Defective insulin signaling in skeletal muscle of the hypertensive TG(mREN2)27 rat

Julie A. Sloniger, Vitoon Saengsirisuwan, Cody J. Diehl, Betsy B. Dokken, Narissara Lailerd, Andrew M. Lemieux, John S. Kim, and Erik J. Henriksen

Department of Physiology, University of Arizona College of Medicine, Tucson, Arizona

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The TG(mREN2)27 rat provides an excellent genetic model of both hypertension and insulin resistance. The TG(mREN2)27 rat is a transgenic animal that harbors the mouse Ren-2 renin gene (25) and develops severe fulminant hypertension, left ventricular hypertrophy, and cardiac failure with a clearly defined monogenetic origin (22–24, 31). Increases in the local renin-angiotensin system, leading to elevated tissue angiotensin II levels (2, 24), likely underlie these cardiovascular defects.

The male heterozygous TG(mREN2)27 rat is also characterized by insulin resistance at the whole body and skeletal muscle levels (18, 19). The TG(mREN2)27 rat displays an exaggerated insulin response during an oral glucose tolerance test and exhibits a significant decrease in whole body insulin sensitivity during this glucose challenge (18, 19). Moreover, insulin-mediated glucose transport activity is significantly decreased in isolated epitrochlearis and soleus muscles of male TG(mREN2)27 rats compared with that in nontransgenic, normotensive Sprague-Dawley controls (19).

METHODS

Animals and treatments. Male heterozygous TG(mREN2)27 rats and male Hannover Sprague-Dawley normotensive control rats were purchased from the Hypertension and Vascular Disease Center of Bowman Gray School of Medicine at Wake Forest University (Winston-Salem, NC) at ~5–6 wk of age. The TG(mREN2)27 rats were derived from Hannover Sprague-Dawley rats housed in the breeding colony at this institute. All animals were housed in a temperature-controlled room (20–22°C) with a 12:12-h light-dark cycle (lights on from 7:00 AM to 7:00 PM) at the Central Animal Facility of the University of Arizona. The animals had free access to chow (Purina, St. Louis, MO) and water, and all of the procedures were approved by the University of Arizona Animal Care and Use Committee.

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Animals were food restricted (4 g of chow given at 5:00 PM the previous evening) and at 8:00 AM underwent an oral glucose tolerance test (OGTT) using a 1 g/kg body wt glucose feeding by gavage. Blood (0.25 ml) was collected from a small cut at the tip of the tail immediately before and at 15, 30, 60, and 120 min after the glucose feeding. Whole blood was mixed thoroughly with EDTA (18 mM final concentration) and centrifuged at 13,000 g to isolate the plasma. The plasma was stored at −80°C and subsequently assayed for glucose (Sigma Chemical, St. Louis, MO), insulin (Linco Research, St. Charles, MO), and free fatty acids (FFA; Wako Chemicals, Richmond, VA). Immediately after completion of the OGTT, all animals received a 2.5-ml subcutaneous injection of 0.9% sterile saline to compensate for plasma loss.

Assessment of muscle glucose transport activity. Seventy-two hours after the OGTT, the animals were again food restricted as described above. At 8:00 AM, the animals were weighed and deeply anesthetized with pentobarbital sodium (50 mg/kg body wt ip). Soleus and epitrochlearis muscles were dissected and prepared for in vitro incubation. In one set of animals, the total amount of abdominal fat was assessed. Whereas the epitrochlearis muscles were incubated intact, the soleus strips (one incubated without insulin and one incubated with insulin) were removed, trimmed of excess fat and connective tissue, quickly frozen in liquid nitrogen, and weighed. These strips were subsequently used for insulin-signaling and other biochemical assessments. The remaining incubated muscles were rinsed for 10 min at 37°C in 3 ml of oxygenated (95% O2-5% CO2) Krebs-Henseleit buffer (KHB) supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% BSA (radioimmunoassay grade, Sigma Chemical). One epitrochlearis muscle and two soleus strips were incubated in the absence of insulin, and the contralateral epitrochlearis muscle and soleus strips were incubated in the presence of a maximally effective concentration of insulin (2 μU/ml Humulin R; Eli Lilly, Indianapolis, IN). After this initial incubation period, two of the soleus strips (one incubated without insulin and one incubated with insulin) were removed, trimmed of excess fat and connective tissue, quickly frozen in liquid nitrogen, and weighed. These strips were used for glucose transport activity (modified by Henriksen et al. (17)).

