Aldosterone Induces Vascular Insulin Resistance by Increasing Insulin-Like Growth Factor-1 Receptor and Hybrid Receptor

Shamshad J. Sherajee, Yoshiko Fujita, Kazi Rafiq, Daisuke Nakano, Hirohito Mori, Tsutomu Masaki, Taiga Hara, Masakazu Kohno, Akira Nishiyama, Hirofumi Hitomi

Objective—We previously showed that aldosterone induces insulin resistance in rat vascular smooth muscle cells (VSMCs). Because insulin-like growth factor-1 receptor (IGF1R) affects insulin signaling, we hypothesized that aldosterone induces vascular insulin resistance and remodeling via upregulation of IGF1R and its hybrid insulin/insulin-like growth factor-1 receptor.

Methods and Results—Hybrid receptor expression was measured by immunoprecipitation. Hypertrophy of VSMCs was evaluated by 3H-labeled leucine incorporation. Aldosterone (10 nmol/L) significantly increased protein and mRNA expression of IGF1R and hybrid receptor in VSMCs but did not affect insulin receptor expression. Mineralocorticoid receptor blockade with eplerenone inhibited aldosterone-induced increases in IGF1R and hybrid receptor. Aldosterone augmented insulin (100 nmol/L)-induced extracellular signal-regulated kinase 1/2 phosphorylation. Insulin-induced leucine incorporation and α-smooth muscle actin expression were also augmented by aldosterone in VSMCs. These aldosterone-induced changes were significantly attenuated by eplerenone or picropodophyllin, an IGF1R inhibitor. Chronic infusion of aldosterone (0.75 µg/hour) increased blood pressure and aggravated glucose metabolism in rats. Expression of hybrid receptor, azan-positive area, and oxidative stress in aorta was increased in aldosterone-infused rats. Spironolactone and tempol prevented these aldosterone-induced changes.

Conclusion—Aldosterone induces vascular remodeling through IGF1R- and hybrid receptor–dependent vascular insulin resistance. Mineralocorticoid receptor blockade may attenuate angiopathy in hypertensive patients with hyperinsulinemia. (Arterioscler Thromb Vasc Biol. 2012;32:257-263.)

Key Words: insulin resistance ■ reactive oxygen species ■ receptors ■ signal transduction ■ aldosterone

Insulin resistance is a major attribute of type 2 diabetes mellitus and metabolic syndrome.1 Cardiovascular complications are often seen in these patients, and vascular insulin resistance is considered to be involved in proatherogenic changes and subsequent cardiovascular events.2 There is growing interest in the role of aldosterone and its receptor, mineralocorticoid receptor (MR), in the pathogenesis of insulin resistance.3 For instance, clinical studies have shown that patients with primary aldosteronism exhibit impaired glucose tolerance.4 Some possible mechanisms of insulin resistance induced by aldosterone have been considered, such as a low serum potassium concentration, and direct effects of aldosterone on insulin signaling.5,6 Previously, we have demonstrated that aldosterone induces insulin resistance in rat vascular smooth muscle cells (VSMCs) via the downregulation of insulin receptor substrate-1, a key molecule of insulin signaling pathway.7 Our data also clearly showed that aldosterone attenuates glucose metabolism in VSMCs. However, the precise molecular mechanisms responsible for aldosterone-induced VSMC insulin resistance and proatherogenic changes have not been identified.

Insulin induces various actions, such as glucose metabolism and normal cell physiology, by binding to the insulin receptor (IR).8 The VSMCs express not only IR but also insulin-like growth factor-1 receptor (IGF1R).8 Compared with IR, IGF1R is more abundant in VSMCs, and expression of IGF1R is increased in aortas of diabetic animals.9 Furthermore, subunits of IR and IGF1R easily form hybrid receptors, depending on the ratio of these receptors’ expressions.10 These hybrid receptors function more like IGF1R in that they have higher affinity for insulin-like growth factor-1 (IGF1) than to insulin.11,12 IGF1 induces hypertrophic changes and insulin resistance via IGF1R in vasculature. Although the affinity of IGF1R for insulin is very low compared with IGF1, high concentrations of insulin, which are often seen in patients with insulin resistance, may affect intracellular
VSMCs were incubated with the indicated concentrations of aldosterone for 48 hours.

