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

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Running title: Azelnidipine and olmesartan improve glucose intolerance

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List of abbreviations: CCB, calcium channel blocker; Ang, angiotensin; ARB, AT_1 receptor blocker; AT_1, angiotensin II type 1; ACE, angiotensin converting enzyme; ROS, reactive oxygen species; EDL, extensor digitorum longus; DHE, dihydroethidium; 2-[^{3}H]DG, 2-[^{3}H]deoxy-D-glucose; GLUT, glucose transporter; IRS-1, insulin receptor substrate-1; SOD, superoxide dismutase; PI3 kinase, phosphatidylinositol 3' kinase

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Abstract

The potential combined effect and mechanism of calcium channel blockers (CCB) and angiotensin II type 1 (AT_1) receptor blockers (ARB) to improve insulin resistance were investigated in type 2 diabetic KK-Ay mice, focusing on their anti-oxidative 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 fed state, attenuated the increase in plasma glucose in oral glucose tolerance test (OGTT), and increased 2-[^{3}H]deoxy-D-glucose (2-[^{3}H]DG) uptake into skeletal muscle with the increase in translocation of 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 fed state and superoxide production in skeletal muscle, GLUT4 translocation to the plasma mrembrane by azelnidipine was not significantly affected by co-administration of an antioxidant, tempol. However, those changes caused by olmesartan were further improved by tempol. Moreover, olmesartan enhanced the insulin-induced tyrosine-phosphorylation of IRS-1 induced in skeletal muscle, whereas azelnidipine did not change it. Co-administration of azelnidipine and olmesartan further decreased the plasma concentrations of glucose and insulin, improved OGTT, and increased

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2-[^{3}H]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, and the clinical possibility that the combination of CCB and ARB could be more effective than monotherapy in the treatment of insulin resistance.

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Introduction

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-[^{3}H]deoxy-D-glucose (2-[^{3}H]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 in 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., 1997; Harada et al., 1999;

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Takada et al., 2001). In previous reports, CCB showed anti-oxidant effect (Mak et al., 1992; Taddei et al., 2001). On the other hand, recent studies also revealed that angiotensin (Ang) II type 1 (AT_1) receptor blocker (ARB) improved diabetes in animal model (Pugsley, 2005) and 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 paper, we examined the possibility that a dihydropyridine CCB, azelnidipine, could improve insulin sensitivity in KK-Ay mice, especially focusing on the anti-oxidative action.

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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 to 18:00. KK-Ay mice result from a cross between glucose-intolerant black KK female mice and male yellow obese A^y 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) orally for 2 weeks and/or olmesartan (RNH-6270; 3 mg/kg/day, donated by Sankyo Pharmaceutical Co.) for 2 weeks using an osmotic minipump implanted intraperitoneally. Tempol (2,2,6,6-tetramethyl-1-piperidinyloxy, radical; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was administered in the drinking water (10 mmol/L) for 14 days. Blood pressure was measured by the indirect tail-cuff method (MK-1030, Muromachi Kikai Co. Ltd., Tokyo, Japan). C57BL/6 mice are generally used as non-diabetic controls for KK-Ay mice. All 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 U.S. National Institutes of Health and with the guidelines of

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the Ehime University School of Medicine Committee on Animals.

Oral Glucose Tolerance Test (OGTT). The OGTT was performed after a 16-hour overnight fasting. Glucose (2 g/kg) was administered orally, and blood was collected from the orbital sinus at 0, 30, 60, and 120 minutes.

Measurement of Rate Constant of Net Tissue Uptake of 2-[^{3}H]deoxy-D-glucose. Uptake of 2-[^{3}H]deoxy-D-glucose (2-[^{3}H]DG) in peripheral tissues was measured as previously reported (Shiuchi et al., 2000; Shiuchi et al., 2004). 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-[^{3}H]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 minutes after the injection and homogenized as previously described (Shiuchi et al., 2002). Equal amounts of protein (0.5-1.0 mg) of

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supernatants were incubated at 4°C with anti-insulin receptor substrate (IRS)-1 antibody (Upstate Biotechnology Inc.) overnight with constant agitation, and then further incubated with protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) for 1 hour as previously described (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-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated with anti-insulin receptor β subunit antibody (Transduction Laboratory), anti-phosphotyrosine antibody (4G10) (Upstate Biotechnology Inc.) or anti-IRS-1 antibody, and then bands were visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech) using Lumino-Image-Analyzer (LAS3000mini, Fuji Photo Film Co., Ltd., Tokyo, Japan) . Plasma membrane fraction was prepared and 40 μg aliquot was applied for immunoblot with anti-glucose transporter 4 (GLUT4) antibody (Santa Cruz Biotechnology) was performed as previously described (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; Insulin measurement kit, Morinaga, Japan, respectively).

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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, purchased from Molecular Probes, Inc., Eugene, Oregon) in phosphate-buffered saline for 30 minutes 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 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., Osaka, Japan) from 20 minutes before and during DHE staining.

Statistical Analysis. All values are expressed as mean ± SE. The effects of the different

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treatments on all 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 with $p < 0.05$ was considered significant.

