Loss of ACE2 Exaggerates High-Calorie Diet–Induced Insulin Resistance by Reduction of GLUT4 in Mice

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ACE type 2 (ACE2) functions as a negative regulator of the renin-angiotensin system by cleaving angiotensin II (AII) into angiotensin 1–7 (A1–7). This study assessed the role of endogenous ACE2 in maintaining insulin sensitivity. Twelve-week-old male ACE2 knockout (ACE2KO) mice had normal insulin sensitivities when fed a standard diet. AII infusion or a high-fat, high-sucrose (HFHS) diet impaired glucose tolerance and insulin sensitivity more severely in ACE2KO mice than in their wild-type (WT) littermates. The strain difference in glucose tolerance was not eliminated by an AII receptor type 1 (AT1) blocker but was eradicated by A1–7 or an AT1 blocker combined with the A1–7 inhibitor (A779). The expression of GLUT4 and a transcriptional factor, myocyte enhancer factor (MEF) 2A, was dramatically reduced in the skeletal muscles of the standard diet–fed ACE2KO mice. The expression of GLUT4 and MEF2A was increased by A1–7 in ACE2KO mice and decreased by A779 in WT mice. A1–7 enhanced upregulation of MEF2A and GLUT4 during differentiation of myoblast cells. In conclusion, ACE2 protects against high-calorie diet–induced insulin resistance in mice. This mechanism may involve the transcriptional regulation of GLUT4 via an A1–7–dependent pathway. ***Diabetes* 62:223–233, 2013**

Accumulating evidence has demonstrated that increased angiotensin II signaling as a consequence of the activation of the renin-angiotensin system (RAS) is strongly associated with insulin resistance, including a meta-analysis that indicated that the pharmacologic inhibition of the RAS prevents the initial onset of diabetes beyond its blood pressure-lowering effect (1). The important role of ACE type 2 (ACE2), which cleaves angiotensin II into angiotensin 1–7, has recently been recognized; it works as a negative regulator of the RAS in multiple disease states (2). Multiple tissues express ACE2 (3), and previous studies using ACE2-deficient mice have demonstrated that endogenous ACE2 may have a protective role in several pathologies, including pressure overload–induced heart failure (4), the development of diabetic nephropathy (5,6) and atherosclerosis (7). ACE2 exhibits its tissue-protective effects not only by decreasing angiotensin II but also by producing angiotensin 1–7, which counteracts angiotensin II signaling through its receptor, Mas (8). Interestingly, Mas-deficient FVB/N mice exhibited glucose intolerance and reduced insulin sensitivity, implying the close relationship between the angiotensin 1–7/Mas axis and glucose metabolism (9). Because ACE2 is expressed in the liver (10), adipose tissue (11), and skeletal muscle (12), which are the primary organs involved in the pathophysiology of insulin resistance (13), the endogenous ACE2 expressed in these organs may be involved in maintaining insulin sensitivity and glucose homeostasis. In this study, we investigated the roles of endogenous ACE2 in maintaining insulin sensitivity and glucose homeostasis that occurs during the metabolic syndrome.

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RESEARCH DESIGN AND METHODS

Experimental animals and protocols. ACE2 knockout (ACE2KO) mice with a C57/BL6 background were generated as described previously (4). At 8 weeks of age, male ACE2KO mice (*Ace2*$^{-/y}$) and their control littermates (*Ace2*$^{+/y}$) were randomly assigned to continue receiving a standard diet (MF, Oriental Yeast) or to begin receiving a high-fat, high-sucrose (HFHS) diet (AIN93G, Oriental Yeast). A schema of the study protocol is displayed in Fig. 1. They were housed individually until an intraperitoneal glucose tolerance test (IPGTT), intraperitoneal insulin tolerance test (IPITT), and tissue extraction were performed at age 12 weeks. Blood pressure was measured in conscious mice using a tail-cuff system (BP-98; Softron). The study protocol was approved by the Osaka University institutional animal care and use committee and was conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

IPGTT and IPITT. Mice were fasted for 16 h and anesthetized with medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). Blood was collected from the tip of the tail vein, and glucose levels were monitored with a glucometer (Roche Diagnostic) at the indicated time points after intraperitoneal injection of glucose (2 g/kg body weight) for IPGTT or human insulin (0.75 units/kg body weight) for IPITT. ACE2KO and WT mice that received the same treatment sequentially underwent the measurements in the same day.