Western Blot Analysis and Immunoprecipitation
Protein samples from VSMCs and aorta were extracted, as previously described.14,15 Hybrid receptor expression was determined by coimmunoprecipitation and Western blotting. Protein samples from VSMCs or aorta (600 μg of protein) were immunoprecipitated by overnight incubation with anti-IRβ-subunit antibody, followed by immunoblot analysis with an antibody against IGF1Rβ-subunit.8,9

Real-Time Reverse Transcription-Polymerase Chain Reaction
The mRNA expressions of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), type I and type III collagen, serum and glucocorticoid-regulated kinase 1 (Sgk1), and IGF1R were analyzed by real-time polymerase chain reaction, as described previously.16–18 Primer sequences for GAPDH, Sgk1, type I and type III collagen, and IGF1R were described previously.16,17 All data were normalized against GAPDH mRNA expression.

[3H]Leucine Incorporation and α-Smooth Muscle Actin Expression
Protein synthesis was evaluated by measurement of [3H]leucine incorporation.19 Expression of α-smooth muscle actin was determined using immunofluorescence, as previously reported.16

Oral Glucose Tolerance Test
Glucose metabolism was evaluated by oral glucose tolerance test, as previously reported.20

Histological Examination
Aortic fibrosis was evaluated by the percentage of azan-positive area in 5 randomly selected microscope fields (×200) in each section. Medial thickness and lumen ratio were measured using a previously described method.21

Statistical Analysis
Results are expressed as mean±SE. Statistical significance was assessed using ANOVA and the Bonferroni post hoc test. Student t tests were performed to compare the means when the experimental design was comprised of 2 individual groups. A value of P<0.05 was considered statistically significant.

Results
In Vitro Study
Effects of Aldosterone on IGF1R, IR, and Hybrid Receptor Expressions in VSMCs
Aldosterone increased IGF1R protein expression in a dose-dependent manner, with a significant increase at 10 nmol/L (Figure 1A), whereas IR protein expression was not affected (Supplemental Figure IA and IB). Aldosterone (10 nmol/L) induced a time-dependent increase in IGF1R protein expression, signaling pathway via IGF1R or hybrid receptors.13 However, the roles of IGF1R and hybrid receptors in insulin resistance are not well understood.

In the present study, we hypothesized that aldosterone induces insulin resistance via upregulation of IGF1R and its hybrid receptors. To test this hypothesis, VSMCs were used to examine the effects of aldosterone on expression of these receptors. We also examined the hypertrophic effect of insulin in aldosterone-treated VSMCs. To confirm these in vitro findings in in vivo experimental models, insulin signaling pathways and vascular remodeling were also evaluated in aortas of aldosterone-infused rats.

Methods
For a detailed description of the procedures, see the expanded Methods section in the Supplemental Data, available online at http://atvb.ahajournals.org.

Cell Culture
The VSMCs were isolated from thoracic aortas of 4-week-old male Sprague-Dawley rats (CLEA Japan, Shizuoka, Japan) by enzymatic digestion as previously described.14 The VSMCs were used from the third to eighth passages.

Animal Preparation
All experimental procedures were performed according to the guidelines for the care and use of animals established by Kagawa University. Sprague-Dawley rats (male, 4 weeks old) were randomly treated with 1 of the following combinations for 6 weeks: group 1, tap water (control; n=6); group 2, 1% NaCl plus 1% KCl in drinking water; n=6); group 3, aldosterone (Aldo, 0.75 μg/hour, SC; n=6); group 4, 1% NaCl plus 1% KCl plus aldosterone (Aldo+salt; n=10); group 5, 1% NaCl plus 0.2% KCl plus aldosterone plus spironolactone, an MR antagonist (200 mg/kg per day, PO; n=8); group 6, 1% NaCl plus 1% KCl plus aldosterone plus tempol, a superoxide dismutase mimetic (Aldo+salt+Temp, 3 mmol/L in drinking water; n=8); group 7, 1% NaCl plus 1% KCl plus aldosterone plus hydralazine, a nonspecific vasodilator (Aldo+salt+hydralazine, 25 mg/kg per day by gavage; n=8).

Statistical Analysis
Results are expressed as mean±SE. Statistical significance was assessed using ANOVA and the Bonferroni post hoc test. Student t tests were performed to compare the means when the experimental design was comprised of 2 individual groups. A value of P<0.05 was considered statistically significant.

