CHRONIC RENIN INHIBITION WITH ALISKIREN IMPROVES GLUCOSE TOLERANCE, INSULIN SENSITIVITY, AND SKELETAL MUSCLE GLUCOSE TRANSPORT ACTIVITY IN OBESE ZUCKER RATS

By

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Chronic renin inhibition with aliskiren improves glucose tolerance, insulin sensitivity, and skeletal muscle glucose transport activity in obese Zucker rats
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Abstract

We have demonstrated previously that overactivity of the renin-angiotensin system (RAS) is associated with whole-body and skeletal muscle insulin resistance in obese Zucker (fa/fa) rats, a model of the cardiometabolic syndrome. Moreover, this obesity-associated insulin resistance is reduced by treatment with angiotensin converting enzyme inhibitors or angiotensin receptor (type 1) blockers. However, it is currently unknown whether specific inhibition of renin itself, the rate-limiting step in RAS functionality, improves insulin action in obesity-associated insulin resistance. Therefore, the purpose of the present study was to assess the effect of chronic treatment with a selective renin inhibitor (aliskiren) on glucose tolerance, whole-body insulin sensitivity, and insulin action on the glucose transport system in skeletal muscle of obese Zucker rats. Female Obese Zucker rats were treated for 21 days with either vehicle or aliskiren (50 mg/kg body wt/day ip). This renin inhibition was associated with a significant lowering (10%, p<0.05) of resting systolic blood pressure and induced reductions in fasting plasma glucose (11%) and free fatty acids (46%) and in the HOMA-IR (13%). Glucose tolerance (glucose area under the curve) and whole-body insulin sensitivity (inverse of the glucose-insulin index) during an oral glucose tolerance test were improved by 15% and 16%, respectively, following chronic renin inhibition. Moreover, insulin-stimulated glucose transport activity in isolated soleus muscle of renin inhibitor-treated animals was increased by 36% and was associated with a 2.2-fold greater Akt ser473 phosphorylation, compared to vehicle-treated obese control animals. In summary, these data provide evidence that chronic selective inhibition of renin activity, the first step in the RAS, leads to improvements in glucose tolerance and whole-body insulin sensitivity in the obese Zucker rat, a model of obesity-associated insulin resistance. Importantly, chronic renin inhibition is associated with upregulation of insulin action on the skeletal muscle glucose transport system, likely due to improved Akt signaling. These data support the strategy of targeting the RAS to improve both blood pressure regulation and insulin action in conditions of insulin resistance.
**Introduction**

Insulin resistance of skeletal muscle glucose transport is a core defect in the metabolic syndrome, often accompanied by hyperinsulinemia, glucose intolerance, visceral obesity, dyslipidemia, essential hypertension, and inflammation (DeFronzo 1991, Henriksen 2007). Insulin is the primary regulator of glucose disposal, and skeletal muscle is the major tissue responsible for insulin-stimulated glucose uptake (Shepherd 1999). Insulin resistance occurs when normal insulin levels do not initiate the expected disposal of blood glucose by skeletal muscle. Insulin resistance increases the risk for the development of type 2 diabetes and cardiovascular disease (Reddy 2010). The prevalence of diabetes and obesity is increasing in the United States population. The Centers for Disease Control and Prevention estimate that in 2011, 25.8 million children and adults in the United States have diabetes, and an additional 79 million are in a prediabetic or insulin-resistant state (CDC 2011). Furthermore, it is estimated that 68% of the population in the United States is considered overweight or obese (BMI ≥ 25) (Flegal 2010). It is therefore critical to understand the etiology of skeletal muscle insulin resistance and to develop appropriate interventions to lessen the impact of diabetes and obesity on the population.

Hypertension is an independent risk factor for the development of insulin resistance. The renin-angiotensin system (RAS) is critical for normal blood pressure regulation. In this system, angiotensinogen is cleaved by the enzyme renin to form angiotensin I, which is further cleaved by angiotensin converting enzyme (ACE) into the potent vasoconstrictor, angiotensin II (Lavoie 2003, Henriksen 2007). Angiotensin II has been shown to negatively affect insulin action (Henriksen 2007). A proposed mechanism is through the production of reactive oxygen species (ROS) due to increased angiotensin II action, causing defects in the insulin signaling pathway (Manrique 2009). In this way, defects in the RAS can increase the risk of both hypertension and insulin resistance. In previous investigations, preventing the formation of angiotensin II by ACE inhibitors and angiotensin II receptor blockers (ARBs) has been shown to produce an insulin-sensitizing effect (Dietze 2008). ACE inhibitors and ARBs are commonly prescribed as antihypertensive agents, and these compounds have been used to demonstrate the critical role of the RAS in the regulation blood pressure and insulin action.

