Calcium Channel Blocker Azelnidipine Reduces Glucose Intolerance in Diabetic Mice via Different Mechanism Than Angiotensin Receptor Blocker Olmesartan

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ABSTRACT

The potential combined effect and mechanism of calcium channel blockers (CCB) and angiotensin II type 1 receptor blockers (ARB) to improve insulin resistance were investigated in type 2 diabetic KK-Ay mice, focusing on their antioxidative action. Treatment of KK-Ay mice with a CCB, azelnidipine (3 mg/kg/day), or with an ARB, olmesartan (3 mg/kg/day), for 2 weeks lowered the plasma concentrations of glucose and insulin in the fed state, attenuated the increase in plasma glucose in the oral glucose tolerance test (OGTT), and increased 2-[3H]deoxy-D-glucose (2-[3H]DG) uptake into skeletal muscle with the increase in translocation of glucose transporter 4 (GLUT4) to the plasma membrane. Both blockers also decreased the in situ superoxide production in skeletal muscle. The decrease in plasma concentrations of glucose and insulin in the fed state and superoxide production in skeletal muscle, as well as GLUT4 translocation to the plasma membrane, after azelnidipine administration was not significantly affected by coadministration of an antioxidant, 2,2,6,6-tetramethyl-1-piperidinyloxy (tempol). However, those changes caused by olmesartan were further improved by tempol. Moreover, olmesartan enhanced the insulin-induced tyrosine phosphorylation of insulin receptor substrate-1 induced in skeletal muscle, whereas azelnidipine did not change it. Coadministration of azelnidipine and olmesartan further decreased the plasma concentrations of glucose and insulin, improved OGTT, and increased 2-[3H]DG uptake in skeletal muscle. These results suggest that azelnidipine improved glucose intolerance mainly through inhibition of oxidative stress and enhanced the inhibitory effects of olmesartan on glucose intolerance, as well as the clinical possibility that the combination of CCB and ARB could be more effective than monotherapy in the treatment of insulin resistance.

Insulin resistance occurs in a wide variety of pathological states and is commonly associated with obesity, type 2 diabetes, accelerated atherosclerosis, and hypertension (Reaven et al., 1996; Zanella et al., 2001), which are often associated with increased oxidative stress (Cai and Harrison, 2000; Dzau, 2001; Taniyama and Griendling, 2003). It has been suggested that reactive oxygen species (ROS) modulate various biological functions by stimulating transduction signals, some of which are involved in the pathogenesis and complications of diabetes, and the increased production of ROS induced by hyperglycemia has also been suggested to be involved in redox regulation of glucose transport in skeletal muscle (Bonnefont-Rousselot, 2002). 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 uptake of 2-[3H]deoxy-D-glucose (2-[3H]DG) in response to insulin was lower and the superoxide production in skeletal muscle was higher in type 2 diabetic KK-Ay mice than in C57BL/6 mice (Shiuchi et al., 2004).

Calcium channel blockers (CCB) have been widely used for the treatment of patients with hypertension. The improvement of insulin sensitivity by CCB in various diabetic models has been reported (Bursztyn et al., 1994; Srinivasan et al., 2001). This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, the Japan Research Foundation for Clinical Pharmacology, the Tokyo Biochemical Research Foundation, and a grant from the Smoking Research Foundation. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.106.108894.

