Mechanisms of Antihyperglycemic Effects of Moxonidine in the Obese Spontaneously Hypertensive Koletsky Rat (SHROB)\(^1\)

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**ABSTRACT**

Increased activity of the sympathetic nervous system may be a critical factor in the development of impaired insulin secretion and insulin resistance. We studied the chronic effects of sympathetic inhibition with moxonidine on glucose metabolism in the spontaneously hypertensive genetically obese rat (SHROB). This unique animal model closely resembles human syndrome X, expressing insulin resistance, genetic obesity, spontaneous hypertension, and hyperlipoproteinemia. Moxonidine, a selective imidazoline receptor agonist, was administered to lean spontaneous hypertensive rats (SHR) and SHROBs for 90 days in food at 8 mg/kg/day and significantly reduced mean blood pressure. Moxonidine treatment reduced fasting insulin levels by 71% in SHROB and lowered plasma free fatty acids by 25%. In SHR, moxonidine treatment decreased free fatty acids by 17% compared with controls. During an oral glucose tolerance test, blood glucose levels in moxonidine-treated SHROB were reduced relative to untreated controls from 60 min onwards. Insulin secretion was facilitated at 30 min (83% greater) and 60 min (67% greater) postchallenge compared with control SHROB. In skeletal muscle, moxonidine treatment increased the expression of the insulin receptor b subunit by 19% in SHROB but was without effect in SHR. The level of insulin receptor substrate-1 (IRS-1) protein was decreased by 60% in SHROB but was without effect in SHR. Moxonidine treatment enhanced the expression and insulin-stimulated phosphorylation of IRS-1 protein in skeletal muscle in SHROB by 74 and 27%, respectively, and in SHR by 40 and 56%, respectively. Moxonidine increased the levels of expression of IRS-1 protein in liver in SHROB by 275% and in SHR by 260%. These findings indicate that chronic inhibition of sympathetic activity with moxonidine therapy can lower free fatty acids and significantly improve insulin secretion, glucose disposal, and expression of key insulin signaling intermediates in an animal model of obese hypertension.

A large body of evidence suggests that essential hypertension is associated with insulin resistance (Zavaroni et al., 1987; Maheux et al., 1994). Although the mechanisms underlying this relationship are not completely understood, recent studies have shown that some, but not all, agents that lower blood pressure in humans and animal models may improve insulin sensitivity (Lithell, 1991). For example, treatment of hypertensives with beta adrenergic blockers or diuretics can worsen insulin sensitivity (Pollare et al., 1989). Moreover, it is now recognized that hypertension and insulin resistance frequently do not appear in isolation and are often part of a complex set of abnormalities including obesity, hyperlipidemia, and diabetes, described as syndrome X or the metabolic syndrome (Reaven, 1988). Therefore, treatments for conditions related to syndrome X such as hypertension must be assessed for their impact on insulin resistance.

Recent clinical studies suggest that treatment of hypertension with the antihypertensive agent moxonidine, a centrally acting \(^1\) imidazoline receptor agonist, may lower glucose levels in hyperglycemic patients (Kaan et al., 1995). Experiments in spontaneous hypertensive obese rats (SHROB) and lean spontaneous hypertensive rats (SHR) (Ernsberger et al., 1996, 1997) and in fructose-fed hypertensive rats (Rosen et al., 1997) suggest that chronic moxonidine therapy improves glucose tolerance and the insulin response to a glucose load. Indeed, chronic moxonidine therapy has been shown by Henrikson et al. (1997) to enhance skeletal muscle glucose transport activity in insulin-resistant obese Zucker rats. The cellular mechanisms for this effect are still unknown. In hypertensive and obese patients, increased sympathetic activity and high catecholamine levels may impair the insulin response to glucose and contribute to insulin resistance (Rob-
eptson et al., 1976; Landsberg, 1993). Catecholamines activate lipolysis in adipose tissue and allow release of free fatty acids directly into the portal circulation (Krotkiewski et al., 1983; Perris et al., 1986). Excess free fatty acids have been shown to stimulate hepatic gluconeogenesis (Boden, 1997), inhibit insulin secretion (Sako and Grill, 1990; Zou and Grill, 1994), and impair muscle glucose uptake, all of which contribute to insulin resistance (Randle et al., 1988; Felley et al., 1989).

Insulin resistance in human obesity and non-insulin-dependent diabetes mellitus (NIDDM) involves cellular defects in insulin signaling, which is compounded in NIDDM by an inappropriate delay in insulin secretion (Kahn, 1994). The intracellular events that couple the stimulation of insulin receptors (IRs) to glucose uptake in skeletal muscle are partially understood. The events include binding of insulin to the IR α subunit on the extracellular surface of the cell, activation of the IR β subunit resulting in autophosphorylation, the subsequent phosphorylation of IR substrate-1 (IRS-1), and the interaction of these substrates with downstream signaling molecules that stimulate the translocation of vesicles to the cell surface containing the glucose transporter-4 (GLUT4) (White and Kahn, 1994). The phosphorylation of IRS-1 on multiple tyrosine residues is critical for coupling signaling from the IR to glucose uptake and has been implicated in NIDDM (Tamemoto et al. 1994; Gooyear et al., 1995).

