Marked Insulin Resistance in Obese Spontaneously Hypertensive Rat Adipocytes Is Ameliorated by in Vivo but Not in Vitro Treatment with Moxonidine

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ABSTRACT
The obese spontaneously hypertensive rat (SHROB) is a model of marked insulin resistance with normoglycemia. We sought to determine whether insulin resistance extends to adipocytes and the impact of an insulin-sensitizing imidazoline, moxonidine (4 mg/kg/days for 21 days). Gonadal adipocytes were isolated from SHROB and lean spontaneously hypertensive rat (SHR) littermates. In lean SHR adipocytes, Akt activation by 100 nM insulin peaked at 3 min at 25-fold, whereas SHROB adipocytes showed only 4-fold activation. In dose-response experiments, the maximal response ($E_{max}$) was markedly reduced 18.8 ± 2.3 versus 3.7 ± 0.8. Insulin sensitivity was also attenuated, with higher concentrations required for responses ($EC_{50}$ = 3.5 ± 0.5 versus 29 ± 3.8 nM). Glucose uptake as determined with [3H]2-deoxyglucose was also less responsive in SHROB relative to lean SHR. Moxonidine had little or no effect when applied acutely in vitro, but adipocytes isolated from SHROB treated with moxonidine in vivo showed significantly improved responses to insulin, both in terms of Akt activation and facilitation of glucose uptake. Chronic but not acute moxonidine treatment partially restores insulin sensitivity in SHROB adipocytes, suggesting an indirect action of this agent.

Obese spontaneously hypertensive rats (SHROB) are markedly insulin-resistant, showing a greater than 20-fold elevation fasting insulin levels in the presence of normal fasting glucose (Friedman et al., 1997; Velliquette et al., 2005). At the cellular level, defects in insulin action have been noted in skeletal muscle and the liver, as indicated by reduced insulin receptor protein, reduced levels of its substrate protein IRS-1, and impaired insulin-induced tyrosine phosphorylation of both proteins (Friedman et al., 1997). In adipocytes and in skeletal muscle, the stimulation of glucose transport by insulin is impaired. However, insulin signaling in adipocytes in the SHROB model has not yet been characterized.

Akt (protein kinase B) is a 57-kDa Ser/Thr kinase that plays a key role in the insulin induced PI3K-Akt pathway (Hanada et al., 2004; Osaki et al., 2004). The binding of insulin to its receptors leads to the phosphorylation of PI3K, which then phosphorylates phosphatidylinositols at the 3-position. Then, Akt is recruited to the inner side of plasma membrane due to the interaction between its pleckstrin homology domain and the phosphatidylinositol-(3,4,5)-trisphosphate produced by PI3K. The Thr308 and Ser473 on Akt are then phosphorylated by 3-phosphoinositide-dependent protein kinase 1/2 and mammalian target of rapamycin (Hresko and Mueckler, 2005). Once activated, Akt regulates many cellular functions related to insulin action (Hanada et al., 2004). As a key element in insulin signaling, Akt could be an efficient indicator for cellular insulin response. Here, we measured the phosphorylation level of Akt in corresponding to insulin stimulation as an indicator of insulin sensitivity in isolated rat adipocytes. We hypothesized that the inherent defect in insulin action at this step downstream in the insulin-signaling cascade might be more impaired than the early stages of insulin signaling studied previously.

Moxonidine is a centrally acting sympatholytic agent that was unexpectedly found to possess insulin-sensitizing actions through largely unknown mechanisms in humans (Haenni and Lithell, 1999; Chazova et al., 2006) and in animal models (Henriksen et al., 1997; Yakubu-Madus et al., 1999; Ernsberger et al., 1996, 1999a). Similar results have been obtained for another imidazoline agonist, rilmenidine (Penicaud et al., 1998; Velliquette and Ernsberger, 2003b;...
Anichkov et al., 2005). Moxonidine is a selective agonist at 1α-imidazoline receptors, while also activating α2-adrenergic receptors (Ernsberger et al., 1993). Whereas both imidazoline and α2-adrenergic receptors contribute to sympathetic actions, only the imidazoline component improves glucose metabolism (Velliquette and Ernsberger, 2003b). Most studies have been carried out with chronic treatment, but acute improvements in glucose tolerance and insulin secretion can be detected under blockade of α2-adrenergic receptors (Velliquette and Ernsberger, 2003b).

