabbits (54). Collectively, these results suggest that mechanisms related to and independent of oxidative stress likely contribute to ANG II-induced hypertension and related pathologies, such as vascular hypertrophy (48), endothelial dysfunction (54), and altered renal hemodynamics (10, 54).

In conclusion, our results suggest an important contribution of oxidative stress to the insulin resistance induced by chronic ANG II overexpression and show that antioxidant intervention aids in improving insulin-stimulated glucose uptake. Therefore, enhanced RAS may mediate the close association between hypertension, impaired glucose metabolism, and cardiovascular diseases through increases in tissue ROS. Furthermore, because hyperglycemia and hyperinsulinemia also contribute to increased ROS (4, 22, 28, 35), a vicious cycle of oxidative stress-insulin resistance-hyperglycemia-oxidative stress is potentially at work, and the value of developing protective antioxidant strategies is reinforced. However, the complex relationships between insulin-stimulated pathways and ROS production are just beginning to emerge. In view of disparate results in studies investigating ANG II effects on PI 3-kinase activity and Akt phosphorylation (38, 41, 52), further
investigation of insulin postreceptor pathways is clearly necessary. Likewise, future investigations should address the connection between local ANG II levels and GLUT4 protein expression and translocation.

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