Aliskiren is a potent inhibitor of renin, an enzyme considered to be the rate-limiting step in the RAS (Habibi 2008). Previous studies involving renin inhibition have been performed in the insulin-resistant state, but none have involved a model of obesity. The obese Zucker rat is a model of obesity-associated insulin resistance. The purpose of the present study is to test the hypothesis that chronic (21 day) in vivo inhibition of renin by aliskiren will improve glucose tolerance, whole-body insulin action, and skeletal muscle glucose transport activity in obese Zucker rats.
Methods

Animals and Treatments

Female obese (fa/fa) Zucker rats (Harlan, Indianapolis, IN) were received at 7-8 weeks of age. Animals were housed in a temperature-controlled room (20-22 °C) at the Arizona Health Sciences Center Animal Care Facility of the University of Arizona with a 12:12 hour light/dark cycle (lights on from 7 AM to 7 PM). The animals had free access to chow (Purina, St Louis, MO) and water. All procedures were approved the University of Arizona Animal Use and Care Committee.

Starting at 8-9 weeks of age (weighing 270 to 300 g), animals were randomly assigned to one of two groups: a vehicle-treated control group or an aliskiren-treated group. Animals were weighed and received 50 mg/kg body weight of either vehicle (0.9% saline) or aliskiren by intraperitoneal (ip) injection for 21 consecutive days.

Blood Pressure and Heart Rate Measurements

Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate were measured non-invasively by tail cuff plethysmography (NIBP-8 Non-invasive Blood Pressure Monitor, Columbus Instruments, Columbus, OH). Baseline blood pressure measurements were obtained on the first day of dosing, and subsequently on days 7, 14, and 19. Prior to baseline blood pressure measurements, animals were acclimated to the plethysmography instrument.

Oral Glucose Tolerance Tests

All animals were food-restricted (4 g of chow given at 5 PM) the evening before the oral glucose tolerance test (OGTT). Between 8:00 and 10:00 AM on day 16 of the study, approximately 12 hours after the most recent treatment, rats were administered a 1 g/kg body weight glucose load by gavage. Blood was drawn from a cut at the tip of the tail before (time 0) and at 15, 30, 60, and 120 min after the glucose feeding. Whole blood was thoroughly mixed with EDTA (18 mM final concentration) and centrifuged at 13,000 X g to separate the plasma. Plasma samples were stored at -80 °C and subsequently analyzed for glucose (Fischer, Houston, TX), insulin (Linco, St. Charles, MO), and free fatty acids (Wako, Richmond, VA). Fasting whole-body insulin sensitivity was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) by using the formula: [fasting plasma glucose (mg/dl) x fasting plasma insulin (μU/ml)]/405 (Matthews et al. 1985). The glucose-insulin index is an inverse measure of whole-body insulin sensitivity during the OGTT and is defined as the product of the glucose area under the curve (AUC) and insulin AUC (Cortez 1991). Immediately after completion of the OGTT, all animals received 2.5 mL of sterile 0.9% saline subcutaneously to compensate for plasma loss. Vehicle or aliskiren treatments were recommenced the following day for five further days.

Glucose Transport Activity
On day 21, animals were food-restricted as described above before the OGTT. Between 8:00 and 10:00 AM, approximately 12 hours after the final chronic aliskiren treatment, animals were deeply anesthetized by ip injection of pentobarbital sodium (50 mg/kg body weight Nembutal, Abbott Laboratories, North Chicago, IL). Both soleus muscles were surgically removed and prepared for in vitro incubation. Muscles were split into 2 paired strips (~40 mg). The muscles were incubated for 1 hour at 37 °C in 3 mL oxygenated (95% O2-5% CO2) Krebs Henseleit buffer (KHB) containing 8 mM glucose, 32 mM mannitol, and 0.1% BSA (radioimmunoassay grade, Sigma Chemical). One soleus strip of the pair was incubated in the absence of insulin, and the other strip in the presence of insulin (5 mU/mL; Humulin R, Indianapolis, IN). Following the initial incubation, one set of soleus muscles were removed and prepared for use in the assay of signaling proteins. These strips were trimmed of connective tissue, quickly frozen in liquid nitrogen, weighed, and stored at -80 °C. The other set of soleus muscles were transferred for 10 minutes into 3 mL of an oxygenated 37 °C KHB rinse containing 40 mM mannitol, 0.1% BSA, and insulin, if present previously. Afterwards, the muscles were transferred into 2 mL oxygenated KHB containing 1mM 2-deoxy[1,2-3H]glucose (300 mCi/mmol; Sigma Chemical), 39 mM [U-14C]mannitol (0.8 mCi/mmol; ICN Radiochemicals, Irvine, CA), 0.1% BSA, and insulin, if present previously, for 20 minutes. Following this final incubation, the muscles were trimmed of connective tissue, frozen in liquid nitrogen, weighed, and dissolved in 0.5 mL of 0.5 N NaOH at 60 °C. After the muscle was completely dissolved, 5 mL of scintillation cocktail were added, and the intracellular accumulation of the glucose analog 2-DG was measured as described previously (Henriksen 1995, 2005). This method for assessing glucose transport activity in isolated muscle has been validated (Gulve 1993, Hansen 1994).