Results
In Vitro Study
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with a significant effect occurring at 48 hours (Figure 1B). To clarify the genomic effect of aldosterone, we measured the effect of aldosterone on IGF1R mRNA expression. Aldosterone treatment at 10 nmol/L resulted in a significant increase in IGF1R mRNA expression after 3 hours, and expression continued to increase for up to 72 hours (Figure 1C).

Next, we examined the effect of aldosterone on hybrid receptor expression using immunoprecipitation in VSMCs. Aldosterone treatment increased hybrid receptor expression, with maximal effect at 10 nmol/L and 48 hours (Figure 2A). The following experiments were therefore performed using 10 nmol/L aldosterone for 48 hours.

**Effects of Eplerenone on Aldosterone-Induced Receptor Expressions in VSMCs**

To investigate the role of MR on aldosterone-induced receptor expressions, VSMCs were pretreated with eplerenone (10 μmol/L), a selective MR antagonist, for 30 minutes. Aldosterone-induced IGF1R (Supplemental Figure IIA and IIB) and hybrid receptor (Figure 2B) expressions were completely inhibited by pretreatment with eplerenone, suggesting that these responses are mediated by MR.

**Effect of Aldosterone on Hypertrophic Changes Induced by Insulin in VSMCs**

VSMCs pretreated with aldosterone showed the concentration-dependent effects of insulin (100 nmol/L, 5 minutes) on extracellular signal-regulated kinase 1/2 phosphorylation (Supplemental Figure IIIA), indicating that aldosterone increased insulin-induced mitogen-activated protein kinase phosphorylation. Aldosterone treatment attenuated Akt phosphorylation induced by insulin (Supplemental Figure IIIB).

Next, we measured leucine incorporation as an index of protein synthesis in VSMCs. Insulin at different concentrations (0–100 nmol/L) for 24 hours showed no effect on leucine incorporation (Figure 3A). However, pretreatment with aldosterone significantly augmented leucine incorporation induced by insulin (100 nmol/L). These aldosterone-induced increases in leucine incorporation were completely inhibited by pretreatment with eplerenone, suggesting that aldosterone-induced protein synthesis is augmented by aldosterone via MR. We also measured α-smooth muscle actin expression, using immunofluorescence staining to confirm that aldosterone induces hypertrophic changes in VSMC. Aldosterone increased insulin-induced α-smooth muscle actin expression, which had been diminished by pretreatment with eplerenone (Figure 3B), suggesting that
α-smooth muscle actin expression induced by insulin is augmented by pretreatment with aldosterone via MR.

Finally, we evaluated the effect of IGF1R on insulin-induced protein synthesis in aldosterone-treated VSMCs. The increased leucine incorporation was completely inhibited by picropodophyllin (1 mmol/L), a selective IGF1R inhibitor (Supplemental Figure IIC); this suggests that aldosterone augments protein synthesis induced by insulin via IGF1R. On the other hand, IGF1 alone (0.1–100 ng/mL) increased leucine incorporation (Supplemental Figure IID).

In Vivo Study

Systolic Blood Pressure in Rats

The systolic blood pressure profile over time is shown in Supplemental Figure IV. There were no significant differences in basal systolic blood pressure levels among the groups. The systolic blood pressure of Aldo+salt-treated rats was significantly higher than that in controls (213±6.0 versus 115±0.4 mm Hg). Treatment with spironolactone or tempol normalized systolic blood pressure in these animals (117±0.8 and 122±0.9 mm Hg, respectively).

Glucose Metabolism in Rats

The oral glucose tolerance test was performed to analyze whole-body insulin sensitivity, in which both blood glucose and insulin concentration were measured and their respective areas under the curve were calculated. Compared with controls, the Aldo+salt rats showed higher glucose (Figure 4A) and insulin (Supplemental Figure VA) levels after administration of oral glucose. Spironolactone and tempol treatment significantly attenuated the increase in glucose and insulin levels seen in the Aldo+salt group. The areas under the curve for blood glucose (Figure 4B) and insulin (Supplemental Figure VB) were significantly larger in Aldo+salt rats and were significantly reduced by the spironolactone and tempol treatment.

Because glucose metabolism mediated by insulin is affected by potassium, we measured serum potassium concentration. There was no difference between potassium concentration in control (4.8±0.1 mmol/L) and Aldo+salt-treated (5.3±0.3 mmol/L) rats. Spironolactone did not affect serum potassium concentrations (5.2±0.2 mmol/L).

