ACE Inhibitor Improves Insulin Resistance in Diabetic Mouse Via Bradykinin and NO

Tetsuya Shiuchi, Tai-Xing Cui, Lan Wu, Hironori Nakagami, Yuko Takeda-Matsubara, Masaru Iwai, Masatsugu Horiuchi

Abstract—Improvement of insulin resistance by ACE inhibitors has been suggested; however, this mechanism has not been proved. We postulated that activation of the bradykinin–nitric oxide (NO) system by an ACE inhibitor enhances glucose uptake in peripheral tissues by means of an increase in translocation of glucose transporter 4 (GLUT4), resulting in improvement of insulin resistance. Administration of an ACE inhibitor, temocapril, significantly decreased plasma glucose and insulin concentrations in type 2 diabetic mouse KK-Ay. Mice treated with temocapril showed a smaller plasma glucose increase after glucose load. We demonstrated that temocapril treatment significantly enhanced 2-[3H]-deoxy-D-glucose (2-DG) uptake in skeletal muscle but not in white adipose tissue. Administration of a bradykinin B2 receptor antagonist, Hoe140, or an NO synthase inhibitor, L-NAME, attenuated the enhanced glucose uptake by temocapril. Moreover, we observed that translocation of GLUT4 to the plasma membrane was significantly enhanced by temocapril treatment without influencing insulin receptor substrate-1 phosphorylation. In L6 skeletal muscle cells, 2-DG uptake was increased by temocapril, and Hoe140 inhibited this effect of temocapril but not that of insulin. These results suggest that temocapril would improve insulin resistance and glucose intolerance through increasing glucose uptake, especially in skeletal muscle at least in part through enhancement of the bradykinin-NO system and consequently GLUT4 translocation. (Hypertension. 2002;40:1111–1118.)

Key Words: angiotensin-converting enzyme ■ bradykinin ■ glucose ■ insulin resistance ■ nitric oxide

The earliest defect in the development of type 2 diabetes is insulin resistance, characterized by decreased glucose transport and metabolism in muscle and adipocytes. Glucose is cleared from the bloodstream by a family of facilitative transporters (glucose transporters, GLUTs). The glucose transporter GLUT4 mediates insulin-stimulated glucose uptake in adipocytes and muscle by rapidly moving from intracellular storage sites to the plasma membrane. In insulin-resistant states such as obesity and type 2 diabetes, GLUT4 expression is decreased in adipose tissue but preserved in muscle.

Insulin-resistant states are often associated with hypertension, and the effects of antihypertensive drugs on insulin resistance have been highlighted. Accumulated data indicate that ACE inhibitors have either no adverse effect on glucose control or insulin sensitivity or may even improve them. The variability of results between studies may relate to differences in experimental design, the degree of glycemia or insulin resistance, potassium balance, and the dose or duration of ACE inhibitor treatment, among others. The actions of ACE inhibitors are due in part to the decrease in angiotensin II formation and accumulation of kinins, and the resulting increase in nitric oxide (NO) production may also play a role.

Kishi et al proposed that bradykinin directly stimulates GLUT4 translocation and increases 2-deoxy-D-glucose (2-DG) uptake through an insulin-independent pathway in both 3T3-L1 adipocytes and L6 myotubes. Interestingly, NO synthase (NOS) is expressed in skeletal muscle, and it is well established that bradykinin increases eNOS activity, which consequently produces NO. We also reported that leptin enhanced bradykinin and/or the NO system, which contributed to enhanced glucose uptake in skeletal muscle, whereas insulin-mediated glucose uptake was not influenced by bradykinin and/or NO. These results suggest that the bradykinin-NO system plays an important role in glucose uptake in skeletal muscle independent of insulin. We postulate that activation of the bradykinin-NO system by ACE inhibitors enhances the translocation of GLUT4 in type 2 diabetic mice, KK-Ay, resulting in improvement of insulin resistance.