Functionality of Signaling Proteins

The frozen strips of soleus muscle (~40 mg) from each animal were homogenized in 8 vol of cold lysis buffer (50 mM HEPES,150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na3VO4, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM MgCl2, 1 mM CaCl2, 10 g/ml aprotinin, 10 g/ml leupeptin, 0.5 g/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride). Homogenates were incubated on ice and then centrifuged at 13,000 g at 4°C. Total protein assay was performed by BCA method (Sigma Chemical). Akt ser473 phosphorylation and GSK3β ser9 phosphorylation expression was determined by immunoblotting with commercially available antibodies, as described previously (Dokken 2005)

Statistical Analysis

All values are expressed as means ± SE. The significance of differences between vehicle-treated and aliskiren-treated groups was assessed by using an unpaired Student’s t-test. A level of p<0.05 was set for statistical significance.
Results

Body Weights

The initial body weights of the vehicle-treated animals (282 ± 4 g) was not different from that of the aliskiren-treated animals (288 ± 3). At several time points during the 21-day period of treatment, animals given aliskiren had a significantly (P<0.05) lower average body weight compared to vehicle-treated controls (Fig. 1). Moreover, the final body weight of the aliskiren-treated animals (337 ± 9.6) (P<0.05) was significantly less than that of the vehicle-treated animals (360 ± 4.6), indicating a potential role of renin inhibition in body weight regulation.

Systolic and Diastolic Blood Pressure

Baseline blood pressure measurements showed no significant differences between the non-hypertensive obese vehicle-treated and aliskiren-treated Zucker rat groups (Fig. 2). By the second week, the aliskiren-treated group showed a significant (P<0.05) lowering of SBP (10%) compared to the vehicle-treated group. There were no differences in DBP (Fig. 2) or heart rate (data not shown) between the two groups.

Plasma Glucose, Insulin, and FFAs

Aliskiren treatment induced a significant reduction (11%) in fasting plasma glucose compared to the vehicle-treated animals (Fig. 3). Chronic renin inhibition did not affect fasting plasma insulin. The homeostasis model assessment of insulin resistance (HOMA-IR), an index of fasting insulin resistance, was 13% less in the aliskiren-treated group than in the vehicle-treated group. In addition, chronic renin inhibition with aliskiren reduced fasting plasma FFA levels by 46% compared to control.

OGTT Responses

Chronic treatment with aliskiren led to a significantly lesser (11%, 10%, 19%, 15%, 13%) glucose response during the OGTT at the respective time points (0, 15, 30, 60, 120) compared to the vehicle-treated group (Fig. 4). The total integrated AUC for glucose was reduced by 15% in the aliskiren-treated group. In contrast, aliskiren treatment had no effect on the insulin response during the OGTT. Chronic renin inhibition significantly lowered the glucose-insulin index by 16% compared with control, indicating a significant enhancement of whole-body insulin sensitivity.

Glucose Transport Activity

Insulin-stimulated glucose transport activity was assessed in isolated soleus muscle strips to determine the cellular locus for the enhancement of insulin action (Fig. 5). Rates of 2-DG uptake in the absence of insulin were not significantly different between the aliskiren-treated and
vehicle-treated groups. Insulin-stimulated glucose transport activity was enhanced by 36% in skeletal muscle from the aliskiren-treated animals compared to controls.

**Insulin Signaling**

The functionality of critical distal elements of the insulin signaling pathway in isolated soleus muscle was determined (Fig. 6). Phosphorylation of Akt ser\(^{473}\) in the presence of insulin was significantly enhanced by 2.2-fold in the chronic aliskiren-treated group compared to the vehicle-treated group. However, ser\(^{9}\) phosphorylation of GSK-3β in the presence of insulin was not different between groups.
Discussion

In the present study, we made the novel observation that chronic renin inhibition with aliskiren leads to improvements in glucose tolerance and whole-body insulin sensitivity in the obese Zucker rat. The obese Zucker rat is a model of obesity-associated insulin resistance, a model reflective of human obesity and the associated insulin resistance. In these obese rats, treatment with aliskiren caused a significant decrease in blood pressure, which was associated with an increase in glucose tolerance and insulin sensitivity and with an increase in insulin action on skeletal muscle glucose transport activity. Whole-body insulin sensitivity, as assessed during the OGTT, was enhanced due to a significantly lesser glucose response in aliskiren-treated animals at all time points. This upregulation of insulin action on glucose transport activity in skeletal muscle of the aliskiren-treated animals can likely be attributed to an increase in insulin-stimulated phosphorylation of Akt. Interestingly, there was no enhancement of GSK-3β, a substrate of Akt. This would have the consequence of not modifying the inhibitory downstream effects of GSK-3 on glycogen metabolism and the negative feedback effect of this serine kinase on IR and IRS-1 functionality (Henriksen 2010).