ABBREVIATIONS: ROS, reactive oxygen species; 2-[3H]DG, 2-[3H]deoxy-D-glucose; CCB, calcium channel blocker(s); Ang, angiotensin; AT1, angiotensin II type 1; ARB, AT1 receptor blocker; OGTT, oral glucose tolerance test; IRS-1, insulin receptor substrate-1; GLUT, glucose transporter; DHE, dihydroethidium; SOD, superoxide dismutase; PI3K, phosphatidylinositol 3′-kinase.
Individuals from 8 weeks of age in plastic cages at 25°C were obtained from Clea Japan, Inc. (Tokyo, Japan) and housed as previously described (Harada et al., 1999; Takada et al., 2001). In previous studies, CCB showed antioxidative effect (Ma et al., 1992; Taddei et al., 2001). On the other hand, recent studies also revealed that angiotensin (Ang) II type 1 (AT₁) receptor blocker (ARB) improved diabetes in an animal model (Pugsley, 2005) and in patients (Lindholm et al., 2002). It has also been reported that both CCB and ARB can attenuate oxidative stress (Ogihara et al., 2002; Jinno et al., 2004). We previously observed that an ARB, valsartan, enhanced insulin-induced signaling but decreased superoxide production in skeletal muscle of KK-Ay mice (Shiuchi et al., 2004).

These results suggest the possibility that combination therapy with an ARB and CCB could more effectively improve insulin sensitivity than monotherapy. In this article, we examined the possibility that a dihydropyridine CCB, azelnidipine, could improve insulin sensitivity in KK-Ay mice, especially focusing on the antioxidative action.

Materials and Methods

Animals and Treatment. Male KK-Ay/Ta and C57BL/6J mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and housed individually from 8 weeks of age in plastic cages at 25 ± 1°C with lighting on from 6:00 AM to 6:00 PM. KK-Ay mice result from a cross between glucose-intolerant black KK female mice and male yellow obese Ay mice and are known to serve as an excellent model of type 2 diabetes (Suto et al., 1998). They were given a standard diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. KK-Ay mice at 8 weeks of age were administered azelnidipine (CS-905; 3 mg/kg/day, donated by Sankyo Pharmaceutical Co., Tokyo, Japan) p.o. for 2 weeks and/or olmesartan (RNI-6270; 3 mg/kg/day, donated by Sankyo Pharmaceutical Co.) for 2 weeks using an osmotic minipump implanted i.p. Tempol (2,2,6,6-tetramethyl-1-piperidinyloxy, radical; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was administered in the drinking water (10 mM) for 14 days. Blood pressure was measured by the indirect tail-cuff method (MK-1030; Muramachi Kikai Co. Ltd., Tokyo, Japan). C57BL/6 mice are generally used as nondiabetic controls for KK-Ay mice. All the experimental procedures were approved and carried out in compliance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (Bethesda, MD) and with the guidelines of the Ehime University School of Medicine Committee on Animals.

Oral Glucose Tolerance Test. The oral glucose tolerance test (OGTT) was performed after a 16-h overnight fasting. Glucose (2 g/kg) was administered p.o., and blood was collected from the orbital sinus at 0, 30, 60, and 120 min.

Measurement of Rate Constant of Net Tissue Uptake of 2-[3H]DG. Uptake of 2-[3H]DG in peripheral tissues was measured as previously reported (Shiuchi et al., 2000, 2004). Skeletal muscles (extensor digitorum longus, 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 (Sudo et al., 1991).

Tissue Protein Sample Extraction, Immunoprecipitation, and Immunoblotting. After overnight fasting, 0.1 ml of insulin (1.0 U/kg) was injected through the portal vein. Hind limb skeletal muscles were removed 3 min after the injection and homogenized as described previously (Shiuchi et al., 2002). Equal amounts of protein (0.5–1.0 mg) of supernatants were incubated at 4°C with anti-insulin receptor substrate (IRS)-1 antibody (Upstate Biotechnology, Lake Placid, NY) overnight with constant agitation and then further incubated with protein G-Sepharose 4 Fast Flow (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h as described previously (Shiuchi et al., 2002). For immunoblotting, whole cell lysates (20 μg for insulin receptor β subunit) or immunoprecipitates (whole fraction for IRS-1) were denatured and subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were incubated with anti-insulin receptor β subunit antibody (Transduction Laboratories, Lexington, KY), antiphosphotyrosine antibody (4G10; Upstate Biotechnology), or anti-IRS-1 antibody, and then bands were visualized with an enhanced chemiluminescence system (GE Healthcare) using Lumino-Image-Analyzer (LAS3000mini; Fuji Photo Film Co., Ltd., Tokyo, Japan). Plasma membrane fraction was prepared, and a 40-μg aliquot was applied for immunoblot with anti-glucose transporter 4 (GLUT4) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and performed as described previously (Shiuchi et al., 2002).