The present study focuses on the effects of moxonidine in the genetically obese SHR Koletsky rat (SHROB), a unique animal model for human syndrome X, expressing genetic obesity, spontaneous hypertension, and hyperlipoproteinemia (Type IV) (Koletsky and Ernsberger, 1992). The obese phenotype results from a nonsense mutation recently identified in the leptin receptor gene, designated fa<sup>α</sup> (Takeya et al., 1996). The fa<sup>α</sup> mutation imposed on a hypertensive backcross (fak/fak) was obtained, and within 30 min at 4°C, followed by centrifugation at 10,000 × g at 4°C, and the supernatant was allowed to clot on ice, centrifuged for 20 min at 5000 × g at 4°C, and the serum frozen at −70°C until assayed for insulin. An insulin radioimmunoassay kit with rat insulin standards and antibodies directed against rat insulin was used (Linco, St. Charles, IL). Assays were conducted in duplicate and the intra-assay coefficient of variation was less than 5%. Serum free fatty acid levels were assayed in blood obtained at the time of sacrifice after an overnight fast using an enzymatic kit from Wako Chemicals (Neuss, Germany). Urinary protein was assayed by the method of Peterson (1977).

**Materials and Methods**

**Reagents.** Human insulin was purchased from Eli Lilly & Co. (Humulin R; Indianapolis, IN). Affinity-purified polyclonal antibodies to IRS-1 and phosphatidylinositol-3 kinase (p85α) were obtained from Upstate Biotechnology (Sarancakes, NY). Monoclonal antiphosphotyrosine antibody and rabbit polyclonal antiserum to the IR (β subunit) were obtained from Transduction Laboratories (Lexington, KY). Rabbit antisera raised against the C-terminal 12 amino acids of rat GLUT4 was kindly supplied by G. Linsky Dohm (East Carolina University, Greenville NC) and was affinity purified before use.

**Animals.** The SHROB (Koletsky rat) arose originally in 1970 at Case Western Reserve University from the mating of a female SHR and male Sprague-Dawley rat. Several obese animals were noted among the offspring, and lean hypertensive littermates from this original mating were then bred to form a closed self-sustaining colony that has been maintained by brother-sister mating for the last 25 years and at least 60 generations. Experiments were conducted on homozygous male and female SHROBs (fa<sup>α</sup>/fa<sup>α</sup>), Age- and sex-matched hypertensive lean SHR littermates (fa<sup>α</sup>/fa<sup>α</sup> or fa<sup>α</sup>/fa<sup>β</sup>) were also used for these studies. SHROBs and lean SHRs were treated with moxonidine at a dose of 8 mg/kg/day in chow for 90 days. Animals were housed individually and were provided food (Purina formula 5008; Ralston Purina, Richmond, IN) and water ad libitum. Animals were on a 12:12-h light/dark cycle (lights on from 7:00 AM–7:00 PM) and the room was maintained at a constant temperature of 21°C. Body weight and food intake were monitored continuously during the experiment. Blood pressure was measured by direct cannulation under urethane anesthesia. These procedures were carried out with the approval of the Case Western Reserve University Animal Care and Use Committee.

**Oral Glucose Tolerance Test.** Oral glucose tolerance tests were carried out in equal numbers of male and female SHRs and SHROBs at 12 to 18 weeks of age. All rats were fasted for 18 h and administered a 50% glucose solution by feeding tube at a dose of 6 g/kg b.wt. Blood (0.2 ml) was obtained from the tail vein of unrestrained, conscious animals at 0, 30, 60, 90, 120, 180, and 240 min and glucose measured in whole blood by colorimetric glucose oxidase assay (One-Touch; LifeSence, Milpitas, CA). The remaining blood sample was allowed to clot on ice, centrifuged for 20 min at 5000 × g at 4°C, and the serum frozen at −70°C until assayed for insulin. An insulin radioimmunoassay kit with rat insulin standards and antibodies directed against rat insulin was used (Linco, St. Charles, IL). Assays were conducted in duplicate and the intra-assay coefficient of variation was less than 5%. Serum free fatty acid levels were assayed in blood obtained at the time of sacrifice after an overnight fast using an enzymatic kit from Wako Chemicals (Neuss, Germany). Urinary protein was assayed by the method of Peterson (1977).