Each muscle was incubated for 30 min at 37°C in 3 ml of oxygenated (95% O2-5% CO2) Krebs-Henseleit buffer (KHB) supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% BSA (radioimmunoassay grade, Sigma Chemical). One epitrochlearis muscle and two soleus strips were incubated in the absence of insulin, and the contralateral epitrochlearis muscle and soleus strips were incubated in the presence of a maximally effective concentration of insulin (2 μU/ml Humulin R; Eli Lilly, Indianapolis, IN). After this initial incubation period, two of the soleus strips (one incubated without insulin and one incubated with insulin) were removed, trimmed of excess fat and connective tissue, quickly frozen in liquid nitrogen, and weighed. These strips were used for glucose transport activity (modified by Henriksen et al. (17)).

Muscle pieces incubated in the absence or presence of insulin were used for the following biochemical assessments. Insulin signaling.

Muscle pieces were homogenized in 1 ml of ice-cold lysis buffer, and the specific intracellular accumulation of [2-3H]DG was determined as described previously (16). This method for assessing glucose transport activity in isolated muscle has been validated (15).

Glycogen synthase activity. Glycogen synthase activity was assessed as the activity ratio (activity in the absence of glucose 6-phosphate divided by the activity in the presence of 5 mMol/l glucose 6-phosphate) using the filter paper assay of Thomas et al. (35), as modified by Henriksen et al. (17).

Biochemical assays. Pieces (~15 mg) of epitrochlearis and soleus muscle were weighed and homogenized in 30 volumes of ice-cold 20 mM HEPES (pH 7.4) containing 1 mM EDTA and 250 mM sucrose. These homogenates were used for the determination of total protein content using the bicinchoninic acid (BCA) method (Sigma Chemical), GLUT4 protein level (16), total hexokinase activity (37), and citrate synthase activity (33). The triglyceride concentration was determined by the processing method of Frayn and Maycock (12), as modified by Denton and Randle (7). Glycerol was ultimately assayed spectrophotometrically using a commercially available kit (Sigma Chemical).

Insulin signaling. The remaining pieces of muscles were homogenized in 8 volumes of ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM Na pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na2VO4, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM MgCl2, 1 mM CaCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mM PMSF). Homogenates were incubated on ice for 20 min and then centrifuged at 13,000 g for 20 min at 4°C. Protein concentration was determined using the BCA method (Sigma Chemical). Insulin-signaling proteins were separated by SDS-PAGE on 7.5 or 12% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. To determine protein expression of insulin-signaling factors, the blots were incubated with the appropriate dilution of commercially available antibodies against IR β-subunit (IRβ), IRS-1, the p85 regulatory subunit of PI 3-kinase, GSK-3α/β (Upstate Biotechnology, Lake Placid, NY), and Akt1/2 (Cell Signaling Technology, Beverly, MA). Activation of protein kinase C (PKC) in lyesates prepared from frozen, unincubated soleus muscle was assessed by determining phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS), a substrate of PKC (30), using a commercially available antibody against ser152/156 of MARCKS (Cell Signaling Technology).

Muscle pieces incubated in the absence or presence of insulin were used for the following biochemical assessments. Insulin signaling.

Muscle pieces were homogenized in 1 ml of ice-cold lysis buffer, and protein concentration was determined. Samples were diluted to 0.5 ml to 0.5 NaOH for assessment of glucose transport activity. After the muscles were completely solubilized, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of [2-3H]DG was determined as described previously (16). This method for assessing glucose transport activity in isolated muscle has been validated (15).