Plasma Glucose and Insulin Concentrations. Plasma glucose and plasma insulin were measured using commercial kits (Glucose B test; Wako Pure Chemical Industries, Ltd., Osaka, Japan, and Insulin measurement kit; Morinaga, Tokyo, Japan, respectively).

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 μM, purchased from Molecular Probes, Inc., Eugene, OR) in phosphate-buffered saline for 30 min at 37°C in a humidified chamber protected from light (Szocs et al., 2002). On the tissue specimen, when superoxide is produced, 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 a fluorescent microscope (Axioskop 2 Plus with AxioCam; Carl Zeiss, Oberkochen, Germany) equipped with a computer-based imaging system. The intensity of red fluorescence was analyzed and quantified using computer-imaging software (Densitograph; ATTO Corporation, Tokyo, Japan). With this software, we could measure the area of the red-colored part and express the value as pixels. For the scavenge treatment experiment, the samples were incubated with superoxide dismutase (SOD; 0.5 mg/ml; Wako Pure Chemical Industries, Ltd.) from 20 min before and during DHE staining.

Statistical Analysis. All the values are expressed as mean ± S.E. The effects of the different treatments on all the data were evaluated with factorial analysis of variance. When a significant effect was found, the results were further compared with Bonferroni’s multiple range test. A difference of p < 0.05 was considered significant.

Results

Effects of Azelnidipine and/or Olmesartan on Glucose Tolerance in KK-Ay Mice. To investigate whether a CCB, azelnidipine, 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/6 mice, which are generally used as nondiabetic controls for KK-Ay mice (Shiuchi et al., 2004). Similar results were observed in OGTT, shown in Fig. 1. We administered a nonhypotensive dose of azelnidipine (3 mg/kg/day) for 2 weeks to KK-Ay mice at 8 weeks of age, which already showed hyperglycemia and hyperinsulinemia. In OGTT using these mice, azelnidipine decreased the peak level of plasma glucose concentration at 30 min after the glucose load, and the plasma glucose concentration decreased more rapidly compared with that in vehicle-treated KK-Ay mice (Fig. 1A). Azelnidipine slightly attenuated the peak level of plasma glucose concentration only at 30 min after the glucose load in C57BL/6 mice. On the other hand, a nonhypotensive dose of olmesartan (3 mg/kg/day), an ARB, also decreased the peak plasma glucose concentration. Plasma glucose level decreased more rapidly with olmesartan than that in vehicle-treated KK-Ay mice (Fig. 1A), as previously reported with an ARB, valsartan (Shiuchi et al., 2004).
et al., 2004). Moreover, coadministration of azelnidipine and olmesartan further decreased the increase in plasma glucose concentration (Fig. 1A), without significant change in blood pressure (100.3 ± 2.5 mm Hg in control, 95.8 ± 2.0 mm Hg in azelnidipine, 96.0 ± 2.2 mm Hg in olmesartan, and 92.9 ± 3.8 mm Hg in the combination group, respectively). Plasma insulin concentration was higher in KK-Ay mice than in C57BL/6 mice (Fig. 1B). However, we observed no significant change in plasma insulin concentrations during OGTT among the experimental groups of KK-Ay mice (Fig. 1B). Basal plasma glucose and insulin concentrations of KK-Ay mice at 10 weeks of age in the fasting condition were similar in each group.