Methods

Animals and Treatment
Male KK-Ay/Ta and C57BL/6J mice were obtained from Nihon Clea (Tokyo, Japan) and housed individually from the age of 8 weeks in plastic cages at 25±1°C, with lighting on from 6:00 AM to 6:00 PM, and given a laboratory diet and water ad libitum. 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 excellent models of type 2 diabetes. 26 C57BL/6J mice are generally used as nondiabetic controls. All experimental procedures were approved and carried out in compliance with the guidelines of the Ehime University School of Medicine Committee on Animals. Temocapril (1 mg/kg per day) and/or L-NAME (30 mg/kg per day) (Funakoshi) were administered by gavage, and Hoe140 (0.1 mg/kg per day) (Pep tide Institute Inc) was administered through an osmotic mini pump (model 1002, Alza Corporation) implanted intraperitoneally. These drugs were administered to mice from 8 weeks of age for 14 days. All experiments were performed in mice at 10 weeks of age. Blood pressure was measured by the undirected tail-cuff method with a blood pressure monitor (MK-1030, Muromachi Kikai Co, Ltd).

**Measurement of Plasma Substrate Concentration**

Plasma glucose (glucose B test, Wako), insulin (insulin assay kit, Morinaga), free fatty acid (FFA) (NEFA C-test, Wako), and triglyceride (Triglyceride E-test, Morinaga) were measured with commercial kits.

**Oral Glucose Tolerance Test**

The oral glucose tolerance test (OGTT) was performed after a 16-hour overnight fast. 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-[3H]-Deoxy-D-Glucose**

2-[3H]-deoxy-d-glucose (2-[3H]DG) uptake in peripheral tissues was measured as previously reported. 23 The rate constant of net tissue uptake of 2-[3H]DG was calculated as described previously. 23

**2-[3H] DG Uptake In Vitro**

L6 skeletal muscle cells were seeded in 24-well plates, and 2-DG uptake assays were performed as described previously with slight modification. After being incubated in serum-free DMEM overnight at 37°C, the cells were stimulated with 100 nmol/L insulin for 30 minutes and/or 10 μmol/L temocapril for 60 minutes, which is the active form of temocapril. Hoe140 (10 μmol/L) was added to L6 cells 10 minutes before stimulation with temocapril or insulin. Glucose uptake was initiated by incubation with 2-[3H]DG, a concentration of 1 μCi/well for 5 minutes at 37°C. Nonspecific glucose uptake was measured in the presence of 10 μmol/L cytochalasin B and was subtracted from total 2-[3H]DG uptake in each assay to obtain specific uptake.

**Membrane Preparation and Western Blot**

After overnight fasting, 0.5 U/kg insulin was injected into KK-Ay mice intraperitoneally. Thirty minutes later, hind limb skeletal muscles of these mice were obtained, and the plasma membrane fraction and total crude membrane were isolated from the muscles by the method previously described. 29 The total crude membrane and the plasma membrane fraction were subjected to SDS-PAGE and transferred to nitrocellulose; immunoblotting was performed with antiphosphotyrosine antibody (Upstate Biotechnology Inc). Proteins were visualized with ECL.

**Tyrosine Phosphorylation of IRS-1**

After overnight fasting, 0.2 mL of insulin (10 U/kg) was injected through the portal vein. Hind limb skeletal muscles were removed 2.5 minutes after injection and supernatant fraction was obtained as previously described. 30 Supernatants of equal amounts of protein were incubated with anti–IRS-1 antibodies (Upstate Biotechnology Inc), then incubated with protein A–sepharose and centrifuged. Immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose; immunoblotting was performed with antiphosphotyrosine antibody (Upstate Biotechnology Inc). Proteins were visualized with ECL.

