Angiotensin II Type-1 Receptor Blocker Valsartan Enhances Insulin Sensitivity in Skeletal Muscles of Diabetic Mice

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Abstract—Angiotensin II has been shown to contribute to the pathogenesis of insulin resistance; however, the mechanism is not well understood. The present study was undertaken to investigate the potential effect of an angiotensin II type-1 (AT₁) receptor blocker, valsartan, to improve insulin resistance and to explore the signaling basis of cross-talk of the AT₁ receptor- and insulin-mediated signaling in type 2 diabetic KK-Ay mice. Treatment of KK-Ay mice with valsartan at a dose of 1 mg/kg per day, which did not influence systolic blood pressure, significantly increased insulin-mediated 2-[³H]deoxy-d-glucose (2-[³H]DG) uptake into skeletal muscle and attenuated the increase in plasma glucose concentration after a glucose load and plasma concentrations of glucose and insulin. In contrast, insulin-mediated 2-[³H]DG uptake into skeletal muscle was not influenced in AT₂ receptor null mice, and an AT₂ receptor blocker, PD123319, did not affect 2-[³H]DG uptake and superoxide production in skeletal muscle of KK-Ay mice. Moreover, we observed that valsartan treatment exaggerated the insulin-induced phosphorylation of IRS-1, the association of IRS-1 with the p85 regulatory subunit of phosphoinositide 3 kinase (PI 3-K), PI 3-K activity, and translocation of GLUT4 to the plasma membrane. It also reduced tumor necrosis factor-α (TNF-α) expression and superoxide production in skeletal muscle of KK-Ay mice. Specific AT₁ receptor blockade increases insulin sensitivity and glucose uptake in skeletal muscle of KK-Ay mice via stimulating the insulin signaling cascade and consequent enhancement of GLUT4 translocation to the plasma membrane. (Hypertension. 2004;43:1003-1010.)

Key Words: angiotensin II ■ insulin ■ glucose ■ diabetes mellitus

The renin-angiotensin system plays an important role in the regulation of cardiovascular and fluid volume homeostasis, and in the control of various hormone secretion, tissue growth and neuronal activity. Angiotensin (Ang) II seems to be involved in the pathogenesis of hypertension and insulin resistance, although few studies have examined the relationship between the two. Insulin resistance occurs in a wide variety of pathological states and is commonly associated with obesity, type 2 diabetes, accelerated atherosclerosis, and hypertension.1,2 Shimamoto et al demonstrated that the insulin sensitivity of fructose-fed rats is improved by treatment with an Ang II receptor blocker (ARB), olmesartan, caused by changes in muscle fiber composition and a decrease in tumor necrosis factor (TNF)-α expression in skeletal muscle.3,4 Henriksen et al5 reported that an ARB, irbesartan, either acutely or chronically, improves glucose tolerance in the obese Zucker rat, at least in part through enhancement of skeletal muscle glucose transport, and the effect of chronic Ang II receptor antagonism on skeletal muscle glucose uptake is associated with an increase in GLUT4 protein expression. Recent large clinical trials revealed that angiotensin II receptor blockade by losartan was associated with a lower risk of development of diabetes.6 However, it remains unclear whether Ang II has a direct effect on the insulin-mediated pathway of glucose metabolism in addition to changes in local blood flow in insulin-sensitive organs such as skeletal muscle.

Recent studies have suggested that Ang II might negatively modulate insulin-mediated actions by regulating multiple levels of the insulin signaling cascade such as the insulin receptor, insulin receptor substrate (IRS), and phosphatidylinositol 3-kinase (PI 3-K).7–9 Therefore, we examined the possibility that an angiotensin II type-1 (AT₁) receptor-specific ARB, valsartan, enhances insulin signaling by activating the insulin signaling cascade, thereby increasing insulin sensitivity in diabetic KK-Ay mice as a model of type 2 diabetes. Recent evidence has revealed that the functions of the AT₁ and AT₂ receptors are mutually antagonistic. The effect of ARB may not be entirely caused by blockade of the AT₁ receptor. When the AT₁ receptor is blocked and unbound Ang II can act on the AT₂ receptor, stimulation of AT₂ receptor might be involved in the effects of ARB. Obviously, the AT₂ receptor plays a role in the pathogenesis and remodeling of cardiovascular and renal diseases.10,11 However, the role of AT₂ receptor stimulation in glucose metabolism is an enigma. In this study, we also used AT₂ receptor...
null (Agtr2−) mice and examined the role of AT2 receptor stimulation in glucose uptake into skeletal muscle.