Oxidative Stress in Aortas

We previously reported that oxidative stress is involved in insulin resistance induced by aldosterone in cultured VSMCs.7 We used dihydroethidium fluorescence to evaluate reactive oxygen species (ROS) formation. Aldo+salt rats showed more intense dihydroethidium staining than did controls; dihydroethidium staining was significantly reduced in aortas of rats treated with spironolactone or tempol (Supplemental Figure VI), suggesting an effect by the MR on ROS formation.

Insulin Signaling in the Aorta

We measured Akt phosphorylation in aortas using insulin incubation under ex vivo conditions. Insulin (100 mmol/L, 30 minutes) increased Akt phosphorylation in the control group (Supplemental Figure VIIA). Insulin-induced Akt phosphorylation was significantly less in the Aldo+salt group compared with controls, indicating that insulin signaling was attenuated by Aldo+salt treatment. Spironolactone and tempol treatment re-

covered Akt phosphorylation. We also measured extracellular signal–regulated kinase phosphorylation under ex vivo conditions (Supplemental Figure VIIIB). Insulin-induced extracellular signal–regulated kinase phosphorylation was significantly augmented in the Aldo+salt group compared with controls.

Fibrotic Changes and Collagen Synthesis in the Aorta

Fibrotic changes in aortas were assessed using azan staining. Aldo+salt-treated aortas showed more azan-positive area than did controls that had been normalized with spironolactone and tempol treatment (Figure 5A). Medial thickness-to-lumen diameter ratio, as an index of vascular hypertrophy, was increased in Aldo+salt-treated aortas and was normalized by spironolactone and tempol treatment (Figure 5B).

Next, we found that mRNA expressions of matrix protein type I (Supplemental Figure VIII A) and type III (Supplemental Figure VIII B) collagens were significantly upregulated in Aldo+salt-treated aortas and were normalized by spironolactone and tempol treatment. Furthermore, mRNA of the MR target gene, Sgk1, was upregulated in Aldo+salt-treated aortas, and was normalized by spironolactone and tempol treatment (Supplemental Figure VIIIIC), suggesting that aldosterone induces a hypertrophic effect via MR.

Figure 4. Glucose metabolism. A, the oral glucose tolerance test was performed to analyze whole-body insulin sensitivity. Aldosterone (Aldo)+salt–treated rats showed higher glucose levels after administration of oral glucose. Spironolactone (Spl) and tempol (Temp) treatment significantly attenuated increases in glucose level. B, AUC indicated area under the curve. Data represent mean±SE (n=6–10). *P<0.05 vs control rats, #P<0.05 vs Aldo+salt–treated rats.
IGF1R and Hybrid Receptor in Aorta

Hybrid receptor expression was significantly increased in Aldo/H11001 salt-treated aorta and was normalized by spironolactone and tempol treatment (Figure 6A). Expression of IGF1R mRNA and protein were also significantly increased in aortic tissues of Aldo/H11001 salt-treated rats (Figure 6B and 6C). The IGF1R inhibitor picropodophyllin (1 mmol/L) improved Akt phosphorylation induced by insulin in Aldo/H11001 salt-treated rats (Supplemental Figure IX).

Discussion

Aldosterone is the final mediator of the renin-angiotensin-aldosterone system; it mediates blood pressure and electrolytic balance. Increasing evidence has indicated that aldosterone is also directly involved in tissue damage, proliferative and hypertrophic changes in the vasculature, and vascular insulin resistance.7,22 Several clinical studies have shown that MR inhibition attenuates cardiovascular complications of diabetes mellitus23,24; however, the molecular mechanism of these protective effects has not been elucidated. The present study showed that aldosterone induces insulin resistance by upregulating IGF1R and hybrid receptor and provides a possible molecular mechanism for atherosclerosis induced by insulin in presence of aldosterone. Our study also showed that aldosterone treatment impaired insulin signaling in the aorta and suppressed whole-body insulin sensitivity, accompanied by upregulation of IGF1R and its hybrid receptor. This is novel information about the role of IGF1R in vasculature; it supports the idea that insulin resistance by aldosterone affects the pathogenesis of vascular remodeling in this model.