**IR and IRS-1 Tyrosine Phosphorylation In Vivo.** Within 3 days after final oral glucose tolerance testing, the animals were prepared for IR studies. Insulin-stimulated tyrosine phosphorylation of the IR and IRS-1 in liver and muscle of intact rats was assayed by a modification of a previous method (Saad et al., 1992). Rats were fasted for at least 12 h, anesthetized with urethane (1 g/kg) or pentobarbital (35 mg/kg), and the abdominal cavity opened and the portal vein exposed. The skin from one hindlimb was removed and a 200-μg sample of the gastrocnemius muscle, followed by a sample of liver, were taken and frozen immediately in liquid nitrogen. A 1-ml bolus of normal saline (0.9% NaCl) with or without insulin (10 U/kg b.wt.) was injected into the portal vein, within 30 s a liver sample was obtained, and within 5 min a sample from the opposite gastrocnemius muscle was obtained. The frozen samples were pulsed in liquid nitrogen and homogenized immediately under denaturing conditions using a Polytron PTA 2057 generator at maximum speed for 30 s in ice-cold 10× volume of homogenization buffer containing phosphatase and protease inhibitors (50 mM HEPES, pH 7.5, 100 mM Na<sub>P</sub>O<sub>4</sub>, 10 mM H<sub>2</sub>O, 100 mM NaF, 10 mM EDTA, and 10 mM Na<sub>2</sub>VO<sub>4</sub>) plus aprotonin (0.1 mg/ml), leupeptin (10 μg/ml), phenylmethylsulfonyl fluoride (PMSF) (34 μg/ml), and 1% Triton X-100. The homogenate was allowed to sit on ice for 30 min at 4°C, followed by centrifugation at 38,000 rpm in a 70 Ti rotor (Beckman Instruments, Fullerton, CA) at 4°C for 30 min to remove insoluble material. The supernatant was collected and assayed for protein concentration (Bradford dye assay; Bio-Rad, Hercules, CA).
Immunoprecipitation and Immunoblotting. Equal amounts of protein from the liver or muscle were immunoprecipitated overnight at 4°C with an antiphosphotyrosine antibody (5 μg of antibody Ab/8 mg of protein) in 1 ml of immunoprecipitation buffer containing 2% Triton X-100, 300 mM NaCl, 200 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.4 mM PMSF, 0.4 mM Na3VO4, and 1% Nonidet P-40. After immunoprecipitation, the samples were mixed with 50 μl of protein-A Sepharose (10% solution) for 4 h at 4°C and the immunoprecipitate was washed in 1 ml of immunoprecipitation buffer, followed by centrifugation at 500g for 1 min at 4°C repeated four times. The washed precipitate was mixed with Laemmli sample buffer (50 μl) and boiled for 5 min, centrifuged for 5 min at 500g, and the supernatant (30 μl) separated on a 7% Tris-polyacrylamide gel electrophoresis (PAGE) gel using a Bio-Rad Mini-Protein gel apparatus. Proteins were then electrotransferred from the gel to polyvinylidene difluoride (PVDF) membrane at 100 V (constant current) for 2 h using a minitransfer apparatus (Idea Scientific, Minneapolis, MN). Gels were stained with Coomassie blue to verify equal protein transfer. To reduce nonspecific protein binding, the membrane was blocked with 5% nonfat dry milk in buffer containing 10 mM Tris-HCl, 150 mM NaCl, with 0.02% Tween 20. The PVDF membranes were incubated with anti-IR β (0.4 μg/ml) or IRS-1 antibody (1.5 μg/ml) in blocking buffer for 4 h at 22°C, followed by extensive washing with 10 mM Tris-HCl, 150 mM NaCl, with 0.02% Tween 20. At the end of the final wash, the blots were incubated with secondary antibody linked to horseradish peroxidase in 10 ml of blocking buffer for 1 h at 22°C and washed again before exposing the membranes to enhanced chemiluminescence (ECL) reagent according to the manufacturer’s instructions (Amersham, Arlington Heights, IL). Autoradiography was carried out using Kodak XAR X-ray film (Eastman Kodak, Rochester, NY). After treatment with the ECL reagent, the exposure time was varied from 1 to 3 min and each exposure was quantified by densitometry. The specific band intensities were quantified by densitometry using a Digiscan scanner (US Biochemical, Cleveland, OH) for integrating the autoradiographic signals. The results shown are expressed as the average signal intensity (arbitrary units) expressed relative to the effect of insulin on phosphorylation of IR and IRS-1 in untreated SHRs.

