Low-Dose Spironolactone Reduces Reactive Oxygen Species Generation and Improves Insulin Stimulated Glucose Transport in Skeletal Muscle in the TG(mRen2)27 Rat

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Running title: Skeletal Muscle Glucose Transport and Oxidative Stress

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Abstract:

Renin-angiotensin-aldosterone system (RAAS) activation mediates increases in reactive oxygen species (ROS) and impaired insulin signaling. The transgenic Ren2 rat manifests increased tissue renin-angiotensin system activity, elevated serum aldosterone, hypertension, and insulin resistance. To explore the role of aldosterone in the pathogenesis of insulin resistance we investigated the impact of in vivo treatment of with a mineralocorticoid receptor (MR) antagonist on insulin sensitivity in Ren2 and aged-matched Sprague Dawley (SD) control rats. Both groups (age 6-8 wks) were implanted with subcutaneous time-release pellets containing spironolactone (0.24 mg/day) or placebo over 21 days. Systolic blood pressure (SBP) and intraperitoneal glucose tolerance test were determined. Soleus muscle IRS-1, tyrosine phosphorylated IRS-1, protein kinase B (Akt) phosphorylation, GLUT4 levels and insulin stimulated 2-deoxyglucose uptake were evaluated in relation to NADPH subunit expression/oxidase activity and ROS production (chemiluminescence and 4-HNE immunostaining). Along with increased soleus muscle NADPH oxidase activity and ROS, there was systemic insulin resistance, and reduced muscle IRS-1 tyrosine phosphorylation, Akt phosphorylation/activation, and GLUT4 expression in the Ren2 group (each p<0.05). Despite not decreasing blood pressure, low dose spironolactone treatment improved soleus muscle insulin signaling parameters and systemic insulin sensitivity in concert with reductions in NADPH oxidase subunit expression/activity and ROS production (each p<0.05). Our findings suggest that aldosterone contributes to insulin resistance in the transgenic Ren2, in part, by increasing NADPH oxidase activity in skeletal muscle tissue.
Key Words: Skeletal Muscle, Glucose Transport, Ren2 Rat, Reactive Oxygen Species, Mineralocorticoid Receptor Blockade

Introduction

The role of insulin resistance in the pathophysiology of type 2 diabetes mellitus (T2DM) and the metabolic syndrome are well established (35, 37), and exploration of the mechanisms leading to diminished insulin sensitivity is a field of active research. Activation of the tissue renin-angiotensin-system (RAS) has been linked to increased production of reactive oxygen species (ROS) through activation of the NADPH oxidase enzymatic complex in numerous tissues, including skeletal muscle (5, 35). Excessive oxidative stress may result in impairment of intracellular insulin signaling, constituting a potential pathway by which RAAS activation induces insulin resistance (5, 14, 31).

There is growing interest in the role of mineralocorticoids in the pathogenesis of insulin resistance. Mineralocorticoids participate in the regulation of blood pressure, water, sodium and potassium homeostasis through interaction with the mineralocorticoid receptor (MR) located in target epithelial cells, translocation to the nucleus, and triggering of specific genomic actions (10). However, mineralocorticoids also exert acute actions in multiple non-epithelial tissues, which appear to be independent of gene transcription (12). These non-genomic actions are particularly relevant in cardiovascular disease (CVD), as they can result in hypertrophy, endothelial dysfunction (4, 17), inflammation (30), fibrosis, apoptosis and cardiovascular remodeling (27). Recently, aldosterone has been reported to suppress insulin metabolic signaling in vascular smooth muscle cells via an oxidative stress mediated mechanism (14). This raises the possibility
that aldosterone, like angiotensin II (Ang II) can suppress insulin mediated glucose uptake in various tissues (5, 35).