**Table, KK-Ay mice also showed higher systolic blood pressure, higher body weight, higher plasma FFA and triglyceride concentrations, and higher food intake than those of C57BL/6J mice. To examine the glucose tolerance of diabetic KK-Ay mice, we performed OGTT after fasting (Figure 1C). As shown in the Table, KK-Ay mice also showed higher systolic blood pressure, higher body weight, higher plasma FFA and triglyceride concentrations, and higher food intake than those of C57BL/6J mice. To examine the glucose tolerance of diabetic KK-Ay mice, we performed OGTT after fasting (Figure 1C). After a glucose load, plasma glucose concentration of C57BL/6J mice increased, reaching a peak at 30 minutes, and**

**Characteristics of Diabetic KK-Ay Mouse at 10 Weeks of Age**

<table>
<thead>
<tr>
<th>Variables</th>
<th>C57BL/6J</th>
<th>KK-Ay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma TG, mg/dL</td>
<td>81.31 ± 4.76</td>
<td>19.74†</td>
</tr>
<tr>
<td>Plasma FFA, mEq/L</td>
<td>0.56 ± 0.05</td>
<td>0.98 ± 0.15*</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td>101.2 ± 2.12</td>
<td>112.3 ± 2.02*</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>24.7 ± 0.68</td>
<td>40.1 ± 0.60†</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>2.793 ± 0.120</td>
<td>5.667 ± 0.235†</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. TG indicates triglyceride; FFA, free fatty acid.

*P<0.05; †P<0.01 vs C57BL/6J. n=6–10.
quickly decreased thereafter. Basal plasma glucose concentration of KK-Ay mice at 10 weeks of age, even in the fasting condition, was higher than that in C57BL/6J mice, and the plasma glucose concentration markedly increased after the glucose load and was maintained at a higher level. To further examine the possibility that insulin-mediated glucose transport into skeletal muscle and adipose tissue is impaired in KK-Ay mice, we investigated 2-[3 H]DG uptake into peripheral tissues in C57BL/6J and KK-Ay mice. The rate constant of 2-[3 H]DG uptake in the basal condition was similar in peripheral tissues in both mouse strains, whereas the rate constant of 2-[3 H]DG uptake in response to insulin was lower in skeletal muscle and adipose tissue in KK-Ay mice (Figure 2), suggesting that KK-Ay mice have severe insulin resistance and glucose intolerance.

**Effect of Temocapril on Insulin Resistance and Glucose Intolerance in KK-Ay Mice**

Administration of a small dose of temocapril (1 mg/kg per day), which did not influence systemic blood pressure, at 8 weeks of age for 2 weeks significantly lowered plasma glucose (231.07 ± 16.29 mg/dL, temocapril versus 445 ± 23.49 mg/dL, vehicle, n = 8, P < 0.01) and insulin (12.90 ± 0.72 ng/mL, temocapril versus 27.69 ± 3.78 ng/mL, vehicle, n = 8, P < 0.05) concentrations compared with those in the vehicle-treated group. To confirm whether temocapril improved glucose intolerance in KK-Ay mice, we performed OGTT (Figure 3) and observed that the mice treated with temocapril showed a lower peak of plasma glucose concentration at 30 minutes after the glucose load and that plasma glucose level declined more rapidly compared with that in vehicle-treated KK-Ay mice without a significant difference in insulin concentrations. To further confirm that temocapril improved insulin resistance, we examined the effects of temocapril administration on glucose uptake in peripheral tissues (Figure 4). We demonstrated that administration of temocapril significantly enhanced 2-[3 H]DG uptake in skeletal muscle but not in white adipose tissue, whereas temocapril treatment tended to enhance 2-[3 H]DG in brown adipose tissue. Next, we examined the involvement of the bradykinin-NO system in temocapril-mediated glucose uptake. Administration of a bradykinin B2 receptor antagonist, Hoe140 (0.1 mg/kg per day), or an NOS inhibitor, L-NAME (30 mg/kg per day), attenuated the effect of temocapril to increase glucose uptake only in skeletal muscle, whereas Hoe140 or L-NAME at these doses did not influence 2-[3 H]DG uptake in the control study without temocapril and did not change systolic blood pressure. These results suggest that temocapril increased glucose uptake, especially in skeletal muscle in diabetic KK-Ay mice, at least partially as the result of the enhancement of the bradykinin-NO system. Moreover, as we previously observed in mice,25 Hoe140 or L-NAME treatment did not influence insulin-mediated 2-[3 H]DG uptake in skeletal muscle and adipose tissue.