Western Blot Analysis of IRβ, IRS-1, p85α, and GLUT4 Protein. Western blot analysis was carried out in samples of gastrocnemius muscle or liver. For GLUT4 determination, total muscle membranes were prepared by homogenization of a portion of the muscle as described previously (Friedman et al., 1997). Each muscle or liver sample was homogenized and aliquoted and an average of three distinct assays was run on separate minigels. Each gel contained an internal standard of a rat heart protein (20 μg aliquot) prepared similar to the skeletal muscle. The samples were homogenized in 10× solubilization buffer containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 0.8 μg/ml aprotinin, 0.6 μg/ml leupeptin, 1 μg/ml pepstatin, and 50 μg/ml PMSF, and the sample was centrifuged at 38,000g for 60 min. The pellet was resuspended in solubilization buffer and 40 μg of protein was treated with Laemmli sample buffer, boiled for 5 min, and resolved on an 8% SDS-PAGE gel. For IRβ, IRS-1, and p85α analysis, frozen samples were homogenized in 10 volumes of solubilization buffer A (50 mM HEPES, pH 7.5, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM Na3VO4, 10 mM Na2P2O7, 10 mM NaF, 2 mM EDTA, 1% NP-40, 10% glycerol, 2 μg/ml aprotinin, 10 μg/ml antipain, 5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 1.5 mg/ml benzamidine, and 34 μg/ml PMSF) using a Polytron PTA 205 generator at maximum speed for 30 s. The homogenate was then centrifuged at 65,000 rpm at 4°C in a model 70 Ti rotor for 60 min to remove insoluble material, and the supernatant was used for analysis. Protein was measured using the Bradford procedure (Bio-Rad). For IRβ, IRS-1, and p85α, 100 μg of homogenate protein was treated with Laemmli sample buffer containing 100 mM dithiothreitol, heated in a boiling water bath for 4 min, and subjected to electrophoresis on a 7% SDS-Tris acrylamide gel using a Bio-Rad Mini-Protein gel apparatus at 100 V for 1 h. Proteins were electrotransferred from the gel to nitrocellulose at 90 V (constant) for 1 h using a minitransfer apparatus. Nonspecific protein binding to the filter was blocked using 5% milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20. The PVDF filter was incubated with antibodies to IRβ, IRS-1, p-85α, or GLUT4 (1.5 μg/ml) diluted in blocking buffer for 4 h at 22°C, followed by extensive washing with Tris-buffered saline (150 mM NaCl, 10 mM Tris + Tween 20). At the end of the final wash, the blots were incubated with secondary antibody linked to horseradish peroxidase in 10 ml of blocking buffer for 1 h at 22°C and washed again before exposing the membranes to the ECL reagent according to the manufacturer’s instructions (Amersham). Autoradiography was carried out using Kodak XAR X-ray film, with exposure time varied from 30 sec to 3 min, and the average specific band intensities from each exposure were quantified by optical density using a Digiscan scanner (US Biochemical) for integrating the autoradiographic signals. The results were expressed as arbitrary units relative to an internal standard sample (rat heart membrane) run together with each blot and the value for the control SHRs set at 100.

Statistical Analysis. Results are presented as means ± S.E.M. for the indicated number of rats. Comparisons between groups were made using Student’s unpaired t test, except for glucose tolerance data, which were analyzed by analysis of variance for repeated measures using Prism (Graph Pad Software, San Diego, CA). Statistical significance was set at P < .05.

Results

Animal Characteristics. At the end of the 90-day moxonidine treatment, body weight was 10% lower in SHROBs compared with aged-matched littermate controls because of a reduced rate of weight gain (Table 1). However, daily food

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>Effects of moxonidine treatment on metabolic parameters in SHRs and SHROBs</td>
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<table>
<thead>
<tr>
<th></th>
<th>SHRs</th>
<th></th>
<th>SHROBs</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td><strong>Moxonidine</strong></td>
<td><strong>Control</strong></td>
<td><strong>Moxonidine</strong></td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>325 ± 31</td>
<td>314 ± 35</td>
<td>623 ± 27a</td>
</tr>
<tr>
<td><strong>Mean blood pressure (mm Hg)</strong></td>
<td>144 ± 4</td>
<td>125 ± 10a</td>
<td>125 ± 4</td>
</tr>
<tr>
<td><strong>Urinary protein (mg/24 h)</strong></td>
<td>45 ± 9</td>
<td>40 ± 12</td>
<td>283 ± 70a</td>
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<tr>
<td><strong>Urinary epinephrine (μg/24 h)</strong></td>
<td>0.33 ± 0.08</td>
<td>0.20 ± 0.10</td>
<td>0.42 ± 0.09</td>
</tr>
<tr>
<td><strong>Urinary norepinephrine (μg/24 h)</strong></td>
<td>1.17 ± 0.10</td>
<td>1.37 ± 0.59</td>
<td>1.72 ± 0.16a</td>
</tr>
<tr>
<td><strong>Blood glucose (mg/dl)</strong></td>
<td>47 ± 6</td>
<td>48 ± 9</td>
<td>54 ± 4</td>
</tr>
<tr>
<td><strong>Plasma insulin (μg/ml)</strong></td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.56 ± 0.11b</td>
</tr>
<tr>
<td><strong>Insulin/glucose ratio</strong></td>
<td>0.01 ± 0.002</td>
<td>0.01 ± 0.004</td>
<td>2.36 ± 0.21a</td>
</tr>
<tr>
<td><strong>Free fatty acids (mM)</strong></td>
<td>1.56 ± 0.07</td>
<td>1.29 ± 0.12b</td>
<td>2.36 ± 0.21a</td>
</tr>
</tbody>
</table>

Data are means ± S.E.M. n = 18 for both SHR and SHROB control groups, and n = 4–7 for both SHRs and SHROBs treated with moxonidine, except for catecholamines in SHR animals (n = 2).