Methods

Animals and Treatment
Male KK-Ay/Ta and C57BL/6J mice were obtained from Nihon Clea and housed individually from age 8 weeks in plastic cages at 25±1°C with lighting on from 06:00 to 18:00. KK-Ay mice result from a cross between glucose-intolerant black KK female mice and male yellow obese A mice and are known to serve as an excellent model of type 2 diabetes.12 Adult male Agtr2+ and Agtr2− mice13 (age 10 to 12 weeks) were also used. They were given a standard diet (MF; Oriental Yeast Co Ltd) and water ad libitum. Ang II (Sigma Aldrich, Japan) was infused for 14 days at a dose of 720 µg/kg per day using osmotic minipump (model 1002; Alza Corporation) implanted in animals at age 8 weeks.14 KK-Ay mice at age 8 weeks were also administered a valsartan (1 mg/kg per day) (provided by Novartis Pharma AG) or an AT2 receptor antagonist, PD123319 (30 mg/kg per day) (Research Biochemicals International), for 14 days using osmotic mini pumps. Blood pressure was measured by the indirect tail-cuff method (MK-1030; Muromachi Kikai Co Ltd.). All experimental procedures were approved and performed in compliance with the guidelines of the Ehime University School of Medicine Committee on Animals.

Oral Glucose Tolerance Test
Oral glucose tolerance test was performed after a 16-hour overnight fast. Glucose (2 g/kg) was administered orally, and a small amount of blood was collected from the orbital sinus at 0, 30, 60, and 120 minutes.

Measurement of Rate Constant of Net Tissue Uptake of 2-[3H]deoxy-D-glucose
Uptake of 2-[3H]deoxy-D-glucose in peripheral tissues was measured as previously reported.15 Interscapular brown adipose tissue, epididymal and retroperitoneal white adipose tissues, and skeletal muscles (extensor digitorum longus [EDL], soleus, and red and white parts of gastrocnemius) were rapidly dissected and weighed. The rate constant of net tissue uptake of 2-[3H] DG was calculated as described previously.16

Tissue Protein Sample Extraction
Hind limb skeletal muscles were removed 3 minutes after intravenous insulin injection and immediately homogenized as previously described.17

Membrane Preparations of Skeletal Muscle
After overnight fasting, 0.2 mL of 0.9% NaCl with or without 1 U/kg insulin was injected. The plasma membrane fraction of hind limb skeletal muscles was isolated from the muscles by the method previously described.18

Immunoprecipitation and Immunoblotting
Equal amounts of protein (0.5 to 1.0 mg) of supernatants were incubated at 4°C with anti-IRS-1 antibodies (Upstate Biotechnology Inc) or anti-insulin receptor β subunit antibodies (Transduction Laboratory) overnight with constant agitation and then further incubated with protein G-Sepharose 4 Fast Flow for 1 hour as previously described.17 For immunoblotting, the plasma membrane fraction (40 µg), whole-cell lysate (25 µg), and immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-GLUT4 antibody (Santa Cruz Biotechnology), anti-phosphotyrosine antibody, or anti-p85 regulatory subunit of PI 3-kinase antibodies (Upstate Biotechnology Inc) and then visualized with an ECL detection kit (Amersham Pharmacia Biotech).

PI 3-Kinase Assay
The samples immunoprecipitated with anti-IRS-1 antibodies were used for measurement of PI 3-kinase activity as previously described.19

Reverse-Transcription Polymerase Chain Reaction
RNA was prepared from the skeletal muscle of the mice using Trizol Reagent (Gibco-BRL, Tokyo, Japan). Reverse-transcription polymerase chain reaction was performed as previously described.20

Plasma Glucose and Insulin Concentrations
Plasma glucose and plasma insulin were measured using commercial kits (glucose B test and insulin measurement kit).