Glycogen synthase activity. Glycogen synthase activity was assessed as the activity ratio (activity in the absence of glucose 6-phosphate divided by the activity in the presence of 5 mMol/l glucose 6-phosphate) using the filter paper assay of Thomas et al. (35), as modified by Henriksen et al. (17).

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Insulin signaling. The remaining pieces of muscles were homogenized in 8 volumes of ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 20 mM Na pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na2VO4, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM MgCl2, 1 mM CaCl2, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 2 mM PMSF). Homogenates were incubated on ice for 20 min and then centrifuged at 13,000 g for 20 min at 4°C. Protein concentration was determined using the BCA method (Sigma Chemical). Insulin-signaling proteins were separated by SDS-PAGE on 7.5 or 12% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. To determine protein expression of insulin-signaling factors, the blots were incubated with the appropriate dilution of commercially available antibodies against IR β-subunit (IRβ), IRS-1, the p85 regulatory subunit of PI 3-kinase, GSK-3α/β (Upstate Biotechnology, Lake Placid, NY), and Akt1/2 (Cell Signaling Technology, Beverly, MA). Activation of protein kinase C (PKC) in lyesates prepared from frozen, unincubated soleus muscle was assessed by determining phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS), a substrate of PKC (30), using a commercially available antibody against ser152/156 of MARCKS (Cell Signaling Technology).
differences between basal and insulin-stimulated values within groups. A level of $P < 0.05$ was set for statistical significance.

**RESULTS**

Whole body insulin resistance of TG(mREN2)27 rats. The final average body weight of the hypertensive male heterozygous TG(mREN2)27 rats did not differ significantly from that of normotensive Sprague-Dawley controls (Table 1). Heart weight, expressed in either absolute (g) or relative (g/100 g body wt) terms, was 33–51% greater ($P < 0.05$) in the TG(mREN2)27 rats than in the normotensive control animals, reflecting the cardiac hypertrophy associated with the hypertensive state of the transgenic animals. Moreover, the amount of total abdominal fat as a percentage of body weight was 18% greater in the TG(mREN2)27 rats (3.71 ± 0.08%) than in the nontransgenic controls (3.15 ± 0.08%, n = 5–6 animals).

Whereas fasting plasma glucose was not significantly different between groups (Table 1), fasting plasma insulin was 74% higher ($P < 0.05$) in the TG(mREN2)27 rats. Fasting plasma FFA concentrations tended to be higher (42%, $P < 0.1$) in the TG(mREN2)27 rats than in the Sprague-Dawley controls.

The glucose value at the 15-min time point was 25% higher ($P < 0.05$) in the TG(mREN2)27 rats than in the Sprague-Dawley group (Fig. 1A). However, the incremental glucose area under the curve (glucose IAUC) did not differ between groups (Fig. 1B). The insulin concentration at this 15-min time point was more than twice as great ($P < 0.05$) in the TG(mREN2)27 animals (Fig. 1A), and the insulin IAUC for the insulin response was also twofold greater ($P < 0.05$) in the TG(mREN2)27 rats compared with the control group (Fig. 1B). The glucose-insulin index is the product of glucose IAUC and insulin IAUC, and an increase in this value reflects a reduction in whole body insulin sensitivity (4). The glucose-insulin index was 88% greater ($P < 0.05$) in the TG(mREN2)27 rats than in the control animals (Fig. 1B).

**Insulin resistance of muscle glucose transport and glycogen synthase in TG(mREN2)27 rats.** Basal rates of 2-DG uptake were not different between TG(mREN2)27 rats and Sprague-

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Heart Weight, g</th>
<th>Heart Weight, g/100 g body wt</th>
<th>Glucose, mg/dl</th>
<th>Insulin, μU/ml</th>
<th>FFA, meq/ml</th>
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<tr>
<td>Sprague-Dawley</td>
<td>320±8</td>
<td>0.929±0.03</td>
<td>0.298±0.004</td>
<td>110±3</td>
<td>10.9±0.3</td>
<td>0.57±0.13</td>
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<tr>
<td>TG(mREN2)27</td>
<td>338±9</td>
<td>1.400±0.03*</td>
<td>0.397±0.003*</td>
<td>116±2</td>
<td>19.0±1.6*</td>
<td>0.81±0.16</td>
</tr>
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Values are means ± SE for 4–10 animals per group. FFA, free fatty acids. *$P < 0.05$ vs. Sprague-Dawley group.