**Stimulation of GLUT4 Translocation by Temocapril**

We observed that GLUT4 translocation in the hind limb muscles of the mice was enhanced by insulin, whereas insulin-mediated GLUT4 translocation in KK-Ay diabetic mice was less than that in control C57BL/6J mice (3.6-fold increase in C57BL/6J versus 1.2-fold increase in KK-Ay mice). Similar results were reported with the use of type 2 diabetic model rats.29 We next examined the effect of temocapril treatment and observed that it also increased GLUT4 translocation in hind limb muscle in KK-Ay mice.
and insulin administration further increased GLUT4 translocation in temocapril-treated KK-Ay mice (Figure 5A). Expression of total GLUT4 protein in skeletal muscle was not changed by these treatments (Figure 5B).

**Figure 4.** Rate constant of 2-[3H]DG uptake in skeletal muscle (A) and adipose tissue (B) in KK-Ay mice. Temocapril (1 mg/kg per day) was administered to KK-Ay mice for 2 weeks, with or without Hoe140 (0.1 mg/kg per day) or L-NAME (30 mg/kg per day) (n=5 to 8). Rate constant of 2-[3H]DG uptake in interscapular BAT, epididymal and retroperitoneal WAT, and skeletal muscles (EDL, soleus, and red and white parts of GASTRO) was determined as described in Methods. Data are mean±SEM. * P<0.05 vs control; #P<0.05 vs temocapril.

**Figure 5.** Effect of temocapril on GLUT4 translocation to plasma membrane (A) and total GLUT4 content (B) in hind limb skeletal muscle of KK-Ay mice. Insulin stimulation significantly increased tyrosine phosphorylation of IRS-1, and the effect of insulin on IRS-1 phosphorylation was weaker in KK-Ay mice (30-fold increase in C57BL/6J mice versus 14.5-fold increase in KK-Ay mice) (data not shown). Similar results were demonstrated previously. However, temocapril did not enhance insulin-induced IRS-1 phosphorylation significantly (Figure 6A) and did not affect on IRS-1 protein content (Figure 6B).

**Effect of Temocapril on Glucose Uptake in L6 Skeletal Muscle Cell**

Based on our in vivo results, skeletal muscle appeared to be one of the most important tissues for the temocapril-mediated improvement of insulin resistance. Therefore, to examine the possibility that temocapril can increase glucose uptake in skeletal muscle directly, we used cultured L6 skeletal muscle cells. As shown in Figure 7, insulin (100 nmol/L) as well as temocaprilat (10 μmol/L) increased 2-[3H]DG uptake significantly in L6 skeletal muscle cells, and temocaprilat further increased insulin-stimulated 2-[3H]DG uptake. Hoe140 (10 μmol/L) attenuated temocaprilat-induced 2-[3H]DG uptake, whereas Hoe140 had no significant effect on insulin-stimulated 2-[3H]DG uptake in L6 cells. These findings suggest the possibility that temocaprilat enhances glucose uptake in L6 skeletal muscle independent of insulin.

**Discussion**

In this study, we focused on the possibility that ACE inhibitors may improve impaired glucose uptake in peripheral tissues in type 2 diabetes and studied the role of the bradykinin-NO system, which is enhanced by ACE inhibitors. We used KK-Ay mice as a model of type 2 diabetes. KK-Ay mice show glucose intolerance and decreased insulin-mediated glucose uptake in skeletal muscle and adipose tissue. Consistent with these results, plasma glucose and insulin concentrations were higher and increased markedly in an age-dependent manner in KK-Ay mice compared with those in C57BL mice. Administration of temocapril enhanced glucose uptake, especially in skeletal muscle in KK-Ay, resulting in a decrease in plasma glucose and insulin concentrations.

