Aldosterone Suppresses Insulin Signaling Via the Downregulation of Insulin Receptor Substrate-1 in Vascular Smooth Muscle Cells

Hirofumi Hitomi, Hideyasu Kiyomoto, Akira Nishiyama, Taiga Haru, Kumiko Moriwaki, Kumiko Kaifu, Genei Ihara, Yoshiko Fujita, Toyomu Ugawa, Masakazu Kohno

Abstract—Clinical reports indicate that patients with primary aldosteronism commonly have impaired glucose tolerance; however, the relationship between aldosterone and insulin signaling pathway has not been clarified. In this study, we examined the effects of aldosterone treatment on insulin receptor substrate-1 expression and insulin signaling pathway including Akt phosphorylation and glucose uptake in rat vascular smooth muscle cells. Insulin receptor substrate-1 protein expression and Akt phosphorylation were determined by Western blot analysis with anti-insulin receptor substrate-1 and phosphorylated-Akt antibodies, respectively. Glucose metabolism was evaluated using 3H-labeled 2-deoxy-d-glucose uptake. Aldosterone (1–100 nmol/L) dose-dependently decreased insulin receptor substrate-1 protein expression with a peak at 18 hours (n=4). Aldosterone-induced degradation of insulin receptor substrate-1 was markedly attenuated by treatment with the selective mineralocorticoid receptor antagonist eplerenone (10 μmol/L; n=4). Furthermore, degradation was blocked by the Src inhibitor PP1 (20 μmol/L; n=4). Treatment with antioxidants, N-acetylcysteine (10 μmol/L), or ebselen (40 μmol/L) also attenuated aldosterone-induced insulin receptor substrate-1 degradation (n=4). In addition, proteasome inhibitor MG132 (1 μmol/L) prevented insulin receptor substrate-1 degradation (n=4). Aldosterone treatment abolished insulin-induced Akt phosphorylation (100 nmol/L; 5 minutes; n=4). Furthermore, aldosterone pretreatment decreased insulin-stimulated (100 nmol/L; 60 minutes; n=4) glucose uptake by 50%, which was reversed by eplerenone (10 μmol/L; n=4). These data indicate that aldosterone decreases insulin receptor substrate-1 expression via Src and reactive oxygen species stimulation by proteasome-dependent degradation in vascular smooth muscle cells; thus, aldosterone may be involved in the pathogenesis of vascular insulin resistance via oxidative stress. (Hypertension. 2007;50:000-000.)

Key Words: aldosterone ■ oxidative stress ■ insulin receptor substrate-1 ■ insulin resistance ■ type 2 diabetes mellitus ■ metabolic syndrome ■ eplerenone

Insulin resistance is a key attribute of type 2 diabetes and the metabolic syndrome.1–3 Systemic glucose metabolism is maintained in the liver and skeletal muscle, and in the insulin resistant state, insulin-stimulated glucose uptake is attenuated and accompanied with subsequent increases in blood glucose concentration. High blood glucose concentration induces secretion of insulin from the pancreas and results in hyperinsulinemia. Both hyperglycemia and hyperinsulinemia affect the vasculature and are associated with microangiopathy, including retinopathy and nephropathy, and macroangiopathy, including cardiovascular disease and atherosclerosis.1 Insulin resistance in the vasculature might also affect systemic glucose metabolism. However, the involvement of normal insulin signaling contributes to arteriosclerosis.5–6 On the other hand, serine phosphorylation and degradation of insulin receptor substrate-1 (IRS-1) is a possible mechanism involved in insulin resistance.6 We have reported previously that the molecular mechanism of insulin resistance in the vasculature involves angiotensin II–mediated phosphorylation of IRS-1 and subsequent degradation of proteins via the ubiquitin-proteasome pathway in a reactive oxygen species (ROS) and Src-sensitive manner.7

Aldosterone is the final mediator of the renin-angiotensin-aldosterone system and mediates blood pressure and electrolyte balance in the kidney. Recent basic and clinical studies indicated that aldosterone not only mediates mineral balance in the kidney but also is directly involved in tissue damage and proliferative changes in the vasculature through the activation of Src and ROS generation by NADPH oxidase.8–10 These data clearly indicate that ROS production by aldosterone is a possible mechanism of arteriosclerosis.
Patients with primary aldosteronism showed impaired glucose tolerance, as assessed by the oral glucose tolerance test. Furthermore, diabetes mellitus committees have advocated that primary aldosteronism is a unique form of diabetes mellitus. Several recent clinical reports indicated that primary aldosteronism is not a rare disease; however, primary aldosteronisms have not yet been considered to be the common diseases. Some possible mechanisms of insulin resistance induced by aldosterone have been considered, such as a low blood potassium concentration and a direct effect of aldosterone on insulin signaling. However, the relationship among aldosterone, glucose metabolism, and insulin resistance is still poorly understood.