High concentrations of insulin may activate intracellular signaling pathway via IGF1R or hybrid receptors, although the affinity of IGF1R for insulin is much lower than IGF1.13 In this study, high concentration (100 nmol/L) of insulin did not affect hypertrophic changes in VSMCs; however, pretreatment with aldosterone significantly increased mitogen-activated protein kinase activation and leucine incorporation, accompanied by increased levels of IGF1R and hybrid receptor. Pretreatment with IGF1R inhibitor attenuated protein synthesis induced by a
high insulin concentration, whereas a lower insulin concentration (2 nmol/L) had no effect on mitogen-activated protein kinase activation and protein synthesis. These results indicate that high concentrations of insulin induce hypertrophic actions via upregulation of IGF1R or hybrid receptor. In addition, previous reports also showed that plasma insulin levels were significantly increased in type 2 diabetes mellitus patients compared with control subjects, and insulin levels were correlated with decreased IRs and increased hybrid receptor proportions.25 These data suggest that decreased IRs due to high plasma insulin levels result in increased hybrid receptors. In the present study, exogenous aldosterone increased expression of IGF1R mRNA and protein, but not IRs leading to hybrid receptor formation, indicating that aldosterone increases hybrid receptor mainly by IGF1R production.

Glucose metabolism mediated by insulin is affected by serum potassium concentration. Hypokalemia is often observed in patients with primary aldosteronism; it is a risk factor for insulin resistance and glucose intolerance in these patients. In this study, drinking water for aldosterone-treated rats was supplemented with potassium chloride to prevent potential hypokalemia. In addition, there was no significant difference in serum potassium levels. Therefore, we consider that the effect of potassium concentration on insulin action might be minimal in this study, and insulin resistance was directly induced by aldosterone itself. On the other hand, systemic glucose metabolism is predominantly regulated in skeletal muscle, adipose tissue, and liver.26 Therefore, glucose metabolism in vasculature might not play a major role in systemic glucose metabolism. Recent studies have indicated that aldosterone impairs insulin and glucose metabolisms via downregulation of insulin receptor substrate-1 in adipocytes.27 Furthermore, other studies also reported that MR blockade improved systemic insulin sensitivity by changing adiponectin, peroxisome proliferator–activated receptor-γ, and proinflammatory adipokines in obese rats.28 These results suggest that aldosterone attenuated not only vascular insulin signaling but also systemic glucose metabolism.

Previous experiments clarified that ROS affect the insulin signaling pathway.29 We have also reported that aldosterone induces insulin receptor substrate-1 degradation and attenuates glucose metabolism through the activation of ROS generation by NADPH oxidase.7 The present study shows that aldosterone treatment increases ROS generation in the aorta, and treatment with an antioxidant, tempol, improved glucose intolerance, as evaluated by oral glucose tolerance test in aldosterone-treated rats. Taken together, these facts indicate that aldosterone attenuates vascular and systemic glucose metabolism by decreased insulin receptor substrate-1 and increased IGF1R via ROS generation. However, the involvement of aldosterone in atherosclerosis in diabetes mellitus is more complicated, because aldosterone both directly affects proatherogenic changes via ROS30 and indirectly augments insulin-induced hypertrophy through IGF1R-mediated hypertrophic signaling pathways, such as mitogen-activated protein kinase activation. In addition, we reported that ROS is involved in insulin resistance by shift of insulin signaling from glucose metabolism into vascular remodeling.31 Aldosterone-augmented IGF1R may affect the insulin signaling pathway and subsequently result in vascular remodeling via ROS.