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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 employed KK-Ay mice as a model of type 2 diabetes in this study. We previously reported that KK-Ay mice showed the impaired glucose tolerance compared to that in C57BL/6 mice, which are generally used as non-diabetic controls for KK-Ay mice (Shiuchi et al., 2004). Similar results were observed in OGTT shown in Figure 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 minutes after the glucose load, and the plasma glucose concentration declined more rapidly compared to that in vehicle-treated KK-Ay mice (Figure 1). Azelnidipine slightly attenuated the peak level of plasma glucose concentration only at 30 minutes 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 declined more rapidly by olmesartan than that in vehicle-treated KK-Ay mice (Figure 1a), as previously

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reported with an ARB, valsartan (Shiuchi et al., 2004). Moreover, coadministration of azelnidipine and olmesartan further decreased the increase in plasma glucose concentration (Figure 1a), without significant change in blood pressure (100.3 ± 2.5 mmHg in control, 95.8 ± 2.0 mmHg in azelnidipine, 96.0 ± 2.2 mmHg in olmesartan and 92.9 ± 3.8 mmHg in combination group, respectively). Plasma insulin concentration was higher in KK-Ay mice than that in C57BL/6 mice (Figure 1b). However, we observed no significant change in plasma insulin concentrations during OGTT among the experimental groups of KK-Ay mice (Figure 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-[^{3}H]DG uptake in skeletal muscle of KK-Ay mice. As shown in Figure 2, azelnidipine significantly increased 2-[^{3}H]DG uptake in skeletal muscles under a fed condition. Next, we examined

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the role of oxidative stress in 2-[^{3}H]DG uptake in skeletal muscle. Administration of an antioxidant, tempol, increased 2-[^{3}H]DG uptake in skeletal muscle, whereas the addition of azelnidipine to tempol did not further increase 2-[^{3}H]DG uptake in skeletal muscle (Figure 2). These results suggest that the increase in 2-[^{3}H]DG uptake in skeletal muscle mediated by azelnidipine is mainly due to a decrease in oxidative stress. In *in situ* detection of superoxide using dihydroethidium, azelnidipine decreased superoxide production in soleus muscle of KK-Ay mice (Figure 3). Addition of tempol to azelnidipine did not further decrease the superoxide production. On the other hand, olmesartan also increased 2-[^{3}H]DG uptake in skeletal muscles (Figure 2). In contrast, the increase in 2-[^{3}H]DG uptake by olmesartan at this dose (3 mg/kg/day) was further enhanced with addition of tempol. Similar enhancement of the increase in 2-[^{3}H]DG uptake was observed by coadministration of azelnidipine with olmesartan (Figure 2). Superoxide production in soleus muscle was also partly inhibited by this dose of olmesartan, while the inhibition was weaker than that by azelnidipine (42% by olmesartan vs. 68% by azelnidipine, respectively; Figure 3). Consistent with these results of 2-[^{3}H]DG uptake, plasma glucose and insulin concentrations decreased with administration of azelnidipine, olmesartan or tempol, and further decreased with coadministration of azelnidipine and olmesartan (Figure 4). Treatment with tempol did

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not significantly affect the inhibitory action of azelnidipine on these parameters, but further enhanced the inhibitory effects of olmesartan (Figure 4). These results suggested that olmesartan could exert additional mechanism such as enhancing IRS-1/PI3 kinase cascade, in addition to anti-oxidative 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 Figure 5a, neither azelnidipine nor tempol influenced tyrosine-phosphorylation of IRS-1. Consistent with our previous observation using 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 (Figure 5b). Addition of tempol did not change tyrosine-phosphorylation of IRS-1 mediated by olmesartan. These results suggest that the olmesartan could enhance tyrosine-phosphorylation of IRS-1 independent of its anti-oxidative stress effect. Total protein levels of the insulin receptor and

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IRS-1 were not changed by these treatments (Figure 5c). We next examined GLUT translocation to the plasma membrane as shown in Figure 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 (Figure 6). Tempol did not affect the action of azelnidipine on GLUT4 translocation, but it further increased the effect of olmesartan on GLUT4 translocation (Figure 6).

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Discussion

Insulin resistant is often associated with hypertension, and the effects of antihypertensive drugs on insulin resistance have been highlighted. In the present paper, we demonstrated that a non-hypotensive 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 non-hypotensive 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 fasted condition was higher than that in C57BL/6 mice (Shiuchi et al., 2004). Moreover, the response of insulin secretion in OGTT was still existed in KK-Ay mice. Similar changes in plasma insulin concentration were also observed in the present study (Figure 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

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reduced plasma insulin concentration in fed state (Figure 4). This decrease may not be due to a direct action of azelnidipine on pancreatic islet, but due to a secondary response correlated with the improvement of plasma glucose level. Therefore, the inhibitory action of azelnidipine and/or olmesartan was not due to elevation of insulin secretion.