The cellular mechanisms for the insulin-sensitizing action of moxonidine are partially known. Chronic treatment with moxonidine increases the expression of insulin receptor and IRS-1 in muscle and liver in insulin-resistant SHROB and partially restores the ability of insulin to induce tyrosine phosphorylation of these proteins (Ernsberger et al., 1999a). The impacts of moxonidine treatment on other steps in the insulin-signaling cascade or in other cell types are not known. In the present study, we focused on insulin signaling in adipocytes through the Akt phosphorylation step, a possible site of insulin resistance in human diabetes type 2 (Karls-son et al., 2005). We hypothesized that insulin-dependent Akt phosphorylation would be deficient in SHROB model of insulin resistance and that long-term treatment with moxonidine would partially restore this defect, through either a direct action on adipocytes or an indirect systemic action.

Materials and Methods

Materials. Moxonidine (free base) was provided by Solvay Pharmaceuticals (Hannover, Germany). Insulin and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animal Procedures. Adult male and female SHROB were obtained from a closed colony that has been continuously inbred since 1973 (Ernsberger et al., 1999b). Because both male and female SHR and SHROB were used in these studies. No sex differences were noted in any experimental parameter, consistent with previous results (Ernsberger et al., 1999a). Animals were not used in any other experiments, and they were housed in pairs and were provided food (Teklad formula 8664; Teklad, Madison WI) and water ad libitum. Animals were on a 12:12-h light/dark cycle (lights on from 7:00 AM to 7:00 PM) and were maintained at a constant temperature of 21°C. Animals were fasted 18 h before sacrifice. Given the 1-h half-life of moxonidine (He et al., 2000), it was expected that no drug would be present at the time of tissue harvesting.

Acute Moxonidine Treatment and Insulin Stimulation. Moxonidine (100 nM) or 0.1% citric acid vehicle was applied into each tube of floating adipocytes 90 min before insulin stimulation. Insulin was applied into the cell incubations at a concentration of 100 nM for various time lengths from 0 to 90 min (in time-course experiments) or at various concentrations from 0.0 to 1.0 μM (in dose-response experiments) for 10 min.

Glucose Uptake Assay. Gonadal adipose tissue was taken from nonfasted rats for each experiment at the same of day (9:00 AM). Following collagenase digestion as described above, the adipocytes were filtered through mesh and rinsed with wash buffer (phosphate-buffered saline, pH 7.4, containing 1 mM sodium pyruvate, 0.1% BSA, 25 mM HEPES, 2.5 mM MgCl2, and 2.5 mM CaCl2) at least three times. Cell suspensions were equally distributed into 20-ml plastic tubes shaking at 100 rpm in a 37°C water bath, each containing 106 cells in a total volume of 1 ml. Various concentrations of insulin (100, 10, 1, or 0.1 nM) or vehicle (wash buffer) were applied to the cell suspensions for 30 min before exposure to [3H]2-deoxy-D-glucose. Nonspecific uptake was determined in the presence of 10 μM cytochalasin-B added 10 min before [3H]2-deoxy-D-glucose to block glucose transport. At the end of incubation, 150 μl of cell suspension from each tube was transferred into a 300-μl elongated centrifuge tube on top of 70 μ1 of mineral oil. Uptake was initiated by adding 50 μ1 of 2.5 mM [3H]2-deoxy-D-glucose containing a total of 0.33 μCi of 3H labeling. In 3 min, the reaction was stopped by spinning 10 s at 5000g to separate cells from media. The cell fraction was removed from the rest of medium by slicing through the oil layer, and the top portion of the tube containing adipocytes was put into a scintillation vial with 4 ml of scintillation fluid (EcoScint A; National Diagnostics, Atlanta, GA). Vials were counted in a scintillation counter for 5 min, and specific glucose uptake was defined as [3H]2-deoxy-D-glucose incorporation minus incorporation in the presence of cytochalasin-B. The rate of uptake was expressed as picomoles per 105 cells per 3 min. Assays were carried out with triplicate cell aliquots, and the results were averaged.

Statistics. Results are presented as means ± standard error of the mean. Dose-response curves were analyzed by nonlinear curve fitting to a logistic equation (Prism 4.0; GraphPad Software Inc., San Diego, CA). Groups were compared by one- or two-way analysis of variance followed by Newman-Keuls tests.
Results

SHROB Adipocytes Show Profound Insulin Resistance. Consistent with previous results, SHROB were significantly heavier than SHR littermates, and this was true for both females (SHROB, 504 ± 30 g; SHR, 328 ± 12 g) and for males (SHROB, 519 ± 14 g; SHR, 403 ± 7 g). There was no sex difference in body weight in SHROB, but SHR males were heavier than SHR females.