![Fig. 1. A: glucose and insulin responses to an oral glucose tolerance test in Sprague-Dawley (SD) and TG(mREN2)27 (TG) rats. B: incremental areas under the curves (IAUC) for glucose and insulin and the glucose-insulin index derived from these data are displayed. Values are means ± SE for 4–5 animals per group. *$P < 0.05$ vs. Sprague-Dawley group.](http://ajpendo.physiology.org/)

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Dawley controls for either epitrochlearis or soleus muscles (Fig. 2). In contrast, insulin-stimulated 2-DG uptake in the epitrochlearis (30%) and soleus (22%) was significantly less 
(P\textless \text{0.05}) in the TG(mREN2)27 rats than in controls. Likewise, the insulin-induced increases in 2-DG uptake above basal (cross-hatched bars in Fig. 2) were 24% smaller in epitrochlearis and 35% smaller in soleus (both P\textless \text{0.05}) muscles of the TG(mREN2)27 rats than in the nontransgenic controls. These data clearly indicate that skeletal muscle of these transgenic animals is insulin-resistant. GLUT4 protein levels were not different between groups, and activities of total hexokinase and citrate synthase were slightly, though not statistically significantly, greater (11–14%) in soleus muscle of the TG(mREN2)27 rats (data not shown). Interestingly, intramuscular triglycerides were 25% greater in soleus muscle of the TG(mREN2)27 rats compared with the normotensive controls (3.05 ± 0.24 vs. 2.44 ± 0.13, n = 8–9 per group). However, the overall activation of PKC in the soleus, as assessed by Ser152/156 phosphorylation of MARCKS, a PKC substrate (30), was not different between groups (data not shown).

Whereas total glycogen synthase activity was not different in the soleus muscle of the TG(mREN2)27 rats compared with the Sprague-Dawley control group (1.99 ± 0.11 vs. 1.91 ± 0.11 nmol·mg muscle\textsuperscript{-1}·min\textsuperscript{-1}, n = 5 per group), it was 39% greater (P\textless \text{0.05}) in the epitrochlearis of the transgenic animals relative to the Sprague-Dawley rats (1.61 ± 0.04 vs. 1.16 ± 0.06 nmol·mg muscle\textsuperscript{-1}·min\textsuperscript{-1}). Insulin stimulation of glycogen synthase activity in skeletal muscle was compared in TG(mREN2)27 rats and normotensive controls (Fig. 3). The soleus glycogen synthase activity ratio in the absence of insulin was 36% greater (P\textless \text{0.05}) in the TG(mREN2)27 rats than in controls. More importantly, the increase in glycogen synthase activation due to insulin was reduced in both the epitrochlearis (46%) and the soleus muscles (64; both P\textless \text{0.05}) of the TG(mREN2)27 rats compared with the Sprague-Dawley rats.