The roles of IGF1R and hybrid receptor in VSMCs are still unclear. Several studies have shown that IGF1 has potential beneficial effects on vasculopathy and glucose metabolism in cardiovascular tissue,22–24 whereas IGF1 reportedly induces vascular remodeling.35,36 Expression of IGF1R is much higher than that of IR, and increased IGF1R forms hybrid receptors in VSMCs.37 Recently, Cascella et al38 reported that aldosterone accelerates the development of atherosclerosis via IGF1-mediated signaling in VSMCs. In addition, Engberding et al39 reported that aortas of diabetic mice expressed significantly more IGF1R in all wall layers than did those of age-matched controls. However, it is methodologically difficult to distinguish the effects of IGF1R and IR, because both receptors partly share a common downstream signaling pathway. Pircropodophyllin, an IGF1R inhibitor, completely diminished leucine incorporation induced by insulin in aldosterone-treated VSMCs and improved Akt phosphorylation in aorta of aldosterone-treated rats in this study, whereas previous studies showed that an small interfering RNA gene knockdown of IGF1R increased glucose uptake, suggesting that IGF1R is involved in insulin resistance and hypertrophic changes in vasculature. The present study showed that treatment with eplerenone significantly prevented increases in IGF1R and hybrid receptor expression. In this regard, we recently showed that mechanical stretch also increased IGF1R expression in VSMCs,39 suggesting that high blood pressure may affect VSMC insulin resistance via IGF1R. On the other hand, the present study showed that nonspecific vasodilator has no effect on glucose metabolism and IGF1R expression, indicating that both MR activation and blood pressure are involved in insulin resistance in vasculature.

In summary, the present findings showed that the hypertrophic effect of insulin is augmented by aldosterone via upregulation of IGF1R and hybrid receptor, and the relevant signal transduction mechanism in VSMCs. Aldosterone induces cellular hypertrophy by activating the extracellular signal–regulated kinase 1/2 pathway in VSMCs, indicating the potential role of aldosterone in the development of the vascular hypertrophy. In aldosterone-infused hypertensive rats, hypertension, insulin resistance, vascular hypertrophy, and fibrosis are associated with increased vascular IGF1R and hybrid receptor expression. These changes were ameliorated by treatment with spironolactone and tempol, suggesting that aldosterone augments vascular hypertrophic effect of insulin through upregulation of IGF1R via an MR- and oxidative stress-mediated pathway. These data indicate that MR inhibition may attenuate angiopathy in hypertensive patients with hyperinsulinemia or hypertensive diabetic patients who are treated with insulin. Therefore, IGF1R may be a novel therapeutic target for insulin resistance and vascular complications in hypertensive patients with diabetes mellitus.

Acknowledgments
We are grateful to Pfizer Inc. (New York, NY) for supplying eplerenone.

Sources of Funding
This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20590253 and 22790792).
Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2012;32:257-263; originally published online December 15, 2011;
doi: 10.1161/ATVBAHA.111.240697

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Figure IX

The figure shows the effect of insulin, picropodophyllin, and Aldo on Akt phosphorylation. The graph indicates that insulin treatment increases Akt phosphorylation, while picropodophyllin and Aldo have a differential effect on Akt phosphorylation. The asterisks (*) indicate statistically significant differences.
Supplemental Figure V

**A**

- Control
- Salt
- Aldo
- Aldo + salt
- Aldo + salt + Spl
- Aldo + salt + Temp

**Plasma insulin (ng/mL)**

- 0 15 30 60 90 (min)

**B**

- Control
- Salt
- Aldo
- Aldo + salt
- Aldo + salt + Spl
- Aldo + salt + Temp

**Plasma insulin (AUC, ng/mL x min)**

- 0 100 150 200

* # #
Supplemental Figure VI

Control  Salt  Aldo  Aldo + salt  Aldo + salt + Spl  Aldo + salt + Temp

Fluorescence intensity (fold)

Control  Salt  Aldo  Aldo + salt  Aldo + salt + Spl  Aldo + salt + Temp
Supplemental Figure VII

A

pAkt
Akt

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Akt phosphorylation (fold)

B

pERK
ERK

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ERK phosphorylation (fold)
Supplemental Figure VIII

C

- **Sgk-1 mRNA (fold)**

- **Control**
- **Salt**
- **Aldo**
- **Aldo + salt**
- **Aldo + salt + Spl**
- **Aldo + salt + Temp**

* * * 

# # #
**A**

Supplemental Figure X

![Graph: Blood pressure (SBP) over time.](image)

- Control
- Aldo+salt
- Aldo+salt+Hydralazine

*SBP (mmHg)*

0 2 4 6 (weeks)

**B**

![Graph: Blood glucose (AUC, mg/dL x min).](image)

- Control
- Aldo
- Aldo+salt
- Aldo+salt+Hydralazine

*Blood glucose (AUC, mg/dL x min)*

Control Aldo Aldo +salt ns

*ns*
Supplemental Figure X

**C**

IGF1R β-subunit mRNA (fold)

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**D**

IGF1R

Insulin receptor

IP: Insulin receptor

Relative intensity (fold)

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