Collection of tissue and plasma samples. Mice at age 12 weeks received an intraperitoneal injection of human insulin (50 mU/g) or 0.9% saline. After 5 min, they were killed and immediately perfused with saline. Epididymal retroperitoneal white adipose, soleus muscle, and liver tissues were collected, weighed, and immediately frozen in liquid nitrogen and stored at −80°C. Trunk blood from the mice that received the saline injection was collected in ice-chilled tubes and centrifuged at 10,000*g* for 10 min. The clear plasma supernatant was immediately separated and stored at −80°C for posterior analyses.

Measurement of plasma insulin concentration and serum triglyceride levels. Enzyme-linked immunosorbent assay kits were used to measure plasma insulin concentrations (Morinaga) and serum triglyceride levels (SRL, Inc.), according to the manufacturers' instructions.

Muscle and liver angiotensin II concentrations. The angiotensin II concentrations in the soleus muscle and liver were measured using a radioimmunoassay with two antibodies that are specific for angiotensin II (SRL, Inc.) and do not cross-react with angiotensin 1–7.

Western blot analysis. Proteins were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat dried milk and incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study were anti–phospho-AKT-Ser473 antibody, anti–phospho-AKT-Thr308 antibody, anti-AKT antibody, anti–phospho-α-AMP–activated protein kinase (AMPK)-Thr172 antibody, anti–α-AMPK antibody, anti-GLUT4 antibody, anti–myocyte enhancer factor-2A (MEF2A) antibody, anti–α-tubulin antibody, anti–β-actin antibody (Cell Signaling Technology), and anti-GLUT2 antibody (Millipore, Billerica, MA).