**Effects of Azelnidipine and/or Olmesartan on Glucose Uptake and Oxidative Stress in Skeletal Muscle of KK-Ay Mice.** It is well known that the impairment of glucose metabolism in peripheral tissues, such as skeletal muscle, plays a critical role in the development of insulin resistance. To further determine whether azelnidipine could enhance glucose transport in skeletal muscle, we examined the effect of azelnidipine on 2-[3H]DG uptake in skeletal muscle of KK-Ay mice. As shown in Fig. 2, azelnidipine significantly increased 2-[3H]DG uptake in skeletal muscle under a fed condition. Next, we examined the role of oxidative stress in 2-[3H]DG uptake in skeletal muscle. Administration of an antioxidant, tempol, increased 2-[3H]DG uptake in skeletal muscle, whereas the addition of azelnidipine to tempol did not further increase 2-[3H]DG uptake in skeletal muscle (Fig. 2). These results suggest that the increase in 2-[3H]DG uptake in skeletal muscle mediated by azelnidipine is mainly caused by a decrease in oxidative stress. With in situ detec-
tion of superoxide using DHE, azelnidipine decreased superoxide production in soleus muscle of KK-Ay mice (Fig. 3). The addition of tempol to azelnidipine did not further decrease the superoxide production. On the other hand, olmesartan also increased 2-[3H]DG uptake in skeletal muscles (Fig. 2). In contrast, the increase in 2-[3H]DG uptake by olmesartan at this dose (3 mg/kg/day) was further enhanced with the addition of tempol. Similar enhancement of the increase in 2-[3H]DG uptake was observed by coadministration of azelnidipine with olmesartan (Fig. 2). Superoxide production in soleus muscle was also partly inhibited by this dose of olmesartan, although the inhibition was weaker than that by azelnidipine (42% by olmesartan versus 68% by azelnidipine, respectively) (Fig. 3). Consistent with these results of 2-[3H]DG uptake, plasma glucose and insulin concentrations decreased with administration of azelnidipine, olmesartan, or tempol and further decreased with coadministration of azelnidipine and olmesartan (Fig. 4). Treatment with tempol did not significantly affect the inhibitory action of azelnidipine on these parameters but further enhanced the inhibitory effects of olmesartan (Fig. 4). These results suggested that olmesartan could exert additional mechanisms, such as enhancing IRS-1/phosphatidylinositol 3'-kinase (PI3K) cascade, in addition to antioxidative effects. This possible mechanism by olmesartan was further examined in the following experiments.

Effects of Azelnidipine and/or Olmesartan on IRS-1 and GLUT4 in KK-Ay Mice. To examine the possibility that azelnidipine enhances insulin-mediated signaling, thereby increasing GLUT4 translocation to the plasma membrane, we focused on the tyrosine phosphorylation of IRS-1 in skeletal muscle of KK-Ay mice. As shown in Fig. 5A, neither azelnidipine nor tempol influenced tyrosine phosphorylation of IRS-1. Consistent with our previous observation using the ARB valsartan (Shiuchi et al., 2004), treatment with olmesartan increased the tyrosine phosphorylation of IRS-1 and further enhanced insulin-induced phosphorylation of IRS-1 in skeletal muscle of KK-Ay (Fig. 5B). The addition of tempol did not change tyrosine phosphorylation of IRS-1 mediated by olmesartan. These results suggest that olmesartan could enhance tyrosine phosphorylation of IRS-1 independent of its antioxidative stress effect. Total protein levels of the insulin receptor and IRS-1 were not changed by these treatments (Fig. 5C). We next examined GLUT translocation to the plasma membrane as shown in Fig. 6. Figure 6 shows the changes in GLUT4 in plasma membrane fraction prepared from skeletal muscle. Azelnidipine and olmesartan increased GLUT4 level in plasma membrane, whereas these drugs did not change GLUT4 protein levels in total cell lysate. Combination of azelnidipine and olmesartan further increased GLUT4 level in plasma membrane. Moreover, tempol also increased GLUT4 level in plasma membrane.
Tempol did not affect the action of azelnidipine on GLUT4 translocation, but it further increased the effect of olmesartan on GLUT4 translocation (Fig. 6).