A representative set of Western blots illustrating the time course of Akt activation by insulin is shown in Fig. 1. Immunoreactivity to the phosphospecific antibody is shown in the top image, and the immunoreactivity for total Akt protein is shown in the corresponding image below, which was obtained from the same blot after stripping. Note the large sustained increase in phosphospecific immunoreactivity, whereas total Akt is relatively constant, indicating equal loading of the lanes. Adipocytes isolated from SHROB show an equivalent amount of total Akt immunoreactivity, but the increase in phosphospecific immunoreactivity elicited by insulin at each time point is noticeably less.

The level of Akt phosphorylation in gonadal adipocytes from lean SHR increased 26.6 ± 3.5-fold compared with basal level in response to 3-min exposure to 100 nM insulin (Fig. 2). The level of Akt activation then slowly declined over time and was still elevated 14.2 ± 5.4-fold at 90 min, the last time point tested. In contrast, adipocytes from SHROB showed a greatly attenuated response to 100 nM insulin stimulation, with a peak activation of only 3.9 ± 1.4-fold at 3 min. This small response was largely maintained at 90 min (3.3 ± 1.4-fold activation).

This marked attenuation of Akt responses to insulin in adipocytes from SHROB was confirmed in dose-response experiments (Figs. 3 and 4). SHR and SHROB adipocytes showed large differences in maximal response (E_max) at 10 min of insulin stimulation: 18.8 ± 2.3 versus 3.7 ± 0.8 (expressed as -fold increase). Also consistent with reduced insulin sensitivity, the EC_{50} for insulin was lower in SHR than in SHROB by nearly a full log unit (3.5 ± 0.5 versus 29 ± 3.8 nM). Thus, higher insulin concentrations were required to induce even a relatively small response in SHR rat adipocytes.

Chronic Moxonidine Treatment Enhances Insulin Sensitivity in Adipocytes from SHROB. To investigate whether chronic moxonidine improves insulin sensitivity in the adipose tissue of SHROB, an animal model of insulin resistance, we treated SHROB moxonidine orally at a dose of 4 mg/kg/day for 21 days. Consistent with previous reports, this dose of moxonidine did not affect body weight in either males (moxonidine-treated, 519 ± 14 g; vehicle-treated, 533 ± 16 g) or females (moxonidine treated, 470 ± 11 g; vehicle-treated, 504 ± 30 g). The adipocytes from treated SHROB showed significantly enhanced Akt responses to 100 nM insulin (Fig. 2): The peak -fold activation at 3 min in-
creased to 11.4 ± 1.5 after treatment, which was more than twice the response in control SHROB treated with vehicle alone. The activation of Akt did not change over time between 3 and 90 min of treatment, similar to control SHROB adipocytes from vehicle-treated animals.

In dose-response experiments, after chronic moxonidine treatment the maximum response ($E_{\text{max}}$) to insulin expressed as fold increase at 10 min rose to 7.5 ± 0.6 compared with 3.7 ± 0.8 from untreated SHROB (Fig. 4). The EC$_{50}$ for insulin also fell to 2.6 ± 0.6 nM from 29 ± 3.8 nM. Thus, moxonidine treatment in vivo increased the maximal response to insulin as well increasing the sensitivity to low concentrations of insulin.

Since all of the data are expressed as a ratio of phosphospecific to total immunoreactivity, it is possible that changes in unstimulated basal Akt phosphorylation may have contributed to the apparent effect of drug treatment. To evaluate this possibility, we compared the ratio of phosphospecific to total Akt immunoreactivity for adipocytes incubated in the absence of insulin. As shown in Fig. 5, adipocytes from SHR, SHROB, and SHROB treated with moxonidine all showed identical levels of unstimulated baseline Akt activation. Thus, the insulin resistance of SHROB adipocytes and the insulin-sensitizing action of chronic in vivo moxonidine were not mediated by changes in baseline activation.

**Insulin Induced Glucose Uptake in SHR and SHROB Adipocytes and the Effect of Moxonidine Treatment.**

To test whether changes in insulin sensitivity detected at the level of Akt signaling correlate with functional changes relevant to metabolism, we assayed adipocyte glucose uptake. First, we compared insulin responses in adipocytes from SHR and SHROB without drug treatment (Fig. 6A). Similar to SHROB, SHR did not show any effect of moxonidine treatment on body weight (moxonidine-treated, 386 ± 9 g; vehicle-treated, 403 ± 7 g). SHR adipocytes showed a basal glucose uptake of 1.4 ± 0.17 nmol of 2-deoxy-D-glucose per 10$^5$ cells in 3 min, and SHROB adipocytes showed similar results with (1.4 ± 0.16 nmol/10$^5$ cells in 3 min). Insulin induced a concentration-dependent increase in glucose uptake up until 10 nM, with 100 nM having less effect in the SHROB groups (indicated by descending dotted lines). Following stimulation for 30 min with 10 nM insulin, glucose uptake in SHR adipocytes increased to 5.3 ± 0.60 nmol/10$^5$ cells/3 min, whereas SHROB adipocytes could only reach 3.1 ± 0.28 nmol/10$^5$ cells/3 min ($p < 0.05$; $t$ test with Bonferroni correction). These data are consistent with insulin resistance in adipocytes of SHROB.