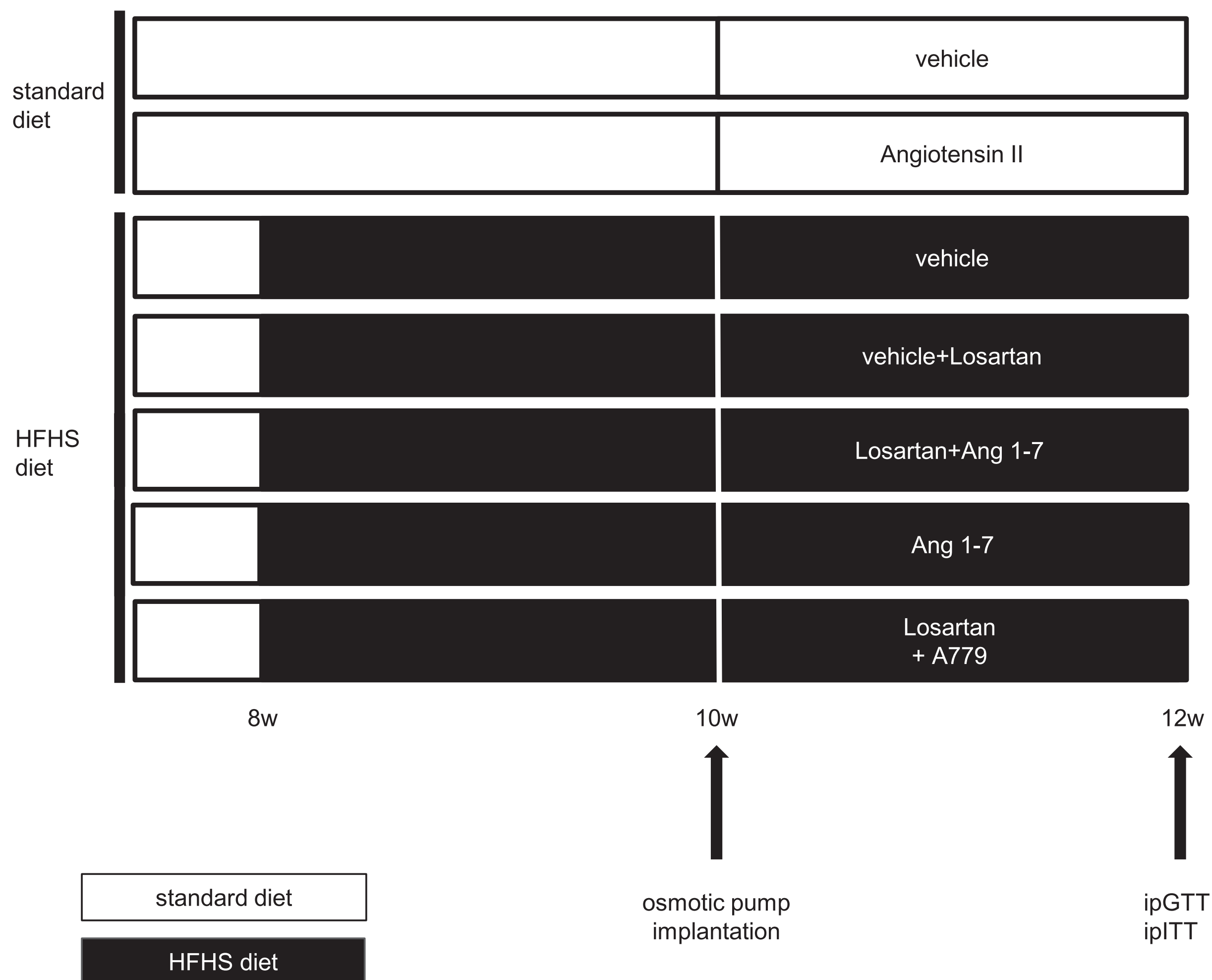


FIG. 1. Experimental protocol. ACE2KO mice and WT mice were divided into seven groups. Vehicle (0.9% saline), angiotensin II (100 ng/kg/min), angiotensin (Ang) 1–7 (100 ng/kg/min), and A779 (300 ng/kg/min) were administered via osmotic pumps. Losartan was administered at 3 mg/kg/day via drinking water. HFHS diet–fed mice that received angiotensin 1–7 alone and HFHS diet–fed mice that received A779, and losartan only underwent IPGTT.

Bands were visualized with a chemiluminescence detection system (LAS-4000 mini, GE) using Chemi-Lumi One Super (Nacalai Tesque).

Cell culture experiment. C2C12 mice myoblast cells were plated in a growth medium consisting of DMEM, 10% FBS, 1% Glutamax (GIBCO), 1% sodium pyruvate, and antibiotics. After attachment, C2C12 cells were exposed to a differentiation medium consisting of DMEM, 1% Glutamax, 1% sodium pyruvate, and 2% horse serum with vehicle or 10^{-8} mol/L angiotensin 1–7. A779 (10^{-5} mol/L) was administrated to cells 30 min before differentiation and added to medium throughout the treatment.

Real-time quantitative (q)PCR. RNA samples were purified using a RNeasy Mini Kit (Qiagen). The RNA samples were converted into cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time qPCR was performed and analyzed on a model 7900 Sequence Detector (Applied Biosystems) using real-time PCR mix (Toyobo) with TaqMan Gene Expression Assay for MEF2A and GLUT4 (Applied Biosystems). The expression level of each gene was normalized by glyceraldehyde-3-phosphate dehydrogenase as an internal control.