Available studies in humans have shown, independent of other components of CMS, an association between increased plasma levels of aldosterone and presence of insulin resistance in the CMS (6, 18). In addition, aldosterone excess in patients with primary aldosteronism is related to impaired glucose homeostasis (11) as well as insulin resistance (9). Likewise, euglycemic hyperinsulinemia results in increased aldosterone production in response to Ang II in an animal model (25), and in healthy humans (32). These detrimental effects have been related to proinflammatory effects exerted by mineralocorticoids (18). However, the impact of signaling through the MR on glucose metabolism in *ex vivo* skeletal muscle has not been investigated.

The transgenic TG(mRen2)27 rat (Ren2), which harbors the mouse renin gene, is an experimental model of excessive tissue local renin-angiotensin system (RAS) activity, which through paracrine adrenal effects leads to increased plasma deoxycorticosterone (DOC), 18-hydroxycorticosterone and aldosterone levels, as well as whole body and skeletal muscle insulin resistance (5, 24, 26, 29). Previous studies from our laboratory have demonstrated that AT$_1$R blockade, and ROS scavenging improves whole body glucose tolerance and skeletal muscle insulin-stimulated glucose transport (5, 35). Since mineralocorticoids promote oxidative stress in cardiovascular tissue (5), we sought to investigate the impact of in vivo MR blockade with low dose spironolactone (SP) on systemic insulin sensitivity, parameters of skeletal muscle insulin metabolic signaling and insulin-stimulated glucose uptake in relation to NADPH oxidase activity/ROS in young insulin resistant Ren2 rats.
Methods

Animals and treatments:

All animal procedures were approved by the University of Missouri animal care and use committees and housed in accordance with NIH guidelines. Ren2 (6-7 weeks of age) and age matched SD littermates were randomly assigned to untreated (Ren2-C and SD-C, respectively), or spironolactone treated (Ren2-Sp and SD-Sp) paradigms. Ren2-Sp and SD-Sp animals were implanted with a 5mg timed release spironolactone or placebo pellet subcutaneously (Innovative Research of America Sarasota, Florida) for 21 days. Pellets were placed, under anesthesia, via a super scapular incision closed with permanent suture.

Systolic Blood Pressure (SBP) and Body Weight:

Restraint conditioning was initiated before blood pressure measurements. SBP was measured in triplicate, on separate occasions throughout the day, using the tail-cuff method (Harvard Systems, Student Oscillometric Recorder) prior to initiation of treatment and on days 19 or 20 prior to sacrifice at 21 days (34, 35). Total body weight was obtained prior to initiation of treatment and at time of sacrifice.

Intraperitoneal glucose tolerance test (IPGTT)

Animals were fasted overnight before the experiment. On day 21, the rats were weighed and anesthetized with nembutal (35 mg/kg intraperitoneally). The femoral artery was carefully dissected and canulated with a 27-gauge angiocath. A 200 µl blood sample was drawn for insulin and glucose measurements. A dose of dextrose (50% solution, 1 g/kg body wt) was injected intraperitoneally, and blood was drawn at 15, 30, 45 and 60
min for insulin and glucose determination. Serum glucose concentrations were determined by means of the glucose oxidase method, and serum was separated analyzed for insulin (ELISA kit; Linco, St. Charles, MO). Insulin Resistance Index was calculated as the product of areas under the glucose and insulin curves \((\text{AUC}_{\text{Glucose}} \times \text{AUC}_{\text{Insulin}})\) as previously described (13).

Oxidative stress:

**NADPH Oxidase Activity:** NADPH oxidase activity was determined in plasma membrane fractions as previously described (5, 33, 34, 35). Aliquots of soleus muscle membrane and cytosolic fractions (12.5-100 mg proteins) were incubated with NADPH (100mM) at 37° C. NADPH oxidase activity was determined by measuring the conversion of Radical Detector (Cayman Chemical) in the absence and presence of NADPH inhibitor diphenylene iodonium sulfate (dpi) (500µM) using spectrophotometric (450nm) techniques.