Superoxide Detection
Frozen, enzymatically intact, 10-µm-thick sections of soleus skeletal muscle in each mouse were incubated at the same time with dihydroethidium (DHE; 1 µmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light.21 DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. For detection of ethidium, samples were examined with an Axioskop microscope (Axioskop 2 plus with AxioCam; Carl Zeiss, Oberkochen, Germany) equipped with a computer-based imaging system. Intensity of the fluorescence was analyzed and quantified using computer imaging software (Densitograph; ATTO Corporation, Tokyo, Japan).

Statistical Analysis
All values are expressed as mean±SE. The effects of the different treatments on all data were evaluated with factorial analysis of variance. When a significant effect was found, the results were further compared with Bonferroni multiple range test. For comparison of 2 means, unpaired Student t test was performed. A difference with P<0.05 was considered significant.

Results

Effect of Valsartan on Plasma Concentrations of Glucose and Insulin, Body Weight in KK-Ay Mice, and Glucose Intolerance in C57BL/6J and KK-Ay Mice
To investigate whether an ARB, valsartan, could improve insulin resistance, we used KK-Ay mice as a model of type 2 diabetes in this study. We previously reported that KK-Ay mice showed impaired glucose tolerance compared with that in C57BL/6J mice, and KK-Ay mice showed high plasma glucose and insulin concentrations even at age 8 weeks, which increased further at age 10 weeks compared with those in C57BL/6J mice (Table),17 which are generally used as nondiabetic controls for KK-Ay mice. We administered nonhypotensive dose of valsartan at a dose of 1 mg/kg per day to KK-Ay mice or C57BL/6J mice (age 8 weeks) for 2 weeks and performed oral glucose tolerance test after 16 hours of fasting. As we previously reported,17 we also observed in this study that the plasma glucose concentration in KK-Ay mice markedly increased after the glucose load and was maintained at a higher level (Figure 1). Administration of valsartan decreased the peak of plasma glucose concentration at 30 minutes after the glucose load in C57BL/6J mice and KK-Ay mice. The plasma glucose level decreased more rapidly compared with that in vehicle-treated KK-Ay mice (Figure 1), with no significant difference in insulin concentration (Figure 1). Basal plasma glucose and insulin concentrations of C57BL/6J and KK-Ay mice at age 10 weeks in the fasting
condition were similar to those in valsartan-administered mice. The administration of valsartan attenuated the age-dependent increase in glucose and insulin (Table). We also observed that Ang II infusion (720 μg/kg per day) for 2 weeks increased basal plasma glucose concentration in C57BL/6J, even after fasting, and increased the peak of plasma glucose concentration at 30 minutes after the glucose load in C57BL/6J mice and KK-Ay mice (Figure 1). There was an increase in blood pressure (C57BL/6J: 133.14 ± 4.10 mm Hg; KK-Ay: 145.26 ± 3.59 mm Hg).

Effect of Valsartan on Glucose Uptake in Skeletal Muscle of KK-Ay Mice

It is well known that impaired glucose metabolism in peripheral tissues such as skeletal muscle plays a critical role in the development of insulin resistance. We previously reported that the rate constant of 2-[3H]DG uptake in response to insulin was lower in skeletal muscle and adipose tissue in KK-Ay mice, suggesting that KK-Ay mice have insulin resistance and glucose intolerance. To further determine whether valsartan could enhance glucose transport in skeletal muscle, we examined the effects of valsartan administration on glucose uptake in skeletal muscle of C57BL/6J and KK-Ay mice. As shown in Figures 2 and 3A, administration of valsartan significantly increased 2-[3H]DG uptake in all peripheral skeletal muscle examined under a fed condition. However, it was not clear from this experiment whether treatment with valsartan actually enhanced the effect of insulin on glucose uptake in skeletal muscle of KK-Ay mice. To further examine this possibility, we performed experiments using the mice after 16 hours of fasting. Under this fasting condition, the plasma concentration of insulin in all experimental mice could be controlled to a very low level without any significant difference between each group (Figure 1B). We observed that insulin-mediated 2-[3H]DG uptake in skeletal muscle was significantly higher in the valsartan-treated group (Figure 3B), suggesting that administration of valsartan could enhance the effect of insulin on glucose uptake into skeletal muscle.