We then tested adipocytes from chronic moxonidine-treated (21 days, 4 mg/kg/day) SHROB and SHR to see whether the insulin-sensitizing effect detected with Akt activation was reflected in glucose uptake. As expected and consistent with Akt activation results, the glucose uptake in SHROB adipocytes stimulated with 10 nM insulin was increased to 3.9 ± 0.41 nmol/10$^5$ cells/3 min by 21 day chronic oral administration of moxonidine, whereas the basal glucose uptake remained almost unchanged: 1.3 ± 0.16 nmol/10$^5$ cells/3 min. However, in contrast to Akt activation, the EC$_{50}$ values for insulin in adipocytes from the three groups were
quite close to each other: the logEC\textsubscript{50} was close to -9.0 for all three groups (Fig. 6A).

We also compared adipocyte glucose uptake between control SHR and SHR-treated with moxonidine for 21 days (Fig. 6B). Results for the untreated control SHR were similar to those of the previous experiment. In contrast to SHROB, SHR treated with moxonidine showed no difference relative to vehicle-treated controls in either basal uptake or insulin-stimulated uptake.

**Effect of Acute Moxonidine Treatment.** To test for a direct effect of moxonidine on Akt activation, we tested the effect of treatment with 100 nM moxonidine for 90 min. This concentration and duration of treatment has previously been shown to trigger multiple cell-signaling events in other cell types (Edwards et al., 2001; Edwards and Ernsberger, 2003). Akt activation in adipocytes treated with moxonidine alone was normalized to the control group treated with vehicle alone. Both SHR and SHROB adipocytes showed no change in basal Akt activation level in response 90 min acute in vitro treatment with moxonidine (Fig. 7). Thus, moxonidine does not directly activate Akt in adipocytes.

Given that in vivo treatment with moxonidine facilitated insulin signaling, we sought to test the possible insulin-sensitizing effect from acute treatment of adipocytes with moxonidine in vitro. Isolated adipocytes from SHR or SHROB were preincubated with or without 100 nM moxonidine for 90 min before being exposed to 100 nM insulin for 0 to 90 min. A representative blot for SHR adipocytes is shown in Fig. 8, and the averaged results for SHR and SHROB are shown in Fig. 9A and B, respectively. Adipocytes from SHR with moxonidine preincubation showed a shifted insulin-response time course with a slightly facilitated maximum -fold response at 5 min: 36.5 ± 7.4 versus 29.0 ± 5.3, but a significantly reduced insulin response at 90 min: 4.4 ± 0.77 versus 11.3 ± 2.6 (Fig. 9A). No significant differences were found between 10 to 60 min. However, adipocytes from SHROB did not show any change in insulin response by acute moxonidine treatment. The peak -fold activation of pretreated and untreated adipocytes from SHROB were very similar (7.8 ± 1.0 versus 8.1 ± 1.3), and there were no significant difference at any other time points through 90 min (Fig. 9B). Similar negative results were obtained with concurrent treatment with moxonidine and insulin (data not shown). Thus, acute exposure to moxonidine in vitro does not reproduce the effects of chronic treatment in vivo, implying a mechanism not involving direct and immediate cellular actions on adipocytes.
Discussion

Previous studies have already demonstrated that SHROB express severe insulin resistance and glucose intolerance at the level of the whole body and in liver and skeletal muscle at the level of the insulin receptor and IRS-1 (Friedman et al., 1997; Ernsberger et al., 1999b). In the present study, we isolated adipocytes from SHROB and SHR to test insulin responses in the absence of the physiological milieu. We assayed Akt activation, which is a key element in insulin cell signaling downstream of both the insulin receptor and IRS-1. Since the results were obtained from isolated cells, the insulin response was independent of influence from blood hormones and should reflect the function of adipose tissue only. Results of this study showed that adipose tissue, one of the major sites of insulin action, expresses profound insulin resistance with impaired Akt activation resulting from insulin stimulation. Insulin-induced uptake of glucose into adipocytes was also impaired, and this cellular defect might contribute to the whole-body insulin resistance syndrome.