**NADPH Oxidase subunit immunostaining:** Harvested soleus muscle tissue was immersed and fixed in 3% paraformaldehyde and prepared as previously described (36). Blocks were sectioned and incubated with 1:100 dilution of primary antibodies in 10 fold diluted blocking agent, and third/fourth sections were washed and kept in the blocker. Over the course of 48 hours, a fifth, sixth, and seventh section was incubated with 1:100 goat gp91\textsuperscript{phox} (NOX2) (Santa Cruz), 1:100 goat p22\textsuperscript{phox}, and 1:100 and p47\textsuperscript{phox} antibody (Upstate Cell Signaling) respectively, in 10 fold diluted blocker. Other sections were incubated with 1:300 Alexa-fluor rabbit anti-goat 647 (Molecular Probe, Eugene, OR) for 4 hours and examined using a laser confocal scanning microscope, images captured by
using Laser-sharp software (Bio-Rad), and signal intensities measured with MetaVue software.

**4-HNE immunostaining:** Anti-4-HNE antibody was used to detect lipid peroxidation as a marker of ROS generation. Cryostat sections (5µm) of the soleus muscle samples were microwaved for antigen retrieval. The sections were incubated overnight at 4ºC with anti-4-HNE antibody. After washing, secondary antibody conjugated with Alex568 (Molecular Probes) was applied. The images were acquired with a Laser-Scanning Confocal Microscope (Olympus IX70). Three sections were examined for each animal studied, 4 animals in each group.

**Reactive Oxygen Species formation:** Levels of reactive oxygen species in skeletal muscle using isolated soleus tissue sections were measured by chemiluminescence. Tissue sections were homogenized in sucrose buffer (250 mM sucrose, 0.5 mM EDTA, 50 mM HEPES, protease inhibitor tablet, pH 7.5) using a glass/glass homogenizer. Homogenates were centrifuged 1500 rcf x 10 min at 4ºC. Supernatants (whole homogenate) were then removed and placed on ice. Whole homogenate (100 uL) was added to 1.4 mL of 50 mM phosphate (KH₂PO₄) buffer (150 mM sucrose, 1mM EGTA, 5 uM lucigenin, 100 uM NADPH, pH 7.0) in dark-adapt counting vials. After dark adaptation for 1 hour, samples were counted every 30 seconds for 10 min on a scintillation counter and the last 5 minutes were averaged. Samples were then normalized to total protein in the whole homogenate. Values are expressed as counts per minute per milligram of protein (cpm/mg).

**Glucose transport:**
2-deoxyglucose transport was measured in isolated soleus strips incubated in the presence or absence of a maximally effective dose of insulin (100 nM) as previously described (5, 29). Soleus muscles were dissected out of anesthetized rats, which had been fasted overnight. The soleus muscle was carefully dissected into longitudinal strips (<40 mg) and incubated for 45 min in 4 mL of pre-incubation buffer that had been brought to 37° C and aerated with 95% O_2-5% CO_2 gas. Pre-incubation buffer consisted of 8 mM glucose, 32 mM mannitol, and 0.1% BSA in modified Kreb’s-Hensleit Buffer (KHB). After additional incubation for 15 min with or without insulin, muscles were transferred to a rinse buffer (40 mM mannitol, 0.1% BSA, in KHB) for 10 min. Thereafter, individual strips were transferred to flasks containing oxygenated incubation buffer (1 mM [^3H]-2-Deoxy-Glucose, 39 mM [^14C]-Mannitol, and 0.1% BSA in KHB) with or without insulin for 20 min. Muscle strips were then removed, trimmed of excess tendon, blotted and snap frozen in liquid nitrogen using aluminum tongs. Weighed samples were placed in 1 mL of 0.1 N NaOH solution, and 1 ml of 0.1 N HCl was added to neutralize the samples before the addition of 15 mL of ScintiVerse SX18-4 (Fisher). Samples were analyzed with a Beckman scintillation counter set for dual channel detection (^3H and ^14C).