In the kidneys, prorenin is transformed into renin and released into circulation. Renin is a peptidase and its action represents the rate-limiting step in the RAS cascade. Renin converts angiotensinogen into angiotensin I, which ACE then acts on to produce the biologically active nonapeptide angiotensin II. Renin is therefore required for production of angiotensin I and angiotensin II, which differentiates renin inhibition from ACE inhibition and ARB treatment (van den Meiracker 2007).

Inhibitors of downstream components of the RAS, such as ACE inhibitors and ARBs, are prescribed by physicians as the first recommended pharmaceutical therapy for hypertensive patients with type 2 diabetes, as these antihypertensive medications have insulin-sensitizing actions (Henriksen 2007, Ismail 2010). Our laboratory has previously worked with RAS inhibitors, including the ACE inhibitors captopril and trandolapril and the ARB irbesartan (Henriksen 1995, Steen 1999, Henriksen 2001). These investigations have demonstrated the negative effect angiotensin II has on insulin action and glucose disposal, as treatments with these RAS inhibitors increased insulin-stimulated glucose transport activity in skeletal muscle and whole-body insulin sensitivity (Henriksen 1995, Steen 1999, Henriksen 2001). Aliskiren is the first renin inhibitor to be approved by the US Food and Drug Administration for the treatment of hypertension (van den Meiracker 2007), and the present study demonstrates beneficial insulin-sensitizing effects that are similar to those of other types of RAS inhibitors.

Similar investigations have been performed using the TG(mREN2)27 rat, a non-obese, hypertensive rat model. The TG(mREN2)27 rat overexpresses the mouse Ren-2 renin transgene, leading to overactivity of the RAS and increased tissue levels of angiotensin II (Habibi 2008). The Ren2 rat exhibits hypertension, insulin resistance, and other damage characteristic of the metabolic syndrome (Henriksen 2007, Habibi 2008). Renin inhibition with aliskiren in these
animals leads to ameliorization of insulin resistance and increased insulin signaling in skeletal muscle and to improvements in organ structure (Habibi 2008).

The binding of angiotensin II to the angiotensin II type 1 receptor initiates vasoconstriction as well as other hypertensive effects including the release of ADH and aldosterone. Reacting with its receptor, angiotensin II generates reactive oxygen species by activation of NADPH oxidase, antagonizing skeletal muscle insulin signaling and glucose transport (Wei 2006, Lastra 2009). Further, bradykinin, a vasodilator and regulator of insulin action, can be degraded by ACE and lead to a lesser production of nitric oxide (Henriksen 2007). In these ways, angiotensin II contributes to the multifactorial etiology of skeletal muscle insulin resistance.

In conclusion, chronic treatment of the obese Zucker rat with the renin inhibitor aliskiren not only reduces blood pressure, but importantly also reduces whole-body and skeletal muscle insulin resistance, likely due to enhanced Akt signaling. The present study provides further support for targeting the RAS as a point of intervention to improve both blood pressure regulation and insulin action in conditions of obesity-associated insulin resistance.
References


Fig. 1. Effect of chronic aliskiren treatment on animal body weights. Values are means ± SE for 4-5 animals per group. *P<0.05, aliskiren vs. vehicle.
Fig. 2. Effect of chronic aliskiren treatment on systolic and diastolic blood pressure. Values are means ± SE for 4-5 animals per group. *P<0.05, aliskiren vs. vehicle.
Fig. 3. Effect of chronic aliskiren treatment on fasting plasma glucose, insulin, and FFAs. HOMA-IR is inversely related to fasting whole-body insulin sensitivity. Values are means ± SE for 4-5 animals per group. *P<0.05, aliskiren vs. vehicle.
Fig. 4. Effect of chronic aliskiren treatment on glucose tolerance and whole-body insulin sensitivity. Values are means ± SE for 4-5 animals per group. *P<0.05, aliskiren vs. vehicle.
Fig. 5. Effect of chronic aliskiren treatment on insulin-stimulated glucose transport activity in soleus muscle. Values are means ± SE for 4-5 animals per group. *P<0.05, aliskiren vs. vehicle.
Fig. 6. Effect of chronic aliskiren treatment on basal and insulin-stimulated ser473 phosphorylation of Akt. Values are means ± SE for 4-5 animals per group. *P<0.05, aliskiren vs. vehicle.
Fig. 7. Effect of chronic aliskiren treatment on basal and insulin-stimulated ser9 phosphorylation of GSK-3β. Values are means ± SE for 4-5 animals per group.