a Significantly different from SHR control, P < .05.
b Significantly different from control, P < .05.
intake was not different in treated and control SHROBs during the last 3 weeks of treatment. There was no significant effect of chronic moxonidine treatment on weight gain or food intake in lean SHRs. Treatment with moxonidine significantly lowered blood pressure in SHROBs and SHRs and reduced urinary protein excretion by 57% in SHROBs. Urinary epinephrine and norepinephrine were decreased by 60% and 54%, respectively, in moxonidine-treated SHROBs. A similar trend was noted in epinephrine in SHRs. Fasting blood glucose levels were not different between SHROBs and SHRs and were not affected by moxonidine treatment. Even though whole blood rather than plasma was used, fasting glucose levels were remarkably low in SHROBs and SHRs, which was in agreement with previous data from rats of this colony (Ernsberger et al., 1997; Friedman et al., 1997). Fasting serum insulin levels were elevated nearly 35-fold in the SHROBs compared with lean SHRs. Moxonidine treatment reduced fasting insulin levels by 71% and lowered plasma free fatty acids by 25% in SHROBs. In SHRs, moxonidine treatment decreased free fatty acids by 17% compared with controls, whereas moxonidine had little effect on the relatively low fasting insulin levels. To calculate the effectiveness of insulin in maintaining normoglycemia, we calculated the fasting insulin-to-glucose ratio. There was a 50-fold greater ratio in the SHROBs compared with SHRs, which was reduced significantly by 73% after moxonidine treatment. There was no significant change in the insulin-to-glucose ratio in the SHRs after moxonidine treatment.

**Effects of Moxonidine on Oral Glucose Tolerance.** In this group of animals, fasting insulin was reduced 2-fold in SHROBs and remained unchanged in SHRs (Fig. 1). Following an oral glucose load, blood glucose levels in moxonidine-treated SHROBs were reduced from 60 min onwards, and there was a sharply higher insulin secretion observed at 30 min (83% greater) and 60 min (67% greater) postchallenge compared with untreated SHROBs. Moxonidine treatment in lean SHRs elevated insulin levels at the 30-, 120-, and 180-min time points during following the glucose challenge test. There was a slightly greater insulin secretion but no significant lowering of already normal glucose tolerance in the SHRs. However, the fall from peak glucose levels at 60 min to the end of the test 180 min later was from 110 mg/dl to 56 mg/dl in moxonidine-treated lean SHRs versus 106 mg/dl to 81 mg/dl in controls. Thus, the fall in glucose between 60 and 240 min was twice as great in treated as in untreated SHRs (54 mg/dl versus 25 mg/dl).

**Profile of IR and IRS-1 Phosphorylation and Protein Expression in Skeletal Muscle from SHRs and SHROBs.** Insulin signaling defects in skeletal muscle are believed to be an underlying cause of insulin resistance to glucose disposal. Therefore, an analysis of IR and IRS-1 expression and phosphorylation was performed on insulin-treated SHRs and SHROBs. Plasma insulin was not measured after IR and IRS-1 phosphorylation in the present study. Previously (Friedman et al., 1997), we tested the conditions for maximal IR- and IRS-1-stimulated receptor phosphorylation in vivo. In pilot studies, we tested 1, 10, and 100 U/kg b.wt. in SHRs and SHROBs. We found that 10 U/kg increased the insulin to levels to >100 ng/ml within 5 min after injections and produced a maximal level of receptor stimulation in the SHRs and SHROBs. These levels are slightly greater than those seen following an oral glucose tolerance test, but are within the high physiological range. Rats were fasted overnight, anesthetized, and a sample of skeletal muscle was biopsied. Within 5 min after insulin administration, a second muscle sample was obtained, homogenized, and supernatant proteins extracted with antiphosphotyrosine antibody (Fig. 2). Aliquots of protein were resolved on a 6% SDS-polyacrylamide gel, transferred to nitrocellulose, and detected with antiphosphotyrosine antibody (Fig. 2). The levels of IR and IRS-1 proteins were quantified by scanning densitometry of the autoradiograph (Fig. 2, C and D). The levels of IR and IRS-1 proteins were reduced significantly by 73% after moxonidine treatment. There was no significant change in the insulin-to-glucose ratio in the SHRs after moxonidine treatment.

**Effects of Moxonidine on IR and IRS-1 Expression in Skeletal Muscle of SHRs and SHROBs.** To evaluate the effects of moxonidine treatment on the levels of IR and IRS-1 protein, Western blot analysis was performed on muscles from eight animals in each group. A representative autoradiogram is shown, and the results of multiple experiments...
were quantified by scanning densitometry (Fig. 3A). There was a 32% decrease in IR protein in skeletal muscle of control SHROBs compared with SHRs. Moxonidine treatment increased the expression of the IR \( \beta \) subunit by 19% in SHROBs, but was without effect in SHRs. The level of IRS-1 protein was decreased by 60% in control SHROBs compared with SHRs. Moxonidine treatment enhanced the expression of IRS-1 protein in skeletal muscle by 74% and 40% in SHROBs and SHRs, respectively.