Signaling Mechanism of Valsartan-Mediated Improvement of Insulin Resistance in KK-Ay Mice

To examine the possibility that valsartan could enhance insulin-mediated signaling, thereby increasing GLUT4 translocation to the plasma membrane, we focused on the insulin receptor/IRS/PI 3-K pathway and GLUT4 translocation in skeletal muscle of KK-Ay mice. Protein extracts prepared from skeletal muscle were immunoprecipitated with anti-insulin receptor β subunit or anti-IRS-1 antibodies and immunoblotted with anti-phosphotyrosine antibodies. As
shown in Figure 4, treatment with valsartan increased the phosphorylation of IRS-1 and further enhanced insulin-induced phosphorylation of IRS-1 in skeletal muscle of KK-Ay, whereas phosphorylation of the insulin receptor was not significantly changed by treatment with valsartan. Next, we examined the effect of valsartan on the insulin-induced association of IRS-1 with the p85 regulatory subunit of PI 3-kinase and PI 3-K activity. We observed that both the basal and insulin-stimulated associations of IRS-1 with the p85 subunit were significantly enhanced in skeletal muscle of mice treated with valsartan (Figure 4B). Consistent with these results, valsartan treatment enhanced basal and insulin-stimulated translocation of GLUT4 in vatsartan-treated mice compared with those in vehicle-treated mice (Figure 4D). Total protein levels of the insulin receptor, IRS-1, the p85 regulatory subunit of PI 3-kinase, and GLUT4 were not changed (Figure 4D and 4E). TNF-α is known to be closely associated with insulin-mediated glucose uptake in peripheral tissues.22 Interestingly, we observed that valsartan treatment decreased TNF-α expression in skeletal muscle of KK-Ay mice (Figure 4F).

Role of AT2 Receptor in Insulin-Mediated Glucose Uptake in Skeletal Muscle
To examine the role of the AT2 receptor in the regulation of insulin-mediated glucose uptake in skeletal muscle, we used AT2 receptor null mice. As shown in Figure 6, no apparent difference in insulin-mediated 2-[3H]DG uptake into skeletal muscle was observed between wild-type and Agr2−/− mice, whereas insulin-induced 2-[3H]DG uptake in white adipose tissues in Agr2−/− mice was significantly lower than that of control mice (Figure 6). Next, we examined the effect of AT2 receptor specific antagonist, PD123319, on insulin sensitivity in KK-Ay mice. We observed that administration of nonhypertensive dose of PD123319 for 2 weeks slightly decreased plasma glucose concentration (Table), whereas this dose of PD123319 did not influence 2-[3H]DG uptake in diabetic KK-Ay mice (Figure 3A).

Superoxide Production in Skeletal Muscle
To examine the superoxide production in skeletal muscle, we used chemical detection of superoxide in freshly frozen tissue with DHE. As shown in Figure 7, superoxide production was higher in soleus muscle of KK-Ay mice than that of C57BL/6J mice. It was significantly suppressed by valsartan but not affected by PD123319 (Figure 7).