It has been indicated that ROS in skeletal muscle may play a pivotal role in the development of insulin resistance (Bonnefont-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 that in non-diabetic 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 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 (Figure 3). This reduction of superoxide production by azelnidipine as well as the improvement of insulin resistance were not influenced by co-administration of tempol (Figures 2 and 3). Previous reports also

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indicate the anti-oxidative 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, due to its anti-oxidative action. In the present study, administration of tempol as well as azelnidipine did not change the tyrosine-phosphorylation of IRS-1 induced by insulin (Figure 2). However, azelnidipine or tempol increased the translocation of GLUT4 to the plasma membrane in skeletal muscle (Figure 6), whereas tempol did enhance the effect of azelnidipine. In contrast, tempol increasd olmesartan-mediated GLUT 4 trasnlocation, 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, since insulin response in OGTT was not significantly changed by these drugs (Figure 1b). However, azelnidipine modulated intracellular signaling of insulin and Ang II by blockade of calcium channel. These intracellular actions of azelnidipine seem to

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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; Folli et al., 1999). The major effects of Ang II are mediated through AT_1 receptor. We have recently reported that an 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 PI 3-kinase, PI 3-kinase activity, and translocation of GLUT4 to the plasma membrane (Shiuchi et al., 2004). Henriksen et al. reported that the another AT_1 receptor blocker, irbesartan, increased GLUT4 protein expression in the skeletal muscle and heart of obese Zucker rats (Henriksen et al., 2001). These results suggest that blockade of AT_1 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 (Ogihara et al., 2002). However, in our study, olmesartan at 3 mg/kg/day reduced superoxide production in skeletal muscle only partially (Figure 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 (Figures 2 and 3). These results suggest that olmesartan improved insulin resistance in KK-Ay mice partly by its

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anti-oxidative action but also by other mechanisms through blockade of AT_1 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 has used various techniques to examine insulin intolerance, and applied an Ang II or ARB for different duration (acutelly or chronically), and employed different experimental models, suggesting that this potential discrepancy would be due to different experimental procedures and/or species difference.

It may be also possible that the improvement of insulin resistance by azelnidipine is caused by the change in muscle blood flow. Previous report suggested that glucose intolerance in SHR is improved through vasodilation caused by a CCB, nitrendipine (Bursztyn et al., 1994). Takada et al. (2001) also reported that a 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, since the dose of these drugs used in our study did not affect systemic blood pressure.

It has been previously reported that the combination of Ca^{2+}-channel blockade by verapamil and ACE inhibition by trandolapril appears to cause possible synergistic effects in

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patients of type II diabetes with essential hypertension (Rett et al., 1994). Other clinical and preclinical animal studies suggest that the combination of an ACE 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 demonstrated that coadminstration 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, due to the different mechanism of action between these drugs, i.e. the effects of azelnidipine are maily mediated by anti-oxidant action, but those of olmesartan includes other pathways through AT_1 receptor blockade resulting in the increase in the increase in IRS-1 phosphorylation, which seemed to be independent of enhanced oxidative stress in this mouse model. Taken together, our results provide evidences of further improvement of glucose intolerance with the combination of azelidipine 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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Footnotes

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Figure Legends

Figure 1. Effect of azelnidipine and olmesartan on oral glucose tolerance test (OGTT) in KK-Ay mice. KK-Ay mice were treated with azelnidipine and/or olmesartan for 2 weeks, and OGTT (2 g glucose/kg) was performed after overnight fasting as described in "Methods". Plasma glucose (a) and insulin (b) concentrations were shown. n= 4-8 for each group. Data are means ± SE. $^{*}p< 0.05$ vs. control of KK-Ay mice. $^{\#}p< 0.05$ vs azelnidipine or olmesartan alone in KK-Ay mice. $^{\dagger}p< 0.05$ vs control of C57BL/6J mice.

Figure 2. Effect of azelnidipine and olmesartan on glucose uptake in skeletal muscle of KK-Ay mice. Animals were treated with azelnidipine and olmesartan for 2 weeks as in Figure 1, and the rate constant of glucose uptake was determined by 2-[^{3}H]deoxyglucose method as described in "Methods". Data are mean ± SE. n=6-7 for each group. GASTRO-W: gastrocnemius white muscle, GASTRO-R: gastrocnemius red muscle, SOLEUS: soleus, EDL: extensor digitorum longus. $^{*,**}p< 0.05, 0.01$ vs. control, respectively. $^{\#}p< 0.05$ vs. olmesartan.

Figure 3. In situ detection of superoxide production in skeletal muscle of KK-Ay mice.

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Tissue samples were obtained from KK-Ay mice after treatment with azelnidipine, olmesaltan and tempol as described in "Methods". Histochemical detection of superoxide anion with dihydroethidium (DHE) was performed in soleus muscle as described in Methods. (a) DHE staining of soleus muscles treated with azelnidipine, olmesartan and tempol. Control with SOD (right lower corner): DHE staining after pretreatment of control tempol (-) sample with superoxide dismutase (SOD) as described in Methods. Magnification x200. (b) Intensity of fluorescence for superoxide in soleus muscles treated with azelnidipine, olmesartan and tempol. Intensity of SOD-treated sample was subtracted from the values in each group. Figures show representative results from five separate experiments. Data are mean ± SE. $^{*}p< 0.05$ vs. control.

Figure 4. Effect of azelnidipine and olmesartan on plasma concentration of glucose (a) and insulin (b) in KK-Ay mice in fed state. Animals were treated with azelnidipine, olmesartan and tempol as in Figure 2. Data are mean ± SE. n= 6-8 for each group. $^{*, **}p<0.05$, 0.01 vs. control, respectively.