Quantification of IRS, Akt, and GLUT4 in Soleus Muscle:

After sacrificing the animals, a strip of soleus muscle was fixed in 3% paraformaldehyde and processed for immunostaining as previously described (33). Then the 4 µm sections were incubated with rabbit anti IRS1 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), anti pIRS1 Tyr 941 1:50 (phosphatidylinositol 3-kinase binding site) (Upstate Biotechnology, Inc., Lake Placid, NY), anti total Akt 1:75 (Cell Signaling
Technology, Charlottesville, VA), anti Akt (Ser\textsuperscript{473}) 1:75 (Cell Signaling Technology) and mouse anti GLUT4 1:100 (Santa Cruz Biotechnology) overnight. Then the sections were washed and incubated with 1:300 Alexa flour donkey anti rabbit 647, except for GLUT4 on which donkey anti mouse was used. After 4 hours the sections were mounted with mowiol, the images were captured and the signals were analyzed as previously described (33).

Statistics:

All results are presented as means ± standard error. Analysis of variance (ANOVA) with Fisher’s Least Significant Differences (LSD) and Dunnett’s multiple post-hoc testing were performed and unpaired t test, as appropriate.

Results

Systolic Blood Pressure (SBP):

There were no significant differences among the study groups with respect to age or weight. At initiation of treatment (6-7 weeks of age), SBP were higher in Ren2-C (145.8±1.7 mmHg) compared with SD controls (129.8±1.1 mmHg, p <0.05) (Fig 1). At the end of the treatment period (9 to 10 wks of age) there was a significant increase in SBP in Ren2-C (192.5±1.2 mmHg) compared to SD-C (142.2±3.3 mmHg, p<0.05). No reduction in SBP was observed with spironolactone treatment in the Ren2-Sp group (194.3±7.0 mmHg, p>0.05).

Whole body insulin sensitivity studies:

Insulin sensitivity was analyzed during an IPGTT, in which both serum insulin and glucose concentrations were determined and their respective AUC were calculated.
Insulin Resistance index ($\text{AUC}_{\text{Glucose}} \times \text{AUC}_{\text{Insulin}}$) analysis demonstrated significantly higher insulin resistance in Ren2 control animals compared to SD controls ($92.2 \pm 22.2 \times 10^3$ versus $27.1 \pm 14.7 \times 10^3$ arbitrary units, $p<0.05$) (Fig 2). In vivo treatment with low dose spironolactone significantly improved whole-body insulin sensitivity in Ren2 rats ($p<0.05$). Indeed, the insulin resistance index in treated Ren2 animals treated with low dose spironolactone was not different from SD controls ($27.1 \pm 14.7 \times 10^3$ versus $33.4 \pm 78.5 \times 10^3$ arbitrary units, $p>0.05$).

Soleus Muscle NADPH oxidase and Oxidative stress:

NADPH oxidase activity was elevated in Ren2 ($6.32 \pm 0.22$ mOD/mg/min) compared to SD controls ($3.84 \pm 0.24$ mOD/mg/min, $p<0.05$), and was normalized in the Ren2-Sp group ($3.98 \pm 0.17$, $p<0.05$) (Fig 3A) to a value comparable to SD-C. There were similar trends in NADPH oxidase subunits NOX2, $p22^{phox}$, and $p47^{phox}$ with increases in the Ren2 control ($56 \pm 4.8$, $44.9 \pm 3.0$, and $75.8 \pm 4.5$ average grey scale intensities, respectively) when compared to SD-C ($44.0 \pm 3.0$, $33.6 \pm 2.0$, and $55.2 \pm 2.8$ average grey scale intensities, respectively; $p<0.05$) (Fig 3B and C). There were improvements in the treated Ren2-Sp ($44.1 \pm 1.0$, $30.2 \pm 1.8$, and $52.5 \pm 3.6$ average grey scale intensities, respectively; $p<0.05$).