**Figure 6.** Effect of temocapril on IRS-1 Phosphorylation

Insulin stimulation significantly increased tyrosine phosphorylation of IRS-1, and the effect of insulin on IRS-1 phosphorylation was weaker in KK-Ay mice (30-fold increase in C57BL/6J mice versus 14.5-fold increase in KK-Ay mice) (data not shown). Similar results were demonstrated previously. However, temocapril did not enhance insulin-induced IRS-1 phosphorylation significantly (Figure 6A) and did not affect on IRS-1 protein content (Figure 6B).
The increase in glucose uptake in skeletal muscle by temocapril was attenuated by Hoe140 or L-NAME, suggesting that inactivation of bradykinin degradation and/or consequent increase in NO production after temocapril administration contribute at least partially to the effects of temocapril to improve insulin resistance in KK-Ay mice. The influence of hemodynamic changes induced by temocapril, L-NAME, and Hoe140 on glucose uptake needs to be addressed. However, we think that the influence was minimal, if present, because systolic arterial pressure was not changed by this dose of temocapril used in our experiments with the increase in glucose uptake in skeletal muscle, and arterial pressure in the Hoe140 and L-NAME groups was comparable to that in the temocapril group with attenuation of temocapril-enhanced glucose uptake. We also observed that temocaprilat increased glucose uptake in L6 cells, and Hoe140 inhibited this effect. We speculate that this NOS activity in skeletal muscle could be higher compared with other tissues such as artery in KK-Ay mouse and that local degradation of bradykinin in skeletal muscle would be decreased, resulting in the higher increase in local bradykinin concentration in skeletal muscle by temocapril. Therefore, bradykinin/NO-mediated GLUT4 translocation to plasma membrane by temocapril would be more sensitive compared with the effect of temocapril-mediated increase in bradykinin/NO on vasodilation, although these possibilities must be examined.

Folli et al\textsuperscript{31} demonstrated that angiotensin II inhibits insulin signaling in aortic smooth muscle cells at multiple levels such as inhibition of phosphoinositide 3-kinase activation associated with IRS-1, suggesting that the decrease in angiotensin II by temocapril may participate in the increase in glucose uptake in peripheral tissues. Recently, angiotensinogen, ACE, and angiotensin II receptors have been reported to be present in adipose tissue.\textsuperscript{32–34} However, in our study, glucose uptake in adipose tissue was not influenced by a low dose of temocapril, which did not affect blood pressure. These results support the notion that the improvement of insulin resistance by temocapril observed in our study is at least partially due to activation of the bradykinin-NO system, but the contribution of the hemodynamic change and increase in angiotensin II appeared to be less.

In contrast to other GLUT isoforms, which are primarily localized to the cell surface membrane, GLUT4 transporter proteins are sequestered into specialized storage vesicles that remain within the cell’s interior under basal conditions. As plasma glucose level rises, the subsequent increase in circulating insulin activates intracellular signaling cascades that ultimately result in translocation of the GLUT4 storage compartments to the plasma membrane. Therefore, insulin-responsive tissues are poised to respond rapidly and efficiently to fluctuations in circulating glucose and insulin level in type 2 diabetes, resulting in hyperglycemia and hyperinsulinemia, with a higher risk of cardiovascular disease. Consistent with this concept, we observed that insulin-mediated IRS-1 phosphorylation and GLUT4 translocation were impaired in diabetic KK-Ay mice. We demonstrated that temocapril enhanced the translocation of GLUT4 to the plasma membrane in skeletal muscle in KK-Ay. Temocapril increased GLUT4 translocation without influencing IRS-1 phosphorylation, suggesting that temocapril may act on downstream of IRS-1, improving insulin resistance and glucose intolerance. Bradykinin/NO would participate in the enhancement of glucose tolerance. Further detailed mecha-
nisms remain to be elucidated in the temocapril-mediated improvement of insulin resistance.

Perspective

Insulin resistance is an important risk factor of cardiovascular disease and often is associated with hypertension. Improvement of insulin resistance by ACE inhibitors has been suggested; however, this mechanism has not been proved. We observed in this study by using type 2 diabetic mouse KK-Ay that the ACE inhibitor temocapril increased glucose uptake, especially in skeletal muscle at least partially as the result of the enhancement of the bradykinin-NO system and consequently GLUT4 translocation. Our observations have been made in the diabetic mouse and therefore we cannot directly extrapolate our results to humans. The more detailed mechanism of ACE inhibitors in the improvement of insulin resistance and the role of the bradykinin-NO system in the pathogenesis of insulin resistance in humans must be addressed. We hope that our studies may lead to new strategies to treat insulin resistance, especially in hypertensive patients.

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