Figure 5. Effect of azelnidipine and olmesartan on insulin-mediated tyrosine-

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phosphorylation of IRS-1 in skeletal muscle of KK-Ay mice. Animals were treated with azelnidipine, olmesartan and tempol for 2 weeks as described in “Methods”. Hind limb muscles were taken 3 minutes after insulin injection, and protein samples were prepared as described in “Methods”. (a) Effect of azelnidipine and tempol on insulin-mediated tyrosine-phosphorylation of IRS-1. (b) Effect of olmesartan and tempol on insulin-mediated tyrosine-phosphorylation of IRS-1. (c) Total protein level of IRS-1 and insulin receptor. Data of densitometric measurements are shown in lower panels of (a) and (b). Data are mean ± SE. IB: immunoblot, IP: immunoprecipitation, p-Tyr: phosphotyrosine. *, $^{**}p<0.05$, 0.01 vs. control, respectively. Figures show representative results from three separate experiments.

Figure 6. Effect of azelnidipine and olmesartan on insulin-mediated translocation of GLUT4 to plasma membrane of skeletal muscle in KK-Ay mice. Animals were treated with azelnidipine, olmesartan and tempol for 2 weeks as described in “Methods”. Hind limb muscles were taken 3 minutes after insulin injection, and plasma membrane fraction was prepared as described in “Methods”. (Upper) Representative result of immunoblot for GLUT4 in plasma membrane fraction and total cell lysate. (Lower) Densitometric

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measurements of GLUT4. Data are mean ± SE for three separate experiments. $^{*, **}p<0.05$, 0.01 vs. control, respectively.