Soleus tissue ROS levels were higher in Ren2 controls ($1805 \pm 343$ cpm/mg) compared to the placebo treated SD group ($701 \pm 175$ cpm/mg, $p<0.05$). There was a similar trend to lower ROS activity observed in the Ren2-Sp treated rat muscles ($1099 \pm 320$ cpm/mg, $p>0.05$) (Fig 3D). 4-HNE immunostaining, a surrogate for ROS-mediated membrane lipid peroxidation, was increased in soleus muscle of Ren2-C ($240.4 \pm 16.0$ average grey scale intensities) compared to SD-C ($100.0 \pm 22.0$ average grey
scale intensities, p<0.05), and improved with low dose spironolactone treatment (126.9±25.0 average grey scale intensities, p<0.05) (Fig 3E).

**Insulin-stimulated glucose uptake measurements:**

Skeletal muscle 2-DOG uptake response to insulin was measured in the absence and presence of maximally effective doses of insulin (100 nM) (Fig 4). There was no significant difference (p>0.05) in basal (not insulin-stimulated) glucose 2-DOG uptake in SD and treated or untreated Ren2 animals. With the addition of insulin, 2-DOG uptake was non-significantly greater in SD (0.78±0.1 mmol/mg) compared to skeletal muscle from untreated Ren2 rats (0.62±0.03 mmol/mg, p>0.05). However, in vivo low dose spironolactone treatment significantly improved insulin-stimulated glucose uptake when compared with placebo treated Ren2 animals (0.89±0.1 mmol/mg, p<0.05).

Insulin-stimulated increment (delta) in glucose uptake in untreated Ren2 was non-significantly lower compared to untreated SD animals (delta: 0.42±0.1 mmol/mg muscle/20 min versus 0.20±0.1 mmol/mg/20 min muscle, p=0.06). Treatment with spironolactone in Ren2 rats increased insulin-stimulated glucose uptake (delta: 0.47±0.1 mmol/mg muscle/20 min, p<0.05) compared to untreated Ren2.

**IRS, Akt, and GLUT4 immunostaining:**

Consistent with glucose uptake studies there were decreases in IRS-1, Akt, and GLUT4 immunostaining in the soleus muscle of Ren2 rats, which improved significantly with low dose spironolactone treatment. Total and Tyrosine (Tyr)$^{941}$ phosphorylated IRS-1 were reduced in the Ren2-C (28.0±2.9 and 24.3±2.9 average grey scale intensities, respectively) compared to SD-C (39.6±5.5 and 44.5±3.9 average grey scale intensities, respectively; p<0.05) (Fig 5A). There were improvements in total and TryIRS-1 in the
Ren2-Sp (44.2±3.6 and 50.7±2.5 average grey scale intensities, respectively; p<0.05). Similarly, there were reductions in total and Serine (Ser)\textsuperscript{473} phosphorylated Akt (34.0±5.6 and 24.9±2.2 average grey scale intensities, respectively) compared to SD-C (54.7±11.7 and 32.0±1.9 average grey scale intensities, respectively; p<0.05). Following spironolactone treatment Akt Ser\textsuperscript{473} phosphorylation was increased, compared to placebo treatment, in the Ren2-Sp (70.6±12.3 and 34.1±1.5 average grey scale intensities, respectively; p<0.05) (Fig 5B). There were reductions in skeletal muscle GLUT4 in the Ren2-C (34.6±4.5 average grey scale intensities) compared to SD-C (70.8±6.4 average grey scale intensities, p<0.05), and GLUT 4 levels increased in the Ren2-Sp (96.9±13.3 average grey scale intensities, p<0.05) (Fig 5C).