Although the clinical relationship between aldosterone and impaired glucose metabolism has already clarified, the molecular mechanisms are still unknown. In this study, we hypothesized that aldosterone interferes with insulin signaling by IRS-1 degradation in a ROS-sensitive manner via a proteasome pathway in vascular smooth muscle cells (VSMCs). The aim of this study was to investigate the effects of aldosterone treatment on IRS-1 expression in VSMCs.

**Materials and Methods**

**Materials**

Aldosterone was purchased from Across Organics (Geel, Belgium). Anti–IRS-1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Phospho-Akt (Ser473) and Akt antibodies were purchased from Cell Signaling Technology, Inc (Beverly, Mass). Eplerenone was provided by Pfizer Inc (New York, NY). All of the other chemicals and reagents, including DMEM with 25 mmol/L of Heps and 4.5 g/L of glucose, were purchased from Sigma (St Louis, Mo).

**Cell Culture**

VSMCs were isolated from the rat thoracic aorta by enzymatic digestion and grown in DMEM, as described previously. For experiments, cells between passages 5 and 10 were used.

**Western Analysis**

Cells at 80% to 90% confluence were made quiescent by incubation with DMEM containing 0.1% calf serum for 24 hours. Cells were stimulated with agonist at 37°C in serum-free DMEM, lysed as described previously, and solubilized proteins were isolated by centrifugation and quantified by the Bradford assay. Proteins were separated using 6% for IRS-1 or 10% for Akt, SDS-PAGE, and transferred to nitrocellulose membranes. After blocking, blots were incubated with primary antibodies, and proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Band intensity was quantified by densitometry of immunoblots using NIH Image software.

**Measurement of Glucose Uptake**

Serum-starved VSMCs were stimulated with vehicle or aldosterone (10 nmol/L) for 18 hours in the presence or absence of eplerenone (10 μmol/L). Cells were then incubated in Krebs-Ringer-Hepes buffer (15 mmol/L of Heps [pH 7.4], 105 mmol/L of NaCl, 5 mmol/L of KCl, 1.4 mmol/L of CaCl₂, 1 mmol/L of KH₂PO₄, 1.4 mmol/L of MgSO₄, and 10 mmol/L of NaHCO₃) for 2 hours. Next, cells were incubated with insulin (100 nmol/L) for 30 minutes, and 0.2 mmol/L 2-deoxy-D-glucose containing 1 μCi/mL 2-deoxy-D-[3H]glucose was added for an additional 30 minutes. Transport was stopped by removal of the buffer, followed by 3 washes with ice-cold PBS. Cells were disrupted with 0.4 mol/L of NaOH, neutralized with HCl, and the amount of labeled glucose taken up was determined by scintillation counting.

**Statistical Analysis**

Results are expressed as mean±SE. Statistical significance was assessed using ANOVA, followed by Bonferroni’s test. A value of P<0.05 was considered to be statistically significant.

**Results**

**Effect of Aldosterone on IRS-1 Protein Expression**

We have shown previously that aldosterone increases ROS production in VSMCs, and ROS is the trigger of IRS-1 degradation. Accordingly to these previous data, we hypothesized that treatment with aldosterone alters IRS-1 protein expression in a ROS-sensitive manner. As shown in Figure 1A, aldosterone induced a time-dependent decrease in IRS-1 protein levels, with the maximal effect occurring at 18 hours (55±11%), but did not affect β-actin levels. We also examined the dose response of aldosterone (Figure 1B). Aldosterone decreased IRS-1 protein expression in dose-dependent...
manner, with a significant decrease at 10 nmol/L. As a result, the following experiments were performed using aldosterone (18 hours; 10 nmol/L). IRS-1 downregulation was completely abolished by eplerenone (Figure 2A; 10 μmol/L), indicating that this response is mediated by the mineralocorticoid receptor. The role of ROS in this response was investigated using VSMCs pretreated with antioxidants, N-acetylcysteine (10 mmol/L), or ebselen (40 μmol/L). Downregulation of IRS-1 by aldosterone was significantly inhibited in these cells, suggesting that ROS is required for the decrease in IRS-1 levels (Figure 2B). Furthermore, we investigated the possible involvement of c-Src, a tyrosine kinase required for generation of ROS, in IRS-1 downregulation using its specific inhibitor PP1 (20 μmol/L), which effectively attenuates Src activation in these cells. Pretreatment of VSMCs with PP1 partially inhibited the ability of aldosterone to induce IRS-1 downregulation (Figure 2C).