**Effects of Moxonidine Treatment on Insulin-Stimulated Phosphorylation of IR and IRS-1 in Skeletal Muscle.** To quantify the effects of moxonidine on insulin-stimulated phosphorylation of the IR and IRS-1, muscle protein extracts from insulin-treated rats were immunoprecipitated with antiphosphotyrosine antibodies and blotted using specific antibodies to the IR \( \beta \) subunit and IRS-1. The level of insulin-stimulated receptor phosphorylation in skeletal muscle of SHROBs was decreased by 36% compared with SHRs (Fig. 3B). Moxonidine treatment increased insulin-stimulated receptor phosphorylation in skeletal muscle of SHROBs by 15%. There was no effect of moxonidine on IR phosphorylation levels in lean SHRs. The level of insulin-stimulated IRS-1 phosphorylation in control SHROBs was modestly reduced by 23% compared with control SHRs. Moxonidine treatment increased insulin-stimulated IRS-1 phosphorylation by 27% in SHROBs and by 56% in SHRs. Both the total mass of IRS-1 and the mass of phosphorylated IRS-1 were increased in moxonidine-treated SHROBs and SHRs. However, the ratio of phosphorylated to unphosphorylated protein was not different. Because the phosphorylated protein is the active species, it is the quantity of this activated protein that is most likely to be relevant.

**Effects of Moxonidine on GLUT4 and p85\( \alpha \) Expression.** In addition to the IR and IRS-1, the levels of p85\( \alpha \) and GLUT4 proteins were determined in muscle from control and moxonidine-treated animals. In control SHROBs, GLUT4 levels were reduced by 45% in skeletal muscle compared with SHRs (Table 2). The cellular content of the IR- associated p85\( \alpha \) was decreased by 32% in the muscle of SHROBs. Treatment with moxonidine had no effect on the expression levels of GLUT4 or p85\( \alpha \) protein in either SHROBs or SHRs.

**Effects of Moxonidine Treatment on IR and IRS-1 in Liver of SHRs and SHROBs.** The liver is an additional important locus of insulin resistance. To determine the effects
of moxonidine therapy on IRs and IRS-1 present in the liver of SHRs and SHROBs, equal amounts of liver protein from the experimental animals were resolved by SDS-PAGE and immunoblotted with anti-IR (β subunit) or IRS-1 antibody (Fig. 4A; a representative autoradiogram is shown). The level of IR protein was reduced by 42% in SHROBs compared with SHRs. Moxonidine treatment had no effect on expression of IRs. The level of IRS-1 protein in liver of control SHROBs was similar to that of control SHRs. Moxonidine increased the levels of expression of IRS-1 protein by 275% in SHROBs and 260% in SHRs.

**Effects of Moxonidine Treatment on Insulin-Stimulated Tyrosine Phosphorylation of IR and IRS-1 in Liver.** Insulin-stimulated phosphorylation of the IR was 51% lower in the liver of untreated SHROBs compared with SHRs. Chronic treatment with moxonidine resulted in 34% greater IR phosphorylation in SHROBs, but had no effect in lean SHRs (Fig. 4B). The level of insulin-stimulated IRS-1 phosphorylation in control SHROBs was similar to SHRs. Moxonidine treatment increased insulin-stimulated IRS-1 phosphorylation by 48% in SHROBs and by 240% in SHRs (P < .05).

**Discussion**

Abnormal glucose metabolism in obesity and NIDDM is characterized by insulin resistance of liver and peripheral tissues and impaired insulin secretion from pancreatic
cells in response to a glucose challenge. In the hyperinsulinemic SHROB model, there is a defect in insulin sensitivity in skeletal muscle and liver and impaired glucose tolerance following an oral glucose load (Friedman et al., 1997). In the present study, chronic administration of moxonidine not only reduced blood pressure but increased the insulin response during the first 30 to 60 min of the oral glucose tolerance test and reduced hyperglycemia from 60 min onward. Among many other influences, insulin secretion is controlled by input by the autonomic nervous system. In NIDDM and obesity, increased sympathetic activity and high catecholamine levels may contribute to the impairment in insulin secretory response to glucose (Robertson et al., 1976). Moxonidine reduces sympathetic activity through a selective action on brain stem I1 imidazoline receptors (Ernsberger et al., 1992). In addition, in vitro studies have shown that several imidazoline compounds act as insulin secretagogues in isolated islets (Kaan et al., 1995; Wang et al., 1995), suggesting that I1 receptors may play a role in the regulation of glucose homeostasis. Our data demonstrate that chronic moxonidine therapy may improve the impairment in insulin secretory response in response to glucose challenge in SHROBs, presumably by the direct activation of I1 imidazoline receptors in the β-cell, by a centrally mediated reduction in sympathetic outflow to the β-cell, or by both mechanisms. Moxonidine facilitates glucose-stimulated insulin release from isolated perfused pancreatic islets, whereas basal release at low glucose concentrations was slightly inhibited by moxonidine (Rosen et al., 1997). These in vitro results are consistent with the present data in SHROBs showing reduced fasting insulin coupled with an increased response to glucose challenge.