**Discussion**

The present investigation explored the effect of a very low, non-blood pressure lowering, dose of the MR blocker spironolactone on oxidative stress and insulin-stimulated glucose transport in skeletal muscle from a rodent model of increased tissue RAS activation and elevated aldosterone levels. In vivo treatment of young Ren2 rats with low dose of spironolactone for 3 weeks improved systemic insulin sensitivity reduced soleus muscle NADPH oxidase activity/ROS production and improved skeletal muscle parameters of insulin metabolic signaling, as well as insulin-stimulated glucose transport. That these beneficial effects were independent of changes in systolic blood pressure suggest direct MR mediated effects on skeletal tissue. Thus, this is the first study that demonstrates that MR antagonism directly affects skeletal muscle insulin metabolic signaling in conjunction with reductions in oxidative stress.
As previously observed (5), oxidative stress as measured by skeletal muscle NADPH oxidase activity, ROS production, and membrane lipid peroxidation, were increased in Ren2 compared to control SD rats. These MR mediated effects on skeletal muscle oxidative stress and insulin metabolic signaling are similar to previously observed effects of AT₁R activation (5, 35). Our laboratory has previously observed that in vivo treatment of young Ren2 rats with a ROS scavenger or AT₁R blocker results in decreases in skeletal muscle NADPH oxidase activity and ROS generation, which was associated with improved systemic and skeletal muscle insulin sensitivity (5, 35). Collectively, these observations suggest that both Ang II and aldosterone reduce skeletal muscle insulin metabolic signaling, in part, through increases in oxidative stress.

NADPH oxidase is a highly regulated membrane-bound enzyme complex that catalyzes the production of superoxide anion ($O_2^-$). Complex components include membrane-bound subunits p22$^{phox}$ and Nox2, as well as cytosolic regulatory subunits p47$^{phox}$, p67$^{phox}$, p40$^{phox}$ and the small GTP-binding protein Rac1/Rac2 all of which are expressed in skeletal muscle tissue (5, 15, 35). Activation of the complex involves the interaction between cytosolic subunits p47$^{phox}$ and p67$^{phox}$, followed by their translocation to the plasma membrane, along with C-terminal-prenylated Rac1 (28), where they interact with plasma membrane-bound subunits (2). There is an emerging body of evidence demonstrating that mineralocorticoids, like Ang II, may activate NADPH oxidase in various tissues (14, 16, 20). Results from the current investigation indicate that MR activation increases skeletal muscle NADPH oxidase activity, in part via activation of membrane-bound Nox2 and p22phox, as well as the cytosolic p47phox subunits. The resulting generation of ROS appears to be associated with reduced IRS-1 levels, reduced
Tyr phosphorylation of IRS-1 and Akt phosphorylation/activation as well as insulin stimulated glucose transport.

The results emanating from this investigation complement observational studies in humans demonstrating an association between increased levels of aldosterone, impaired glucose homeostasis, and insulin resistance. For example, patients with primary aldosteronism, either tumor-induced or idiopathic, manifest a greater insulin response to an oral glucose load as well as reduced systemic insulin sensitivity, compared to age, gender and body mass index-matched normotensive patients (9). Surgical removal of aldosterone producing adenoma or medical therapy with spironolactone restored insulin sensitivity, further supporting a direct role of aldosterone in mediating systemic insulin resistance.

There are several potential mechanisms by which MR activation may reduce insulin metabolic signaling in skeletal muscle tissue. One potential mechanism for aldosterone-induced insulin resistance appears to be the transcriptional down regulation of the insulin receptor, in addition to impairment of the intracellular insulin signaling (8). Glucocorticoid response elements (GRE) have been identified in the promoter of the insulin receptor gene (19) and interactions between activated MR and GRE may result in a negative transcriptional effect of the aldosterone over the insulin receptor gene (8). Aldosterone also activates glucocorticoid receptors in extra-renal tissue (38), which has direct negative effects on insulin metabolic signaling (23). A recent report suggests that aldosterone suppresses insulin metabolic signaling via proteosomal degradation of the IRS-1 docking protein in vascular smooth muscle cells (14). Commensurate with our results, in vitro treatment with a MR antagonist abolished the reduction in IRS-1
expression, Akt phosphorylation, and glucose transport in vascular smooth muscle cells. It was also observed that treatment with antioxidants such as N-acetylcysteine or an inhibitor of the ubiquitin proteosomal pathway prevented aldosterone induced reductions of IRS-1(14). Reductions in Try phosphorylated IRS-1, as observed in the current investigation, would, in turn, result in less engagement of IRS-1 docking protein with phosphoinositol kinase (PI3-K) and decreased downstream Akt phosphorylation/activation as was observed in this study. Collectively, these observations suggest that aldosterone, likely acts through MR mediated stimulation of redox sensitive serine kinase signaling pathways (3, 14) to promote proteosomal degradation of IRS-1 with consequent reductions in insulin metabolic signaling through the PI3-K/Akt and GLUT4 facilitated glucose uptake.