The insulin-sensitizing effect of chronic moxonidine treatment has been observed in both animal and human experiments (Velliquette and Ernsberger, 2003a; Jacob et al., 2004). Results of this study were quite consistent with those studies in that chronic moxonidine treatment shows significant beneficial effects on the sensitivity and responsiveness to insulin. As in other recent studies, moxonidine at a dose of 4 mg/kg/day had no effect on body weight or fat depot size in either SHR or SHROB, ruling out any effect due to loss or gain of weight.

Lean SHR adipocytes did not show an enhancement of insulin action on glucose uptake. This is consistent with the much smaller effect of chronic moxonidine treatment on insulin resistance in SHR than in SHROB (Ernsberger et al., 1999a; Velliquette and Ernsberger, 2003a). Furthermore, the lipid-lowering actions of moxonidine can be detected in SHROB but not in SHR (Velliquette et al., 2006). In human studies, beneficial metabolic effects have been detected in diabetic and insulin-resistant subjects, but in unselected hypertensive subjects (Kaan et al., 1995; Lithell, 1998). Similar results have been obtained with another imidazoline agonist, rilmenidine (Meredith and Reid, 2004; Anichkov et al., 2005).

Both male and female animals were used in the present study. No sex differences were observed in any variable (data not shown). This finding is consistent with all previous studies in the SHROB model, which have consistently failed to find significant differences in metabolic syndrome traits between males and females (Ernsberger et al., 1999b). The SHROB model is unusual in this regard, because most rodent models of obesity and insulin resistance affect one gender more than the other (Kava et al., 1992). The lack of gender differences probably stems from severe hypogonadism (Ko-
Moxonidine treatment in vivo as a change in gene expression. This conclusion is supported by a durable effect of the treatment on adipocytes such as the central nervous system or the endothemic pancreas in a short period such as 15 min. Although definite conclusions cannot yet be made, the results of the present study suggested that the effects of acute moxonidine treatment are not mediated by direction action on adipocytes. The site of action of acute moxonidine may be located within the pancreas, where it acts to facilitate insulin secretion and inhibit glucagon secretion (Velliquette and Ernsberger, 2003b). A number of other groups have characterized the actions of imidazolone agonists within the endothemic pancreas (Morgan and Chan, 2001). Moxonidine may alter adipocyte gene expression indirectly by affecting another organ, such as the pancreas or the liver. Recently, we have shown that moxonidine has a direct action on the liver to reduce the production and secretion of triglycerides into the plasma (Velliquette et al., 2006). Reduced delivery of triglycerides to adipocytes might produce long-lasting changes in their insulin sensitivity. The influence of plasma triglycerides on adipocyte insulin resistance has been shown in humans (Yki-Jarvinen and Taskinen, 1988).

There is a slight discrepancy in the results in that the moxonidine pretreatment experiments (Fig. 9) showed a 5-fold Akt activations in SHR and SHROB were both higher than the corresponding 5-min levels in Fig. 2. This seemed to be a consequence of the 90-min pretreatment period, which reduced the basal Akt activation as a result of additional exposure to serum-free medium. In addition, whereas cell-signal responses to insulin were dose-dependent up to 1.0 μM, the activation of glucose transport fell off at 100 nM insulin for some experimental groups. This might reflect desensitization of insulin signaling pathways in vitro from prolonged exposure to higher concentrations. Differences in the susceptibility of SHR and SHROB adipocytes to insulin desensitization should be examined in future studies.

In conclusion, insulin resistance in SHROB adipocytes persists upon isolation and challenge with insulin in vitro. The phosphorylation of Akt is a step in the insulin signaling cascade that shows much stronger evidence of insulin resistance than tyrosine phosphorylation of the insulin receptor or IRS-1 we have described previously (Friedman et al., 1997; Ernsberger et al., 1999b; Velliquette et al., 2005). Thus, the SHROB adipocyte is a potential in vitro model of insulin resistance. In vivo treatment with moxonidine for 21 days increased the sensitivity of SHROB adipocytes to insulin, despite withdrawal of treatment 18 h before tissue harvesting and extensive washing during the isolation process. This suggests a durable effect of the treatment on adipocytes such as a gene expression change. This conclusion is supported by the lack of an acute effect of moxonidine on insulin-dependent activation of Akt. Moxonidine treatment in vivo also facilitated the glucose uptake response of adipocytes to insulin, suggesting that facilitated Akt signaling may have consequences of insulin sensitivity in the whole organism, particularly in the extremely obese SHROB model where adipose tissue makes up a significant fraction of the body mass.

References


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