In addition to an increased insulin secretory response, moxonidine lowered fasting insulin and free fatty acid levels in SHROBs, reflecting an improved insulin-stimulated skeletal muscle glucose transport capacity (Henriksen et al., 1997). The mechanisms for insulin resistance in obesity and hypertension are not completely understood. In skeletal muscle of obese humans, IRS-1 protein is reduced along with tyrosine-phosphorylated IRS-1 after insulin stimulation, implicating this key signaling is intermediate in the postrecep-

![Insulin Receptor β Protein - Liver](image1)

![Insulin Receptor Phosphorylation in Liver](image2)

**Fig. 4.** A, effect of chronic moxonidine treatment on levels of IR and IRS-1 in liver of SHRs and SHROBs. To measure the total quantity of IRs or IRS-1, equivalent amounts of solubilized liver protein were resolved by SDS-PAGE, transferred to PVDF membranes, detected with antibodies to anti-IR (β subunit) or IRS-1 by Western blotting, and subjected to autoradiography. A representative autoradiogram is shown. Lanes 1 and 2, SHR control; lanes 3 and 4, SHROB control; lanes 5 and 6, SHR moxonidine treated; lanes 7 and 8, SHROB moxonidine treated. Scanning densitometry of multiple autoradiograms was performed according to procedures outlined in Materials and Methods and were obtained from eight different animals in each group. The graphs show the relative quantity of IR β subunit and IRS-1 in the liver from each group expressed relative to SHR controls. The data are expressed as mean ± S.E.M. *P < .05 versus SHR; **P < .05 versus control. B, effect of chronic moxonidine treatment on tyrosine phosphorylation of IR and IRS-1 in intact liver from SHRs and SHROBs. A sample of liver was obtained before (−) and 30 s after insulin injection (+) as described in Materials and Methods. Livers were homogenized with extraction buffer under denaturing conditions at 4°C, and aliquots containing the same amount of protein were incubated at 4°C with pTy antibodies and protein A-Sepharose. The immunoprecipitated proteins were run on SDS-PAGE and analyzed by Western blotting with either anti IR (β-subunit) antibody (not shown) or anti-IRS-1 antibodies and subjected to autoradiography. A representative autoradiogram is shown. Lanes 1 and 2, SHR control; lanes 3 and 4, SHROB control; lanes 5 and 6, SHR moxonidine treated; lanes 7 and 8, SHROB moxonidine treated. Scanning densitometry of multiple autoradiograms was performed according to procedures outlined in Materials and Methods from experiments on eight different animals in each group. The graph shows the net increase in tyrosine phosphorylation in the IR β subunit and IRS-1 in liver from multiple experiments. The data are expressed as mean ± S.E.M. relative to lean SHR. *P < .05 versus lean SHR; **P < .05 versus control.
tor insulin resistance (Goodyear et al., 1995). The failure of insulin to fully stimulate IR and IRS-1 phosphorylation in muscle of the obese SHRs and the reduced levels of these signaling proteins may contribute to lowered glucose uptake and severe hyperinsulinemia in the SHROBs. Moxonidine treatment did not increase the levels of GLUT-4 or p85α in either SHROBs or SHRs, suggesting that moxonidine treatment may improve disposal of an oral glucose load by a mechanism that does not involve increased synthesis of either of these two proteins. One possible explanation for increased glucose disposal in rats treated with moxonidine is that the subcellular distribution of GLUT4 could be altered such that more resides in the plasma membrane or that activation of existing transporters by insulin could be increased as a result of moxonidine treatment. This most likely can occur by improved insulin signaling through the IR and IRS-1 proteins. Moxonidine treatment significantly increased the levels of IR and IRS-1 proteins in muscle and liver of the SHRs and SHROBs. From these data it is implied that the IRS-1 pathway may be contributing to some degree to greater glucose disposal after moxonidine treatment. Although these changes may seem small, it is important that the levels of IRS-1 phosphorylation were corrected to levels seen in the control SHRs and may therefore have contributed to the chronic effect of moxonidine on improved insulin signal transduction. Additional mechanisms other than IRS-1 phosphorylation may underlie some of the improvements in glucose tolerance. It is also possible that decreased circulating catecholamines may have played a role. Because moxonidine reduced norepinephrine and epinephrine, some of the effects on glucose tolerance may have been secondary to the reduction in free fatty acids brought about by reduced catecholamines. It may also be that other metabolic adaptations were possible, such as improved GLUT4 trafficking to the cell surface. These effects remain to be investigated. The mechanisms underlying moxonidine’s positive effects on IR and IRS-1 expression in lean and obese animals is not yet known. Hyperinsulinemia can cause internalization and degradation of the IR (Knutson, 1991) and IRS-1 in 3T3-L1 cells in culture (Rice et al., 1993). The reduced body weight gain in the SHROBs may have also contributed to the lower insulin levels and metabolic improvements, even though food intake had returned to control levels. However, many of the same changes in insulin signaling and reduced fatty acids were noted in the moxonidine-treated lean SHRs in the absence of an effect on weight gain, suggesting a specific drug action.