Insulin resistance of muscle insulin signaling in TG(mREN2)27 rats. The ability of insulin to activate elements of the insulin-signaling cascade critical for regulation of glucose transport activity and glycogen synthase activity was assessed. There were no differences in the protein expression of IRβ, IRS-1, p85, Akt1/2, and GSK-3β in epitrochlearis and soleus muscles between the transgenic and control groups (data not shown). However, in both epitrochlearis and soleus muscles, insulin stimulation of tyrosine phosphorylation of IR (Fig. 4A) and IRS-1 (Fig. 4B) was significantly reduced by 26–45% (P\textless \text{0.05}) in the hypertensive TG(mREN2)27 group compared with controls.
Fig. 4. Tyrosine phosphorylation of insulin receptor (IR; A), tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1; B), and IRS-1 associated with the p85 subunit of phosphatidylinositol (PI) 3-kinase (C) in epitrochlearis and soleus muscles of Sprague-Dawley and TG(mREN2)27 rats. Muscles were incubated in the absence (−) and presence (+) of insulin (2 mU/ml). Representative bands from the autoradiograph are displayed at the top of each panel. Values are means ± SE for 4–8 animals per group. *P < 0.05 vs. Sprague-Dawley group of same incubation condition.
pared with the normotensive Sprague-Dawley animals. Likewise, the insulin stimulation of IRS-1 associated with the p85 subunit of PI 3-kinase, a surrogate measure of PI 3-kinase activity, was reduced ($P < 0.05$) in the TG(mREN2)27 rats compared with the normotensive rats in both the epitrochlearis (37%) and the soleus (50%; Fig. 4C).

Downstream elements of the insulin-signaling pathway were also assessed. Insulin-stimulated Ser$^{473}$ phosphorylation of Akt1/2 was reduced ($P < 0.05$) in both the epitrochlearis (31%) and soleus (54%) muscles of the transgenic vs. control rats (Fig. 5A). The insulin stimulation of Ser$^{9}$ phosphorylation of GSK-3, a target of Akt phosphorylation and a regulator of glycogen synthase activity (5, 34), was also diminished by ∼30% in the epitrochlearis and soleus (both $P < 0.05$) muscles of the TG(mREN2)27 rats (Fig. 5B).

**DISCUSSION**

In the present study, we have demonstrated that the hypertensive male heterozygous TG(mREN2)27 rat displays characteristics of insulin resistance at the whole body and skeletal muscle levels. These animals exhibited impaired glucose tolerance (Fig. 1), decreased whole body insulin sensitivity (Fig. 1), fasting hyperinsulinemia and dyslipidemia (Table 1), and reduced insulin action of skeletal muscle glucose transport (Fig. 2), confirming previous findings of our research group (19) and others (18). Moreover, we have presented the new finding that insulin resistance in skeletal muscle of the TG(mREN2)27 rat is observed not only for the glucose transport process but is also manifested for the activation of glycogen synthase (Fig. 3), the rate-limiting enzyme in the pathway for glycogenesis in muscle.

We have presented further novel information indicating that the skeletal muscle of the TG(mREN2)27 rat is also characterized by defects in insulin-signaling elements, including reduced insulin action on IR and IRS-1 tyrosine phosphorylation (Fig. 4), IRS-1 associated with the p85 regulatory subunit of PI 3-kinase (Fig. 4), and serine phosphorylation of Akt and GSK-3 (Fig. 5). Our results are consistent with the interpretation that a defect in an upstream component of the insulin-signaling cascade exists, which manifests itself in reduced functionality of downstream signaling elements. The defects in the insulin stimulation of the association of IRS-1 with p85 and...
of the serine phosphorylation of Akt appear to be of particular importance as a possible mechanism for the insulin resistance of glucose transport activity observed in skeletal muscle of the TG(mREN2)27 rats (Fig. 2 and Ref. 19), as the functionality of these factors is critical for the normal translocation of GLUT4 and an increase in facilitated glucose transport (reviewed in Ref. 40; also see Refs. 14, 20, and 36).

Moreover, our new data support a potential mechanistic connection between the reductions in serine phosphorylation of Akt and GSK-3β (Fig. 5) and the lesser insulin stimulation of glycogen synthase activity in the skeletal muscle of the TG(mREN2)27 rat (Fig. 3). A reduced insulin-stimulated Akt activity (as reflected by the diminished serine phosphorylation state) would allow GSK-3 to remain in a more active state, thereby increasing the capacity of GSK-3 to inactivate glycogen synthase, a direct target of GSK-3 action (5). Our findings therefore provide further evidence supporting an important physiological role of GSK-3 in the regulation of glycogen synthase activity and of glycogen metabolism in mammalian skeletal muscle.