Discussion
In clinical studies, blockade of the renin-angiotensin system seemed to improve insulin resistance and reduce the occurrence of diabetes. Consistent with these studies, previous reports suggested that Ang II negatively regulates the insulin signaling pathway in cultured vascular smooth muscle cells and perfused rat heart.7,8 However, the detailed mechanism of direct cross-talk of insulin and Ang II-mediated signaling via the AT1 and AT2 receptor subtypes in glucose metabolism is still unclear. Change in blood flow is one important factor in insulin-mediated glucose uptake in peripheral tissues. There-
Therefore, we used a low dose of an ARB, valsartan, which did not influence blood pressure. We demonstrated that selective blockade of the AT$_1$ receptor by valsartan effectively attenuated hyperglycemia and hyperinsulinemia and improved glucose tolerance in KK-Ay mice, an animal model of type 2 diabetes mellitus. We also observed that valsartan enhanced insulin-mediated 2-$[^{3}H]$DG uptake in skeletal muscle in diabetic KK-Ay mice. In contrast, blockade of the AT$_2$ receptor by PD123319 did not influence 2-$[^{3}H]$DG uptake and superoxide production in skeletal muscle of KK-Ay mice, and insulin-mediated glucose uptake in skeletal muscle was similar in $^{1}$Agtr2$^{-/-}$ and $^{2}$Agtr2$^{+/+}$ mice. These results suggest that the AT$_1$ receptor does not appear to be involved in insulin-mediated glucose uptake in skeletal muscle. Consistent with these results, we observed AT$_1$ receptor expression in the soleus skeletal muscle by immunohistochemical staining, whereas AT$_2$ receptor expression in skeletal muscle was very low (data not shown).

In our study, Ang II infusion decreased insulin sensitivity in diabetic KK-Ay mice and nondiabetic C57BL/6J mice. In addition, administration of valsartan increased insulin sensitivity in KK-Ay mice. However, clinical studies have previously suggested that Ang II may increase the insulin sensitivity. Townsend et al reported that acute administration of Ang II in pressor doses increases insulin-mediated glucose disposal and oxidation in lean normotensive men. Buchanan et al demonstrated that acute Ang II infusion enhanced whole-body glucose utilization during hyperinsulinemia in normotensive humans. However, these effects of Ang II seemed to be mediated by the increase in local blood flow and/or local insulin concentration, but not by direct stimulation of glucose transport in skeletal muscle in normotensive humans. In contrast, Ogihara et al reported that the chronic infusion of Ang II decreased insulin-induced glucose uptake in rat soleus muscle. Moreover, irbesartan, an ARB, caused a dose-dependent increase in glucose tolerance and insulin-mediated glucose transport in isolated soleus muscle in obese Zucker rats. Such differences about the effects of Ang II on insulin sensitivity in human and rodents might be caused not only by the species difference, probably depending on the

**Figure 4.** Effect of valsartan on insulin-mediated signaling pathway in skeletal muscle of KK-Ay mice. A, Tyrosine phosphorylation of IRS-1. B, Association between IRS-1 and p85 subunit of PI 3-kinase. C, Tyrosine phosphorylation of insulin receptor. D, GLUT4 translocation to plasma membrane. Upper panels show a representative immunoblot of 4 separate experiments. Data of densitometric measurements are shown in lower panels (A, B, C, and D). Data are mean±SE. *P<0.05 versus control, **P<0.01 vs control. E, Protein level of insulin receptor, IRS-1, and p85 subunit of PI 3-kinase. F, TNF-α expression in skeletal muscle of mice with or without valsartan treatment. E and F, Representative data from 3 separate experiments.
relative efficiency between AT1 and AT2 receptor stimulations, but also by the duration of treatment. Further studies are needed to prove this hypothesis.

To explore the mechanism of improvement of insulin sensitivity by valsartan, we focused on the possibility that selective AT1 receptor blockade could directly enhance insulin signaling, thereby increasing GLUT4 translocation to the plasma membrane. We observed that valsartan treatment enhanced insulin-induced tyrosine phosphorylation of IRS-1, coupling of IRS-1 with the p85 regulatory subunit of PI 3-kinase, activity of PI-3 kinase, and GLUT4 translocation to the plasma membrane in skeletal muscle of KK-Ay mice. In addition, protein content in the insulin signaling pathway including the insulin receptor, IRS-1, p85 subunit of PI-3 kinase, and GLUT4 in diabetic skeletal muscle was not changed by valsartan administration. These results support a previous report that the Ang II signaling cascade effectively modulates phosphorylation at the site of IRS-1 in the insulin signaling pathway. In contrast, Ogihara et al reported that Ang II-induced insulin resistance in the rat cannot be attributed to impairment of early insulin-signaling steps and that increased oxidative stress, possibly through impaired insulin signaling located downstream from PI 3-kinase activation, is involved in Ang II-induced insulin resistance. Henriksen et al reported that administration of an AT1 receptor blocker, irbesartan, increased GLUT4 protein expression in the skeletal muscle and heart of obese Zucker rats. These results suggest that Ang II might regulate insulin sensitivity at multiple sites in various diabetic animal models.