A second mechanism for improved glucose tolerance in moxonidine-treated rats may be a secondary effect of lowering the levels of circulating plasma fatty acids in the animal. SHRs have previously been shown to have elevated free fatty acids relative to normotensive Wistar controls, consistent with their elevated levels of sympathetic tone (Swislocki and Tsuki, 1993). In the SHROBs, the elevated free fatty acid levels are very similar to those seen in other obese strains such as the Zucker rat (Henriksen et al., 1997) and in other previous study of SHROBs (Hiraoka et al., 1997). In addition to increased free fatty acids, fasting levels of serum triglycerides in obese SHRs are elevated 10-fold relative to lean SHRs, and chronic moxonidine treatment reduced the levels of triglycerides and cholesterol by 70% and 35%, respectively, in obese SHR (Ernsberger et al., 1996). By lowering circulating free fatty acids, triglycerides, and cholesterol, the fatty acid composition of the plasma membrane phospholipids could be altered, thus restoring normal insulin action or normal transport of glucose across the membrane (Storelín et al., 1991; Borkman et al., 1993). Furthermore, there is evidence that decreasing plasma-free fatty acids can improve insulin-stimulated glucose uptake in muscle (Vaag et al., 1991; Worm et al., 1994), possibly due to increased glucose oxidation (Randle et al., 1963).

Given the marked fasting hyperinsulinemia in the SHROBs necessary to maintain fasting normoglycemia, insulin resistance is likely to be present in the liver as well as in skeletal muscle. The current study shows a large decrease in IR autophosphorylation in liver of the SHROBs, whereas IRS-1 protein and phosphorylation appears to be normal. In the present study, moxonidine treatment brought about a substantial increase in IRS-1 protein expression in both lean and obese animals, probably increasing the efficiency of signaling between the IR and its substrate. The importance of different sites of insulin resistance to the etiology of cardiovascular diseases has not been studied. These defects could contribute to impaired glucose tolerance and reduced ability of insulin to inhibit hepatic gluconeogenesis (Belivacqua et al., 1987).

Our experiments in hypertensive SHROBs and SHRs, and those of others in fructose-fed hypertensive rats (Rosen et al., 1997) and obese Zucker rats (Henriksen et al., 1997), suggests chronic moxonidine therapy may improve glucose tolerance in two fundamental ways: by enhancing glucose-stimulated insulin secretion, and by potentiating insulin-stimulated glucose disposal. The latter may possibly be mediated through lowering of circulating free fatty acids and increased expression of insulin- signaling proteins in skeletal muscle and liver. The glucose-lowering mechanisms of moxonidine in hypertension and obesity could result from the combined indirect effects of inhibition of sympathetic nervous system effects on liver, muscle, and adipose tissue, as well as direct metabolic effects of moxonidine on the pancreas and other tissues. It is worth noting that agmatine, believed to be the natural ligand for the I1 imidazoline receptor, has been shown in vitro to stimulate glucose uptake in diaphragm muscle and inhibit lipolysis in adipocytes (Kaan et al., 1995). It is not known whether skeletal muscle, liver, or adipose tissues expresses the I1 imidazoline receptor, however, this hypothesis should be readily testable. Future studies should also address whether the positive changes in expression and phosphorylation of insulin signaling proteins with moxonidine occur in liver and muscle cells in culture.

The causes of hypertension in the SHRs and SHROBs are considered to be polygenic and multifactorial. Hyperinsulinemia and insulin resistance have been implicated as causes of hypertension in humans (Reaven, 1988). However, insulin itself is a direct vasodilator and in many studies causes an acute fall in blood pressure (Anderson et al., 1991). Furthermore, humans with insulinomas show profound elevations in circulating insulin with no increase in catecholamines or blood pressure. In the SHROB model, insulin levels are increased 20-fold compared with their lean SHR littermates, yet blood pressures in SHROBs are slightly lower than those in SHRs. These findings argue that insulin resistance and hypertension may segregate as independent phenotypes and do not show synergism in the pathogenesis of either insulin resistance or hypertension. Because the sympatholytic agent...
moxonidine lowered blood pressure and improved insulin resistance in SHROBs, this suggests that excess sympathetic activity might contribute to both hypertension and insulin resistance in the SHROB.

Based on the current results showing that the central sympathetic agent moxonidine reduces blood pressure, lowers blood lipids, and improves insulin resistance, we suggest that increased sympathetic activity might be a common element that links insulin resistance and hypertension in this model of syndrome X. The use of moxonidine should therefore be considered a useful intervention in the treatment multiple metabolic abnormalities associated with syndrome X.

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References


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