There are a number of possible scenarios that can help explain the etiology of the defective insulin signaling in skeletal muscle of the TG(mREN2)27 rat. Angiotensin II, which is locally elevated in tissues of these hypertensive transgenic rats (24), has been shown to induce an insulin-resistant state when infused into both rats (26) and dogs (29). Direct negative effects of angiotensin II on insulin signaling in myocytes have been demonstrated. Acute exposure of heart tissue (11, 38) and cultured aortic smooth muscle cells (10, 11) to angiotensin II caused increased serine phosphorylation of IRS-1 by Janus kinase-2 (associated with the AT1 receptor), leading to a reduction in insulin-stimulated docking of IRS with the p85 regulatory subunit of PI 3-kinase. This is consistent with our finding of reduced insulin-stimulated IRS-1 associated with p85 in skeletal muscle of the TG(mREN2)27 rat (Fig. 4). Collectively, these findings suggest that the angiotensin II-induced reduction in PI 3-kinase activity occurs via cross talk between the angiotensin II and insulin-signaling pathways.

In contrast, Ogihara et al. (26) have presented evidence that the negative effects of angiotensin II on insulin action are manifested distal to the insulin-signaling pathway, possibly by affecting GLUT4 translocation in a oxidative stress-dependent mechanism. These investigators demonstrated that chronic angiotensin II infusion into normal rats induced the accumulation of plasma cholesterol ester hydroperoxides, indicative of increased oxidative stress, and that this alteration was normalized by treatment with tempol, the membrane-permeable superoxide dismutase mimetic. Moreover, the antioxidant treatment ameliorated the insulin resistance of insulin-stimulated glucose transport and PI 3-kinase activity in the angiotensin II-infused rats (26). These findings suggest that the mechanism underlying angiotensin II-induced insulin resistance involves oxidative stress, which may impair insulin signaling at a point distal to PI 3-kinase activation.

Finally, the roles of augmented plasma FFAs and muscle lipids as potential contributors to the defective insulin signaling in skeletal muscle of the TG(mREN2)27 rat merit discussion. A greater degree of central adiposity was detected in the TG(mREN2)27 rats compared with the nontransgenic controls (see RESULTS). Importantly, plasma FFAs are increased in the male heterozygous TG(mEN2)27 rat (Ref. 19 and Table 1), and the connection between elevated plasma FFAs and insulin resistance has long been recognized (1). Increasing evidence supports the effect of increased long-chain fatty acyl-CoAs in the induction of defective IR and IRS-1 functionality (3, 32, 39), possibly via an upregulation of PKC (3, 32, 39) and serine phosphorylation of these signaling elements (39). However, in the present study we were unable to detect an upregulation of overall PKC activity, as assessed by phosphorylation of the PKC substrate MARCKS. We did observe a significant 25% elevation of intramuscular triglycerides (and presumably fatty acid derivatives in the soleus muscle of the transgenic animals), and this may have been sufficient to induce at least part of the defective IR and IRS-1 functionality and insulin resistance of glucose transport observed in these muscles. In addition, the hyperinsulinemia present in the TG(mREN2)27 animals (Table 1) may have also contributed to the development of this skeletal muscle insulin resistance (27). It is clear that further study is needed to clarify the molecular mechanisms responsible for the defective insulin signaling seen in skeletal muscle of this hypertensive rodent model.

In summary, the present investigation has demonstrated that the hypertensive male heterozygous TG(mREN2)27 rat displays glucose intolerance, reduced whole body insulin sensitivity, and a marked reduction in insulin-stimulated glucose transport activity and glycogen synthase activity. Moreover, the defects in these physiological variables are associated with an increase in central adiposity and muscular lipid and a reduced insulin stimulation of insulin-signaling elements in skeletal muscle, including tyrosine phosphorylation of IR and IRS-1, IRS associated with PI 3-kinase, and serine phosphorylation of Akt1/2 and GSK-3β. These results further support the close association between hypertension and insulin resistance, possibly due to local elevations in angiotensin II.

GRANTS

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