Glucose uptake measurement. After serum starvation for 4 h, cells were washed with HEPES-buffered saline (HBS) and then incubated without or with 100 nmol/L insulin for 20 min in HBS. Then, cells were incubated with HBS containing 2-deoxy-[^{3}H]glucose (0.1 mmol/L, 0.5 μCi/mL; PerkinElmer Life and Analytical Science) for 4 min, washed three times with ice-cold PBS, and then lysed in 0.1% SDS. The lysate was mixed with 10 mL scintillation fluid, and glucose uptake was assessed by scintillation counting. Nonspecific deoxyglucose uptake was measured in the presence 20 μmol/L cytochalasin B and subtracted from the total uptake.

Statistical analyses. Data are expressed as means ± SEM. To compare multiple treatments, statistical analyses were performed, including a one-way ANOVA and post hoc analyses. A Student *t* test was performed for between-group comparisons, and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Role of ACE2 deficiency in angiotensin II–induced impaired glucose tolerance and insulin resistance. At age 12 weeks, male ACE2KO mice fed the standard diet and WT mice did not differ in body weight, blood pressure, or fasting glucose levels (Table 1). The ACE2KO mice demonstrated normal glucose tolerances and insulin sensitivities, as indicated by the IPGTT and IPITT, respectively (Fig. 2, *A*). The areas under the curve (AUC) of IPGTT and IPITT were similar for the WT and ACE2KO mice (AUC of IPGTT [mg/dL × 120 min]: 22,528 ± 645 vs. 22,873 ± 474; AUC of IPITT: 6,454 ± 777 vs. 6,471 ± 451 for WT vs. ACE2KO mice, respectively; Fig. 4). A 2-week infusion of angiotensin II at 100 ng/kg/min did not alter the body weights, blood pressures, or fasting serum triglyceride levels of either mouse type, but fasting glucose levels were higher in the ACE2KO mice than in the WT mice (Table 1). Angiotensin II significantly increased fasting plasma insulin concentrations in the ACE2KO mice but not in the WT mice (Table 1). The IPGTT and IPITT

results indicated impaired glucose tolerance and insulin resistance in both types of mice but greater levels of impairment in ACE2KO compared with the WT mice (Fig. 2*B*, Fig. 4) (AUC of IPGTT: 35,050 ± 2,409 vs. 44,166 ± 2,132; AUC of IPITT: 9,465 ± 443 vs. 13,329 ± 930 for WT vs. ACE2KO mice, respectively).

Role of ACE2 deficiency in high-calorie diet–induced impaired glucose tolerance and insulin resistance. To investigate the role of ACE2 in high-caloric load–induced impairments in glucose homeostasis, mice were fed an HFHS diet from age 8 to 12 weeks. The ACE2KO and WT mice experienced equivalent increases in body weight at age 12 weeks (Table 1). There were no changes in blood pressures, fasting triglyceride levels, or fasting glucose levels in either type of mouse (Table 1). Compared with the standard diet, the HFHS diet tended to increase fasting insulin levels in the ACE2KO and WT mice, but the difference was not statistically significant. As illustrated in Fig. 3 and Fig. 4, the ACE2KO mice experienced impaired glucose tolerance and insulin resistance to a greater degree than the WT mice, as assessed using IPGTT and IPITT, respectively (AUC of IPGTT [mg/dL × 120 min]: 25,641 ± 2,364 vs. 37,078 ± 3,241; AUC of IPITT: 7,993 ± 584 vs. 10,251 ± 651 for WT vs. ACE2KO mice, respectively).