It is known that TNF-α inhibits insulin signaling linked to GLUT translocation to the plasma membrane. Moreover, activation of the renin-angiotensin system is thought to be one of the factors upregulating skeletal muscle TNF-α. Therefore, we examined the effect of AT1 receptor blockade on local production of TNF-α in skeletal muscle of KK-Ay mice. Consistent with a previous report, we observed that valsartan decreased TNF-α expression in skeletal muscle of diabetic KK-Ay mice. These results suggest that inhibition of local production of TNF-α in skeletal muscle contributes at least partly to enhancement of insulin sensitivity in skeletal muscle by ARB.

It has been indicated that reactive oxygen species play a pivotal role in the development of insulin resistance and that Ang II is involved in the regulation of reactive oxygen species production. Furthermore, recent evidences suggested that the increase in oxidative stress is involved in the effects of Ang II on pressor response, vascular injury, and insulin resistance. In our study, administration of valsartan significantly decreased superoxide production in soleus muscle of KK-Ay mice, whereas treatment of PD123319 had no effect. These results suggest that the tissue superoxide production is regulated by AT1 receptor stimulation, whereas AT2 receptor stimulation does not play an important role.

Adipose tissue is another target organ influencing glucose metabolism, and AT1 and AT2 receptors are expressed in adipocytes. Therefore, it is possible that administration of

Figure 5. Effect of valsartan on insulin-induced PI 3-kinase activity in skeletal muscle of KK-Ay mice. PI 3-kinase assay was performed as described in Methods. Upper panel shows representative data from 4 separate experiments. Data are mean±SE. *P<0.05 vs control, **P<0.01 vs control.

Figure 6. Rate constant of 2-[3H]DG uptake in skeletal muscle and adipose tissue of AT2 receptor null mice. Insulin or saline was injected through a cardiac catheter 15 minutes before the injection of radioisotopes. Rate constant of 2-[3H]DG uptake was determined as described in Methods. Data are mean±SE; n=5. *P<0.05 vs control, **P<0.01 vs control, respectively. Agtr2+ indicates wild-type mice; Agtr2−, AT2 receptor null mice.
an ARB could regulate glucose uptake in adipose tissue by inhibition of AT$_1$ receptor signaling and stimulation of the uninhibited AT$_2$ receptor by unbound Ang II. It has been demonstrated that AT$_1$ receptor stimulation increases adipose cell size, whereas stimulation of the AT$_2$ receptor induces preadipocyte differentiation. Adipose tissue in obesity and type 2 diabetes tends to be abnormally hypertrophied and secretes Ang II and several cytokines, producing insulin resistance. In contrast, recent evidence indicates that Ang II inhibits in vitro adipogenic differentiation of human preadipocytes via the AT$_1$ receptor without effective AT$_2$ receptor stimulation and expression, whereas blockade of the AT$_2$ receptor leads to marked augmentation of adipogenesis. These interesting possibilities need to be investigated further to understand the roles of the AT$_1$ and AT$_2$ receptor subtypes in glucose metabolism.

**Perspectives**

Specific AT$_1$ receptor blockade by valsartan improved glucose tolerance and attenuated hyperglycemia and hyperinsulinemia in diabetic KK-Ay mice, associated with an increase in insulin-induced phosphorylation of IRS-1, PI 3-K activity, and translocation of GLUT4 to the plasma membrane in skeletal muscle, suggesting a direct interaction of signaling of the AT$_1$ receptor and insulin receptor in the pathogenesis of insulin resistance. Our results provide further information to understand the clinical relevance of the effect of ARB on insulin sensitivity and the onset of diabetes, thereby preventing cardiovascular events associated with insulin resistance.

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