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a

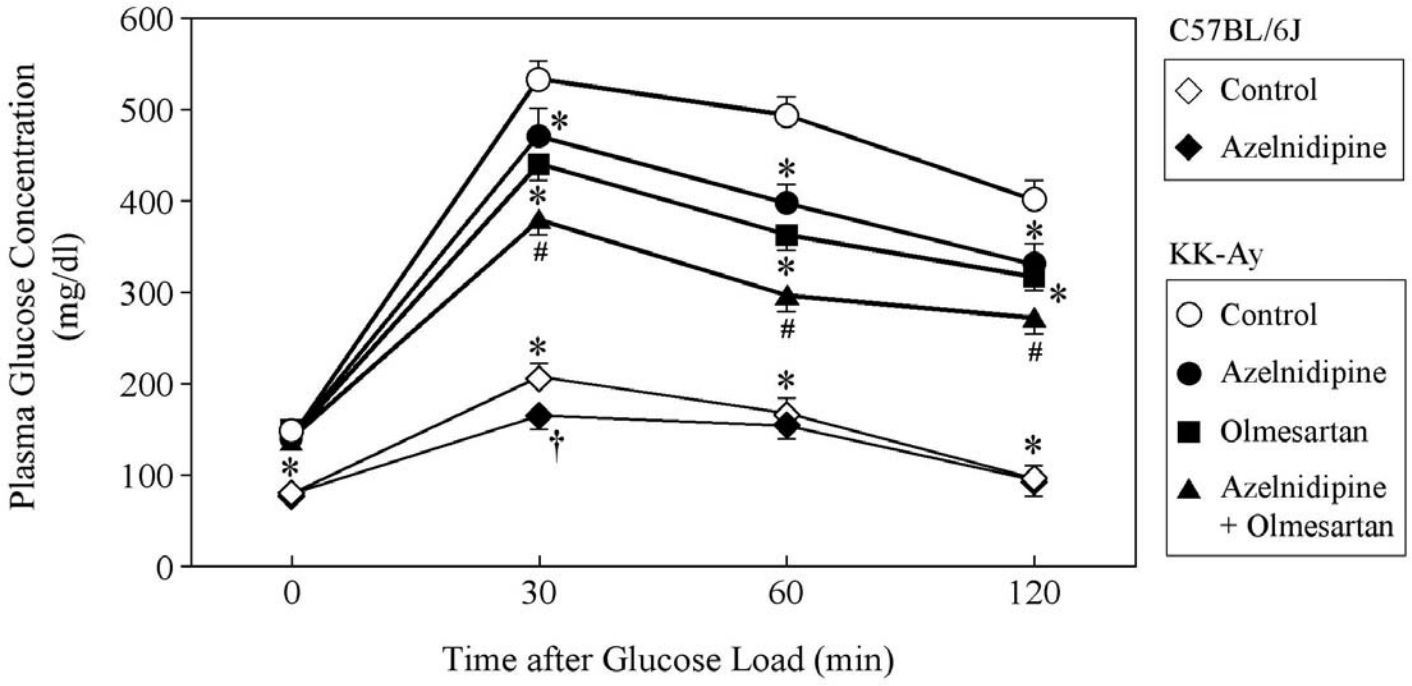

Figure 1a

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b

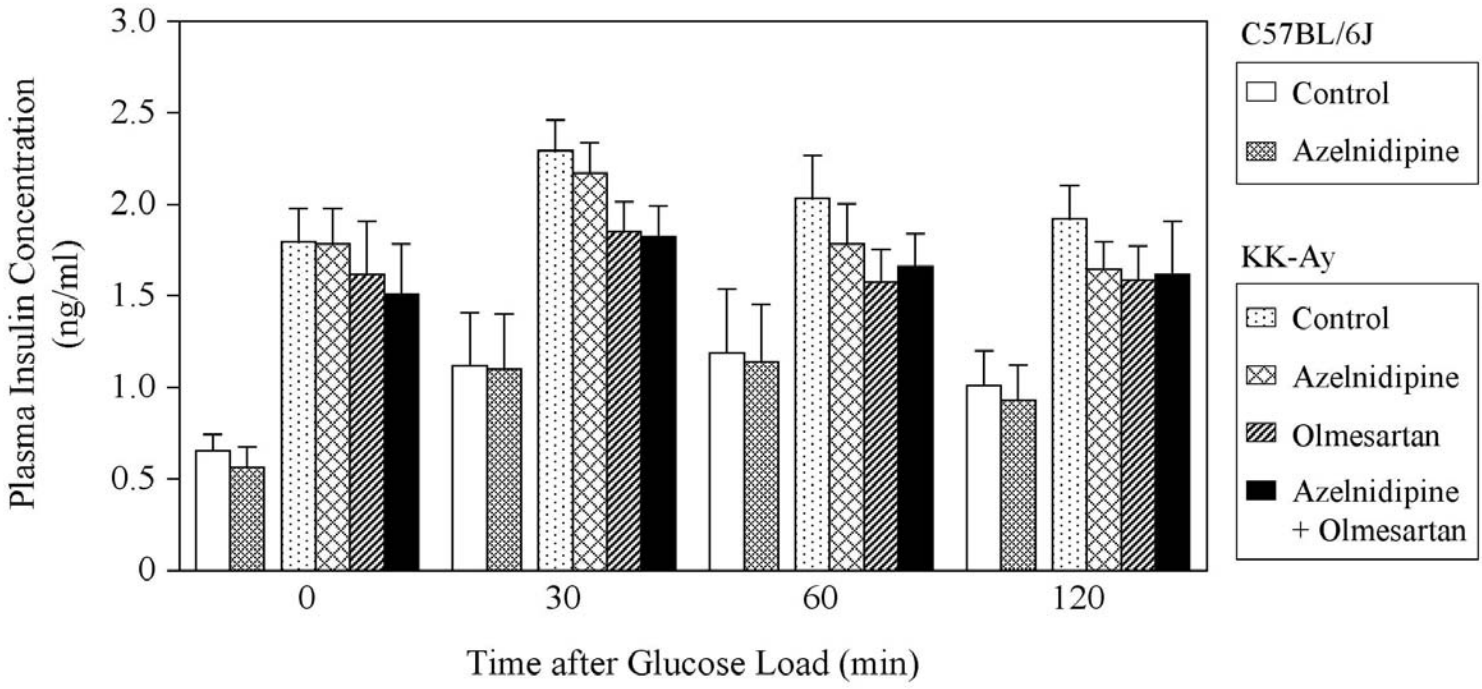

Figure 1b

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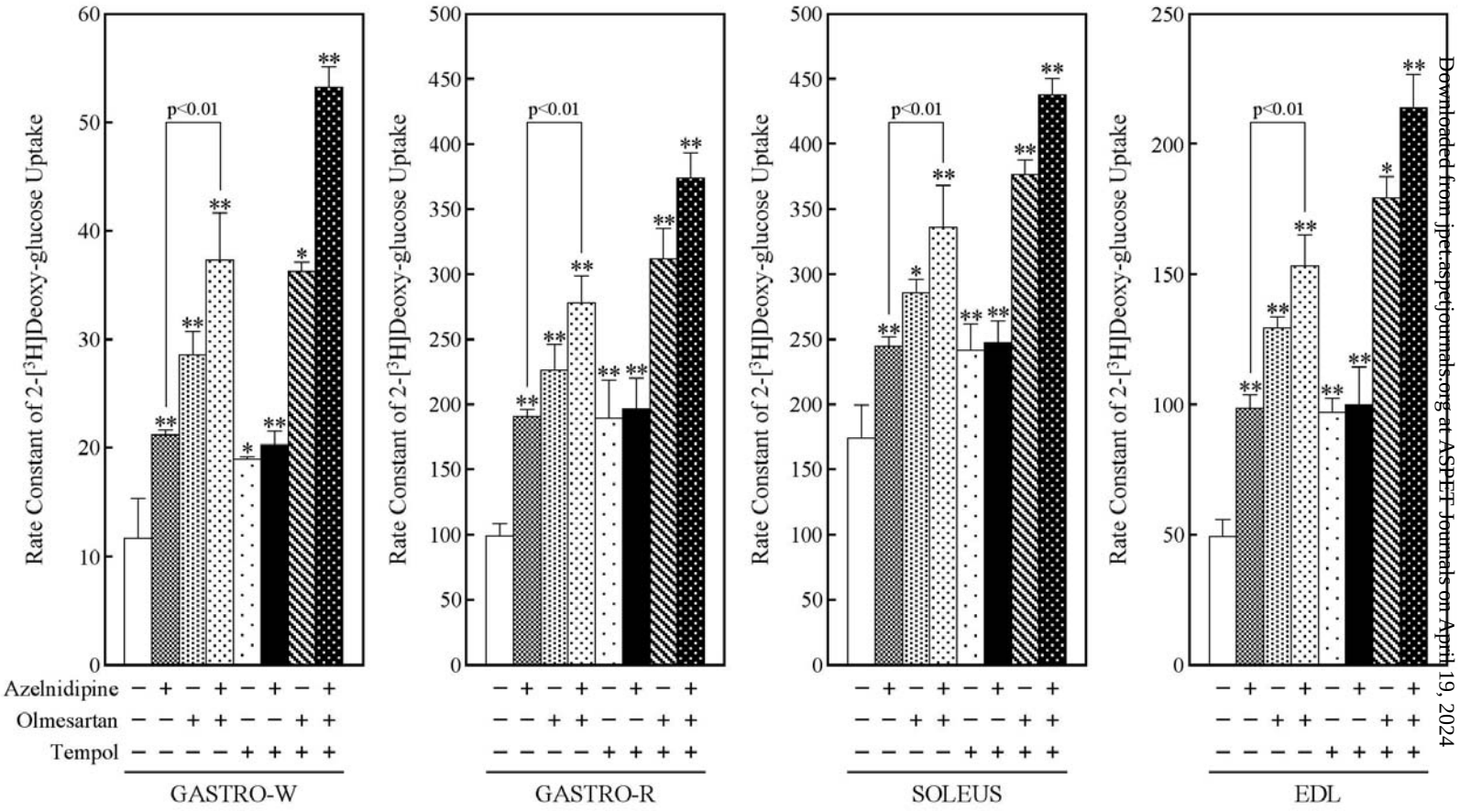