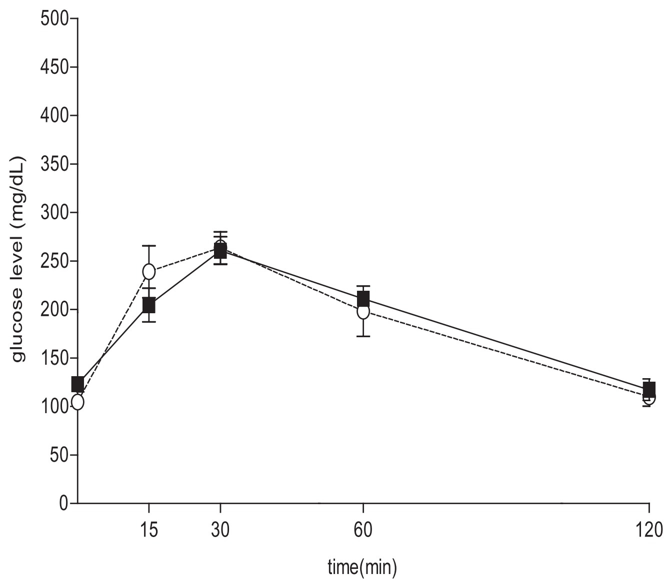
Involvement of angiotensin II and angiotensin 1–7 signaling in the influence of ACE2 on glucose homeostasis. Given the findings of the mice that received the angiotensin II infusion and of those fed the HFHS diet, we speculated that the increased angiotensin II and the decreased angiotensin 1–7 were both involved in the observed effects of ACE2 deficiency. Indeed, the ACE2KO mice fed the HFHS diet had higher concentrations of angiotensin II than the WT mice in their liver and skeletal muscle tissues, which are the primary organs involved in the pathogenesis of insulin resistance (Fig. 5). Thus, losartan, an angiotensin II receptor type 1 (AT1) blocker, was administered for 2 weeks to mice fed the HFHS diet to observe the effects of angiotensin II signaling on the observed manifestations of ACE2 deficiency in glucose tolerance and insulin sensitivity. Losartan did not alter blood pressure in the ACE2KO and WT mice (Table 1). Mice fed the HFHS diet with losartan exhibited normal glucose tolerances and insulin sensitivities as measured by comparing their IPGTT and IPITT AUCs with those of mice fed the standard diet. Losartan modestly improved the glucose tolerances and insulin sensitivities of the ACE2KO mice fed the HFHS diet, but the ACE2KO mice still exhibited glucose intolerance and insulin resistance compared with the WT mice (Fig. 3 and Fig. 4) (AUC of IPGTT [mg/dL × 120 min]: 18,933 ± 594 vs. 27,910 ± 1,920; AUC of IPITT: 6,784 ± 796 vs. 9,330 ± 158 for WT vs. ACE2KO mice, respectively).

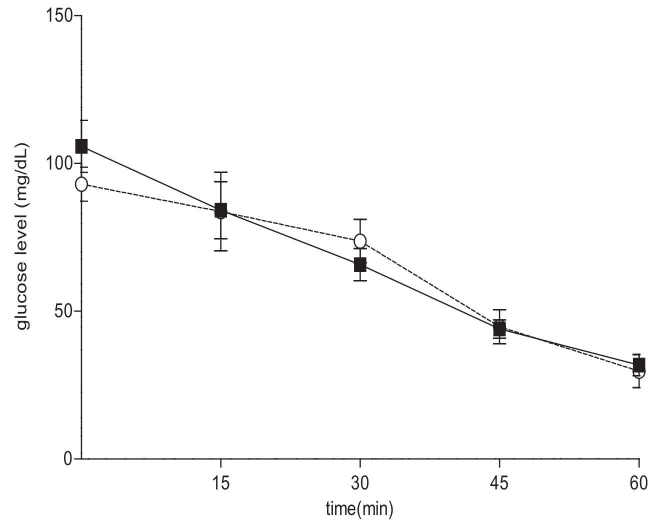
To reveal the role of ACE2 in the metabolism of both angiotensin II and angiotensin 1–7, losartan and angiotensin 1–7 were simultaneously administered for 2 weeks. The coadministration of losartan and angiotensin 1–7 eliminated the differences in IPGTT and IPITT between the ACE2KO and WT mice (Fig. 3 and Fig. 4). The IPGTT and IPITT AUCs of these mice were equivalent to or lower than those of the mice fed the standard diet (AUC IPGTT [mg/dL × 120 min]: 15,328 ± 39 vs. 16,753 ± 811 and IPITT: 6,025 ± 922 vs. 6,870 ± 576 for WT vs. ACE2KO mice, respectively). Moreover, the difference in glucose tolerance between HFHS diet–fed ACE2KO and WT mice was eradicated by infusion of angiotensin 1–7 alone (AUC of