Discussion

Insulin resistance is often associated with hypertension, and the effects of antihypertensive drugs on insulin resistance have been highlighted. In the present article, we showed that a nonhypotensive dose of a CCB, azelnidipine, improved glucose intolerance and superoxide production in skeletal muscle of KK-Ay mice without change in IRS-1 tyrosine phosphorylation. Similar results were observed with a nonhypotensive dose of an ARB, olmesartan, with the increase in IRS-1 phosphorylation. Moreover, the combination of azelnidipine and olmesartan improved further the glucose intolerance and glucose uptake in skeletal muscle more than each drug alone. In our study, KK-Ay mice at this age did not show the defect of insulin secretion; rather, they showed an increase in insulin secretion at 10 weeks of age. In our previous study, plasma insulin concentration of KK-Ay mice in a fasted condition was higher than that in C57BL/6 mice (Shiuchi et al., 2004). Moreover, the response of insulin secretion in OGTT still existed in KK-Ay mice. Similar changes in plasma insulin concentration were also observed in the present study (Fig. 1B). These results suggest that insulin secretion in KK-Ay mice is not significantly impaired. Moreover, plasma insulin concentration was not significantly influenced by azelnidipine, olmesartan, and the combination. In the present study, however, azelnidipine reduced plasma insulin concentration in the fed state (Fig. 4). This decrease may not be the result of a direct action of azelnidipine on pancreatic islet but rather caused by a secondary response correlated with the improvement of plasma glucose level. Therefore, the inhibitory action of azelnidipine and/or olmesartan was not caused by elevation of insulin secretion.

It has been indicated that ROS in skeletal muscle may play a pivotal role in the development of insulin resistance (Bonfond-Rousselot, 2002). In skeletal muscle, sarcoplasmic reticulum contains an NADH-dependent oxidase that reduces molecular oxygen to generate superoxide (Xia et al., 2003). We also reported that in situ superoxide production in soleus muscle was higher in KK-Ay mice than in nondiabetic C57BL/6 mice (Shiuchi et al., 2004). In the present study, treatment of mice with an antioxidant, tempol, alone reduced in situ superoxide production in skeletal muscle and enhanced glucose uptake into skeletal muscle in KK-Ay mice. Tempol also decreased plasma concentration of glucose and insulin in the fed state. These results suggest that the inhibition of superoxide production improves insulin resistance.
in KK-Ay mice. Similar to tempol, azelnidipine reduced in situ superoxide production in skeletal muscle (Fig. 3). This reduction of superoxide production by azelnidipine, as well as the improvement of insulin resistance, was not influenced by coadministration of tempol (Figs. 2 and 3). Previous reports also indicate the antioxidative action of azelnidipine (Shinomiya et al., 2004; Yamagishi et al., 2004; Suzuki et al., 2005). Azelnidipine could reduce the expression of subunits of NADPH oxidase (Suzuki et al., 2005), a key enzyme in superoxide production, or NADPH oxidase-mediated ROS generation (Yamagishi et al., 2004). These results suggest that oxidative stress is involved in the pathogenesis of insulin resistance in KK-Ay mice and that the improvement of glucose intolerance by azelnidipine is, at least partly, because of its antioxidative action. In the present study, administration of tempol and azelnidipine did not change the tyrosine phosphorylation of IRS-1 induced by insulin (Fig. 2). However, azelnidipine or tempol increased the translocation of GLUT4 to the plasma membrane in skeletal muscle (Fig. 6), whereas tempol enhanced the effect of azelnidipine. In contrast, tempol increased olmesartan-mediated GLUT4 translocation, suggesting that oxidative stress could impair the insulin signaling at the point downstream from IRS-1 activation in KK-Ay mice. In the improvement of glucose intolerance, neither azelnidipine nor olmesartan seemed to act directly on pancreatic islet or on the regulation of insulin secretion because insulin response in OGTT was not significantly changed by these drugs (Fig. 1B). However, azelnidipine modulated intracellular signaling of insulin and Ang II by blockade of calcium channel. These intracellular actions of azelnidipine seem to be, at least partly, mediated by the inhibition of oxidative stress.