**Proteasome-Dependent Degradation of IRS-1 by Aldosterone**

The downregulation of IRS-1 protein by aldosterone could occur by protein synthesis inhibition, increasing protein degradation or both. Aldosterone treatment had no effect on IRS-1 mRNA expression over 18 hours (data not shown), suggesting that the effect of aldosterone is posttranslational. IRS-1 is degraded by a ubiquitin-proteasome pathway in response to insulin. Inhibition of the 26S proteasome with MG132 (0.1 μmol/L) attenuated the ability of aldosterone to downregulate IRS-1 (Figure 3).

**Effects of IRS-1 Downregulation by Angiotensin II on Insulin Signaling**

Next, to test whether IRS-1 downregulation by aldosterone affects insulin signal transduction, we examined insulin-induced Akt phosphorylation and glucose uptake. As shown in Figure 4, insulin increased Akt phosphorylation. However, when VSMCs were pretreated with aldosterone (10 nmol/L) for 18 hours, a time at which aldosterone-stimulated Akt phosphorylation is reverted to baseline levels, insulin was no longer able to stimulate Akt phosphorylation. Pretreatment of eplerenone reversed Akt activation induced by insulin. Furthermore, aldosterone pretreatment completely inhibited insulin-stimulated glucose uptake, and this decrease was reversed by the mineralocorticoid receptor inhibitor eplerenone (Figure 5). Insulin had no effect on 3H-mannitol uptake, suggesting that the effect of insulin on glucose uptake does not depend on osmotic pressure. Thus, downregulation of IRS-1 by aldosterone has profound effects on the cellular response to insulin.
Discussion

In this study, we examined the effects of aldosterone on the insulin signaling pathway in VSMCs. The renin-angiotensin-aldosterone system is a crucial part of the physiological and pathological response of the cardiovascular system. Numerous signaling pathways in response to the renin-angiotensin-aldosterone system are mediated by ROS mainly via NADPH oxidase activation. ROS, including superoxide and H₂O₂, are able to regulate cysteine-based phosphatases, such as protein tyrosine phosphatases and lipid phosphatases, and directly influence cell signaling pathways. We and others have reported previously that aldosterone also augments ROS production in VSMCs. Callera et al. have already reported that aldosterone (100 nM; 60 minutes) increased NADPH oxidase activation via Src and p38 mitogen-activated protein kinases. Eplerenone inhibited Src activation, indicating that Src is downstream of the mineralocorticoid receptor. Eplerenone and Src inhibition completely blocked NADPH oxidase activation induced by aldosterone, suggesting that ROS production is mediated by mineralocorticoid receptor and Src. As shown in Figure 2B, antioxidants inhibit the IRS-1 degradation, suggesting that aldosterone-induced IRS-1 downregulation is ROS sensitive. ROS also activate several tyrosine kinases, including Src and phosphoinositide-dependent kinase. Aldosterone activates Src via the mineralocorticoid receptor and subsequently enhances activation of other kinases, including mitogen-activated protein kinase. PP1, a specific Src inhibitor, attenuates IRS-1 degradation, indicating that downregulation is partially mediated via the Src-dependent pathway. Aldosterone also activates the glucocorticoid receptor, and many reports have shown a relationship between glucocorticoid and glucose metabolism. Aldosterone (10 nmol/L), used in this study, may activate the glucocorticoid receptor and affects glucose metabolism in VSMCs. To determine whether aldosterone decreased IRS-1 protein by activation of the glucocorticoid receptor, we used RU486, an inhibitor of the glucocorticoid receptor. However, RU486 had no effects on aldosterone-induced IRS-1 degradation (data not shown), whereas the mineralocorticoid receptor inhibitor eplerenone completely inhibited IRS-1 degradation (Figure 2A) and recovered insulin-stimulated Akt phosphorylation (Figure 4) and glucose uptake (Figure 5), indicating that aldosterone attenuates insulin signaling via the mineralocorticoid receptor. Furthermore, many reports indicated that glucose transporter-4 has an important role in glucose metabolism in skeletal muscle and VSMCs. Our data regarding Akt phosphorylation (Figure 4) and glucose incorporation (Figure 5) suggest that glucose transporter-4 may play a role in this study. Further studies will be required to investigate the roles of glucose transporter-4 in mediating aldosterone-mediated insulin resistance.