Figure 2

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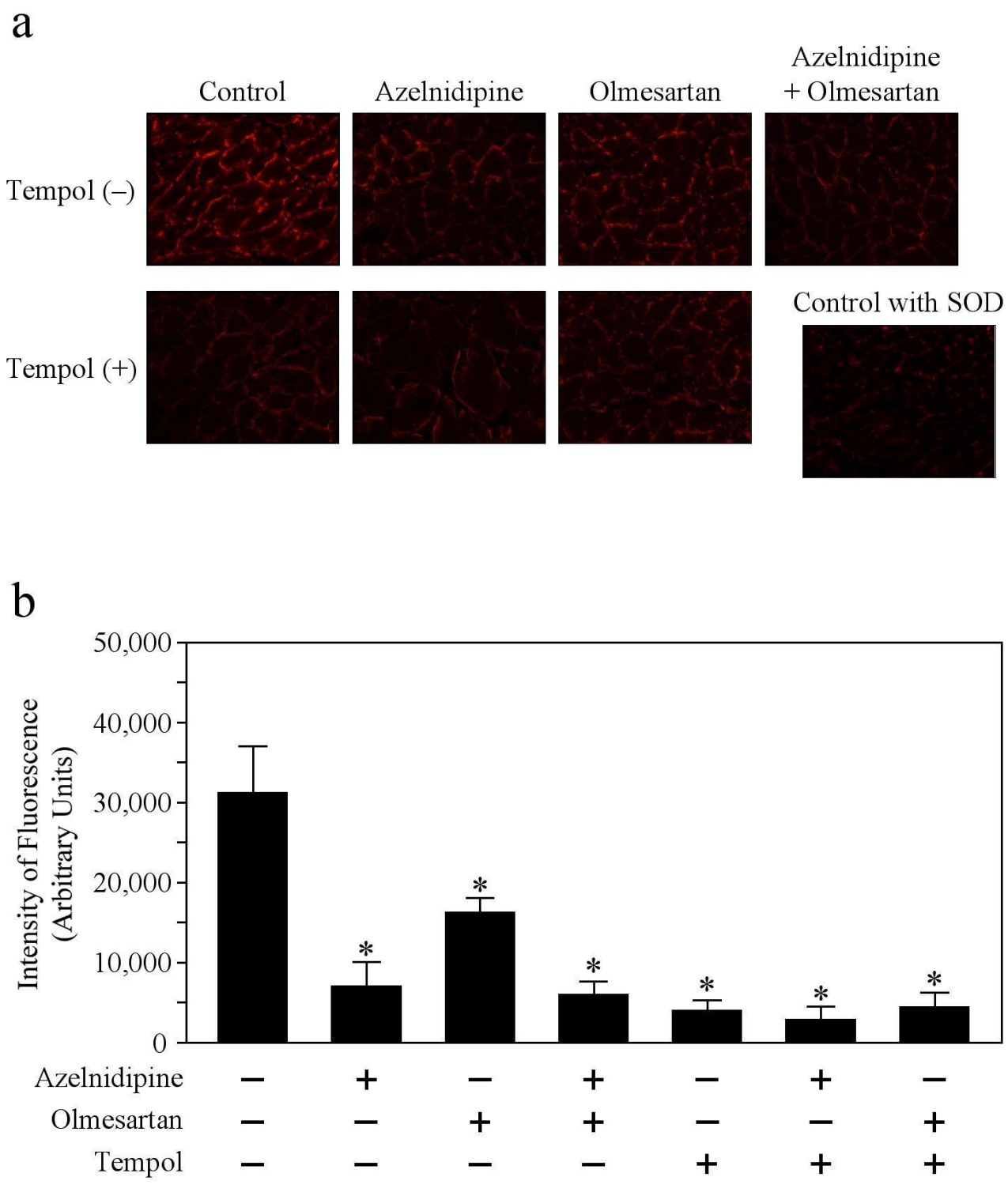