Recent studies suggest that Ang II might negatively modulate insulin-mediated actions by regulating insulin signaling (Velloso et al., 1996; Folli et al., 1997, 1999). The major effects of Ang II are mediated through AT1 receptor. We have recently reported that the ARB valsartan improves glucose intolerance in KK-Ay mice by enhancing phosphorylation of IRS-1 induced by insulin, the association of IRS-1 with the p85 regulatory subunit of PI3K, PI3K activity, and translocation of GLUT4 to the plasma membrane (Shiuchi et al., 2004). Henriksen et al. (2001) reported that another ARB, irbesartan, increased GLUT4 protein expression in the skeletal muscle and heart of obese Zucker rats. These results suggest that blockade of AT1 receptor stimulation increases insulin action on glucose utilization. It is suggested that the oxidative stress is involved in the impairment of the insulin signaling induced by Ang II (Oghara et al., 2002). However, in our study, olmesartan at 3 mg/kg/day reduced superoxide production in skeletal muscle only partially (Fig. 3). The inhibitory effects of olmesartan on in situ superoxide production and glucose uptake in skeletal muscle were further improved or tended to be improved by addition of tempol (Figs. 2 and 3). These results suggest that olmesartan improved insulin resistance in KK-Ay mice partly by its antioxidative action but also by other mechanisms through blockade of AT1 receptor-mediated signaling. Indeed, we observed that olmesartan increased insulin-mediated tyrosine phosphorylation of IRS-1 in skeletal muscle, which was not influenced by tempol. Previous reports, including our study, have used various techniques to examine insulin intolerance and applied an Ang II or ARB for different duration (acutely or chronically) and used different experimental models, suggesting that this potential discrepancy would be the result of different experimental procedures and/or species difference.

It also may be possible that the improvement of insulin resistance by azelnidipine is caused by the change in muscle blood flow. A previous report suggested that glucose intolerance in spontaneously hypertensive rats is improved through vasodilation caused by the CCB nitrendipine (Bursztyn et al., 1994). Takada et al. (2001) also reported that the CCB cilnidipine may improve insulin resistance in fructose-fed rats by increasing muscle blood flow. However, the change in blood flow may not play an important role in the improvement of glucose intolerance by azelnidipine or olmesartan in our study because the dose of these drugs used in our study did not affect systemic blood pressure.

It has been previously reported that the combination of Ca2+-channel blockade by verapamil and angiotensin-converting enzyme inhibition by trandolapril seems to cause possible synergistic effects in patients with type 2 diabetes with essential hypertension (Rett et al., 1994). Other clinical and preclinical animal studies suggest that the combination of an angiotensin-converting enzyme inhibitor and a CCB may be effective in improving insulin resistance (Dal Ponte et al., 1998, Lender et al., 1999). In the present study, we showed that coadministration of azelnidipine and olmesartan further improved glucose intolerance in diabetic KK-Ay mice compared with the single use of each drug. Moreover, the combined action of azelnidipine and olmesartan is, at least partly, caused by the different mechanism of action between these drugs; i.e., the effects of azelnidipine are mainly mediated by antioxidant action, but those of olmesartan include other pathways through AT1 receptor blockade resulting in the increase of IRS-1 phosphorylation, which seemed to be independent of enhanced oxidative stress in this mouse model. Taken together, our results provide evidence of further improvement of glucose intolerance with the combination of azelnidipine and olmesartan, and further extend the clinical notion that the combination of CCB and ARB could be more effective in the treatment of insulin resistance and hypertension than monotherapy.

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