Patients with primary aldosteronism (54%; n = 39) had impaired glucose tolerance, as assessed by an oral glucose tolerance test. Recent clinical reports have shown that primary aldosteronism is a possible cause of glucose intolerance and might be a cause of diabetes mellitus. Experimental evidence has shown a relationship between mineralocorticoid receptor and glucose metabolism in VSMCs. The renin-angiotensin-aldosterone system is a crucial part of the physiological and pathological response of the cardiovascular system. Numerous signaling pathways in response to the renin-angiotensin-aldosterone system are mediated by ROS mainly via NADPH oxidase activation. ROS, including superoxide and H₂O₂, are able to regulate cysteine-based phosphatases, such as protein tyrosine phosphatases and lipid phosphatases, and directly influence cell signaling pathways. We and others have reported previously that aldosterone also augments ROS production in VSMCs. Callera et al. have already reported that aldosterone (100 nM; 60 minutes) increased NADPH oxidase activation via Src and p38 mitogen-activated protein kinases in VSMCs. Eplerenone inhibited Src activation, indicating that Src is downstream of the mineralocorticoid receptor. Eplerenone and Src inhibition completely blocked NADPH oxidase activation induced by aldosterone, suggesting that ROS production is mediated by mineralocorticoid receptor and Src. As shown in Figure 2B, antioxidants inhibit the IRS-1 degradation, suggesting that aldosterone-induced IRS-1 downregulation is ROS sensitive. ROS also activate several tyrosine kinases, including Src and phosphoinositide-dependent kinase. Aldosterone activates Src via the mineralocorticoid receptor and subsequently enhances activation of other kinases, including mitogen-activated protein kinase. PP1, a specific Src inhibitor, attenuates IRS-1 degradation, indicating that downregulation is partially mediated via the Src-dependent pathway. Aldosterone also activates the glucocorticoid receptor, and many reports have shown a relationship between glucocorticoid and glucose metabolism. Aldosterone (10 nmol/L), used in this study, may activate the glucocorticoid receptor and affects glucose metabolism in VSMCs. To determine whether aldosterone decreased IRS-1 protein by activation of the glucocorticoid receptor, we used RU486, an inhibitor of the glucocorticoid receptor. However, RU486 had no effects on aldosterone-induced IRS-1 degradation (data not shown), whereas the mineralocorticoid receptor inhibitor eplerenone completely inhibited IRS-1 degradation (Figure 2A) and recovered insulin-stimulated Akt phosphorylation (Figure 4) and glucose uptake (Figure 5), indicating that aldosterone attenuates insulin signaling via the mineralocorticoid receptor. Furthermore, many reports indicated that glucose transporter-4 has an important role in glucose metabolism in skeletal muscle and VSMCs. Our data regarding Akt phosphorylation (Figure 4) and glucose incorporation (Figure 5) suggest that glucose transporter-4 may play a role in this study. Further studies will be required to investigate the roles of glucose transporter-4 in mediating aldosterone-mediated insulin resistance.

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corticoid hormones and insulin.29 Aldosterone induces serum potassium depletion, which could modulate both insulin secretion and insulin receptor function.14 Recent studies indicate that aldosterone might have direct effects on insulin receptor function along with decreased insulin sensitivity in human adipocytes.30,31 On the other hand, Catena et al32 reported that primary aldosteronism is an insulin-resistant condition that is independent of plasma potassium levels. However, the molecular mechanisms and the relationship among aldosterone, glucose metabolism, and insulin resistance are poorly understood. Our data provide insight into the signaling pathway leading to IRS-1 degradation via the mineralocorticoid receptor, ROS generation, and Src activation by aldosterone. We considered that this signaling pathway might be one of the possible mechanisms of glucose intolerance in patients with primary aldosteronism.

The effect of insulin resistance in the vasculature on systemic glucose metabolism is still controversial, because blood glucose levels are mainly maintained at skeletal muscle, liver, and adipocytes. Numerous reports have clearly shown that diabetes-induced vascular complications, including cardiovascular and renal disease, are the most critical risk factors in diabetes patients.33,34 Insulin resistance is the initial step and is a common pathway in diabetes mellitus and its complications, and involvement of normal insulin signaling in insulin resistance condition might contribute to arteriosclerosis. Therefore, we considered that the inhibition of insulin resistance in the vasculature can prevent diabetes complications.

In summary, we show that aldosterone induces Src- and ROS-mediated IRS-1 degradation via the 26S proteasome. These results indicate a novel mechanism of aldosterone-induced insulin resistance and suggest that aldosterone may be involved in the pathogenesis of vascular insulin resistance.

Perspectives

The clinical observations support that the renin-angiotensin system is involved in vascular complications and inhibition of the renin-angiotensin system by angiotensin type-1 receptor blockers mitigates the development of type 2 diabetes and its associated vascular complications.18 The present study provides evidence that aldosterone attenuates insulin signaling in VSMCs. These results support that not only the renin-angiotensin system but also the renin-angiotensin-aldosterone system inhibition is efficient therapy for diabetes and its vascular complications. Future studies are required to elucidate the in vivo and clinical effects of aldosterone inhibition on insulin signaling and progression of type 2 diabetes mellitus.

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Disclosures

Eplerenone was supplied to H.H. from Pfizer Inc in an amount less than $10 000. The authors have nothing else to disclose.

References


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