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Figure 3

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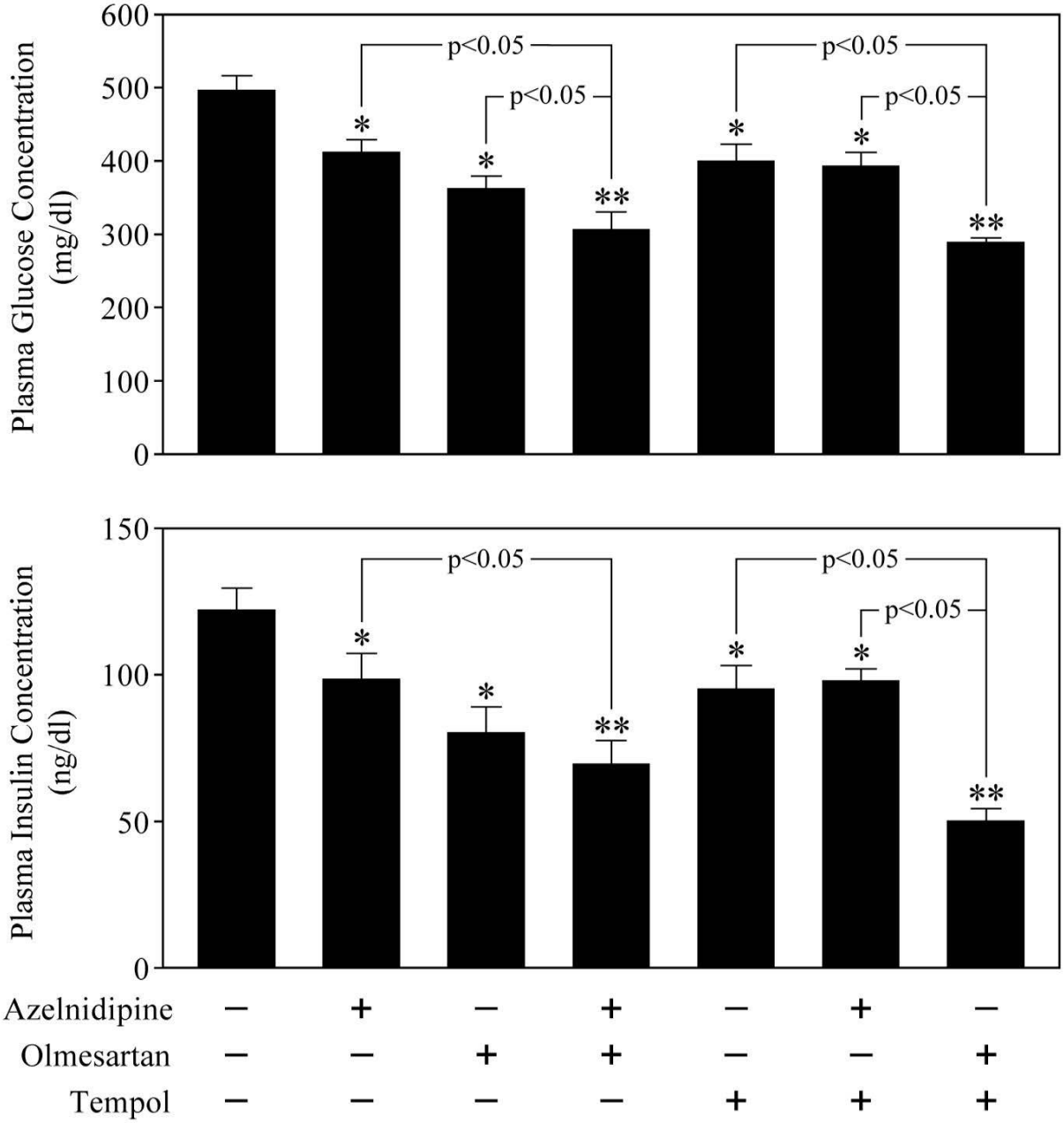

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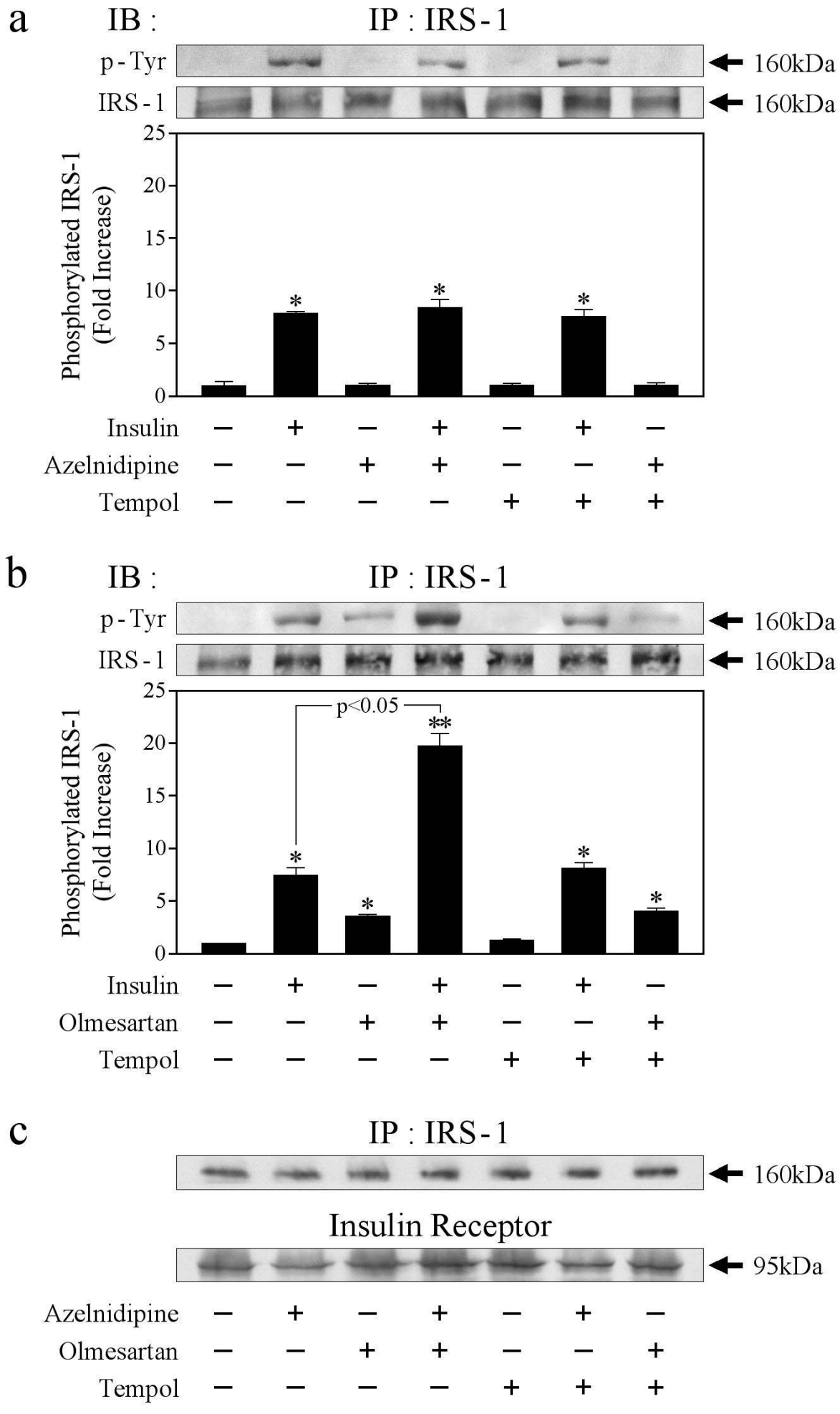