A limitation of our study is the absence of measurements of plasma spironolactone concentrations, which would have assessed the absorption from the pellets. However, use of spironolactone pellets is supported in the available literature, and it is estimated that the absorption is adequate to elicit biological activity (1, 7). Also, previous studies in our laboratory have demonstrated that in cultured myocytes (L6 cells) Ang II is linked to increased activity of NADPH oxidase, production of ROS as well as impairment of intracellular insulin signaling mediated through IRS-1 and Akt pathways (35), which were reversed by blocking AT1R and ROS scavenging. However, this study did not specifically include measurements of mineralocorticoids, and thus we cannot conclude about the impact of these strategies on mineralocorticoid activity in cultured myocytes.
Our data support the hypothesis that MR antagonism can reduce oxidative stress and improve insulin sensitivity in a rodent model of activated RAAS activity and insulin resistance. These effects were obtained in skeletal muscle, a classical target tissue of insulin action using low-dose SP, and were independent of changes in systolic blood pressure. These data add to previous observations that increased oxidative stress leads to impaired insulin sensitivity in skeletal muscle, and that RAAS interruption using AT_1R blockade and/or Ang converting enzyme inhibition attenuates these changes (5, 35). The specific mechanisms of the beneficial effects of MR blockade remain to be fully elucidated, and potentially include local actions in skeletal muscle tissue in addition to reduced NADPH oxidase-mediated oxidative stress. Further, in addition to direct effects on skeletal muscle tissue, systemic MR antagonism and consequent effects on other mineralocorticoid-target tissues implicated in energy homeostasis, such as adipose and brain tissues may be important in mediating improvements in systemic insulin sensitivity.

Interruption of MR signaling is particularly attractive, as increased activation of these receptors is linked to multiple pathologic mechanisms that lead to CVD, including hypertension, oxidative stress, inflammation, apoptosis and fibrosis in cardiovascular and renal tissue. Moreover, MR antagonism has provided solid benefits in terms of cardiovascular morbidity and mortality, as demonstrated in the RALES and EPHESUS trials (21, 22). A better understanding of the mechanisms underlying the participation of MR activation in oxidative stress and insulin resistance could thus identify new therapeutic targets and potentially reduce the disease burden imposed by insulin resistance which frequently accompanies hypertension (37).
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Figure Legends:

Figure 1. Low-dose Spironolactone does not reduce Systolic Blood Pressure (SBP) in the Ren2. SBP were measured prior to starting the experimental protocol and at days 19-20 prior to sacrifice (day 21). Sprague Dawley control (SD-C, n=6), Sprague Dawley treated with spironolactone (SD-SP, n=4), Ren2 control (Ren2-C, n=5), Ren2 treated with spironolactone (Ren2-Sp, n=5). Values are presented as means ± standard error. *, p<0.05 when compared to SD-C; #, p>0.05 when compared to Ren2-C.

Figure 2: Low-dose Spironolactone improves Insulin Resistance in the Ren2. Insulin sensitivity measured during an intraperitoneal glucose tolerance test (IPGTT) performed after overnight fast on day 21. Samples for serum insulin (panel A) and glucose (panel B) were obtained at 0, 15, 30, 45 and 60 minutes after administering 50% dextrose 1 g/Kg intraperitoneally. Areas under the Curve (AUC) were calculated for insulin and glucose concentrations, and the Insulin Resistance Index (panel C) was calculated as the
product of the AUC for glucose and insulin. Sprague Dawley control (SD-C, n=4), Sprague Dawley treated with spironolactone (SD-Sp, n=4), Ren2 control (Ren2-C, n=7), Ren2 treated with spironolactone (Ren2-SP, n=4). Values presented as means ± standard error. **, p<0.05 when compared to Ren2-C.

Figure 3: Low-dose Spironolactone improves measures of Oxidative Stress in Ren2.
A) NADPH oxidase activity. B and C) NADPH oxidase subunits. D) ROS formation by chemiluminescence. E) 4-hydroxy-2-nonenal (4-HNE) immunostaining was used to detect lipid peroxidation as a marker of reactive oxygen species (ROS). Sprague Dawley control (SD-C; n=6 for NADPH oxidase activity, NADPH oxidase subunits and ROS, n=4 for 4-HNE), Sprague Dawley treated with spironolactone (SD-Sp; n=6 for NADPH oxidase activity, NADPH oxidase subunits and ROS, n=4 for 4-HNE), Ren2 control (Ren2-C; n=5 for NADPH oxidase, NADPH oxidase subunits and ROS, n=4 for 4-HNE), and Ren2 treated with spironolactone (Ren2-Sp; n=5 for NADPH oxidase, NADPH oxidase subunits and ROS, n=4 for 4-HNE). Values presented as means ± standard error. *, p<0.01 when compared to SD-C; **, p<0.05 when compared to Ren2-C. Scale bar = 50µm.

Figure 4. Low-dose Spironolactone improves Glucose Transport in the Ren2. 2-deoxyglucose uptake analyzed in ex vivo soleus muscle strips in the absence and presence of a maximally effective dose of insulin. Sprague Dawley control (SD-C, n=6), Sprague Dawley treated with spironolactone (SD-Sp, n=4), Ren2 control (Ren2-C, n=5)
and Ren2 treated with spironolactone (Ren2-Sp, n=5). Values are expressed as means ±
standard error. *, p<0.05 when compared to SD-C; **, p<0.05 when compared to Ren2-C.

Figure 5: Low-dose Spironolactone improves IRS, GLUT4, and Akt in the Ren2. A) Representative fluorescent images of total IRS-1 and Tyrosine (Tyr)$^{941}$ phosphorylated (PO$_4$) IRS-1 and quantification of converted signal intensities in average gray scale intensities to the right. B) Representative fluorescent images total and Serine (Ser)$^{473}$ phosphorylated (PO$_4$) Akt and quantification of converted signal intensities in average gray scale intensities to the right. C) Representative fluorescent images of GLUT4 and quantification of converted signal intensities in average gray scale intensities to the right. Sprague Dawley control (SD-C, n=6), Sprague Dawley treated with spironolactone (SD-Sp, n=4), Ren2 control (Ren2-C, n=5) and Ren2 treated with spironolactone (Ren2-Sp, n=5). *, p<0.05 when compared to SD-C; **, p<0.05 when compared to Ren2-C. Scale bar = 50µm.

References


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Figure 2

A

B

C

Insulin (mU/mL)

Glucose (mg/dL)

Insulin Resistance Index (Arbitrary Units)

Time (min)

Time (min)

SD-C

SD-Sp

Ren2-C

Ren2-Sp
**Figure 3**

**A:**
- NADPH oxidase activity (nmol/min/mg)
- Comparison of SD-C, SD-Sp, Ren2-C, and Ren2-Sp

**B:**
- Average grey scale intensities
- Comparison of SD-C, SD-Sp, Ren2-C, and Ren2-Sp

**C:**
- Images showing NOX2 expression in SD-C, SD-Sp, Ren2-C, and Ren2-Sp

**D:**
- ROS (cpm/mg)
- Comparison of SD-C, SD-Sp, Ren2-C, and Ren2-Sp

**E:**
- Images showing p22phox and p47phox expression in SD-C and SD-Sp
Figure 4
Figure 5

A: SD-C SD-Sp Ren2-C Ren2-Sp
IRS-1
PO4 IRS-1 (Tyr^941)

B: SD-C SD-Sp Ren2-C Ren2-Sp
Akt
PO4 Akt (Ser^473)

C: SD-C SD-Sp Ren2-C Ren2-Sp
GLUT4