Figure 5

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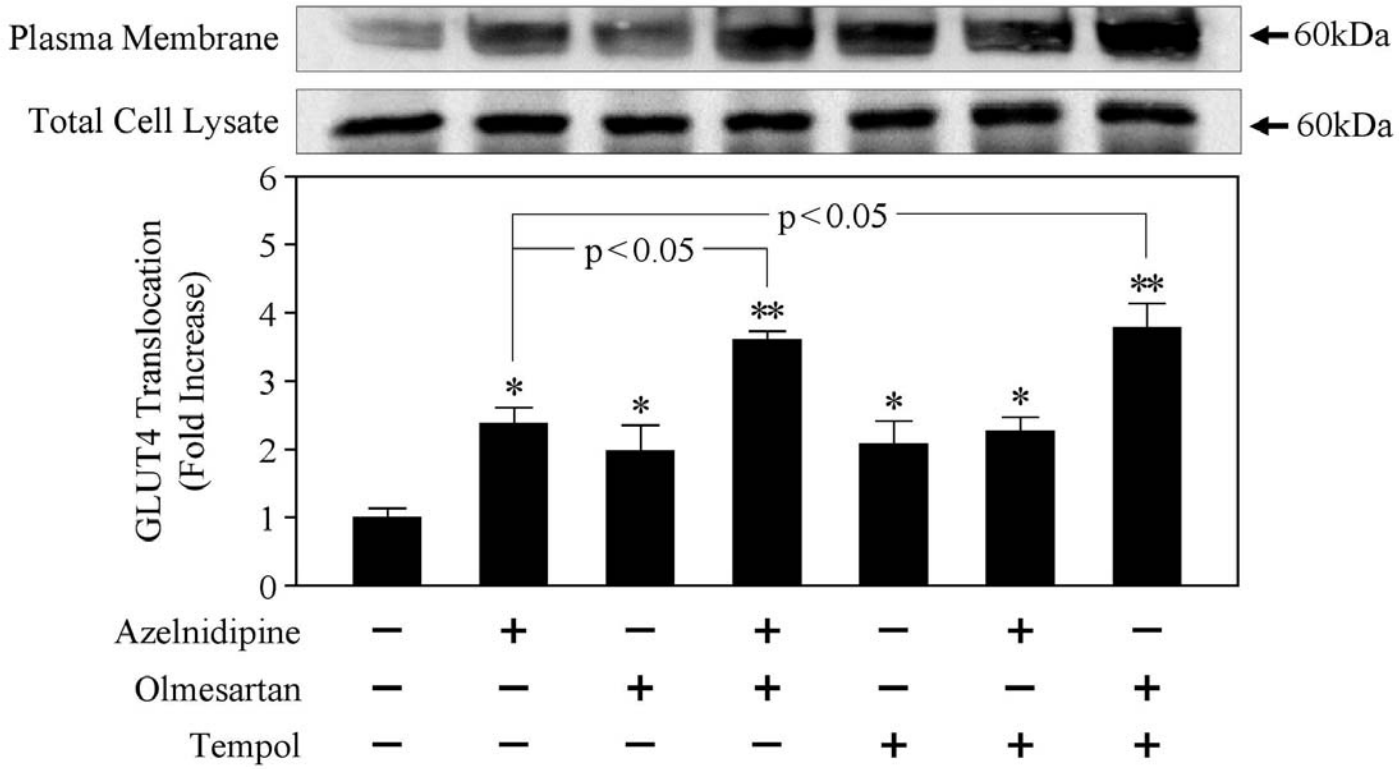

Figure 6

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