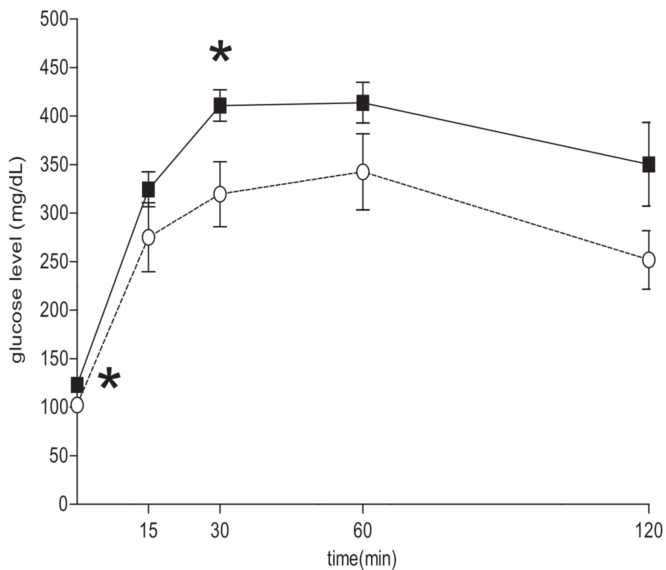
TABLE 1
Physiologic and metabolic parameters of mice

	Standard diet				HFHS diet									
	Vehicle		Ang II		Vehicle		Losartan		Losartan + Ang 1–7		Ang 1–7		Losartan + A779	
Characteristics	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
Body weight (g)	21.1 ± 0.4	22.0 ± 0.4	21.8 ± 0.3	21.2 ± 0.6	25.9 ± 0.9†	25.9 ± 0.5†	25.5 ± 0.6†	24.8 ± 0.5†	24.3 ± 0.3†	25.3 ± 0.3†	24.3 ± 0.3†	25.7 ± 0.7†	26.5 ± 0.9†	24.5 ± 0.5†
Mean blood pressure (mmHg)	69 ± 1	70 ± 5	74 ± 4	75 ± 9	64 ± 2	64 ± 5	65 ± 2	65 ± 7	55 ± 7	58 ± 4	68 ± 3	66 ± 4	67 ± 5	68 ± 4
Fasting														
Glucose (mg/dL)	93 ± 6	110 ± 6	99 ± 5	123 ± 4*	107 ± 3	102 ± 5	109 ± 4	99 ± 6	109 ± 5	107 ± 5	100 ± 4	92 ± 2	95 ± 5	101 ± 6
Insulin (pg/mL)	163 ± 6	171 ± 6	236 ± 42	341 ± 76†	233 ± 38	318 ± 63	201 ± 19	243 ± 5	173 ± 9	186 ± 9	NA	NA	NA	NA
Triglyceride (mg/dL)	105 ± 9	106 ± 17	98 ± 8	96 ± 11	101 ± 10	113 ± 14	83 ± 8	83 ± 16	77 ± 7	85 ± 26	NA	NA	NA	NA

Ang, angiotensin; NA, not applicable. *$P < 0.05$ vs. WT mice that received the same treatment by Student t test. †$P < 0.05$ vs. genotype-matched, standard diet–fed mice with vehicle infusion by Student t test.







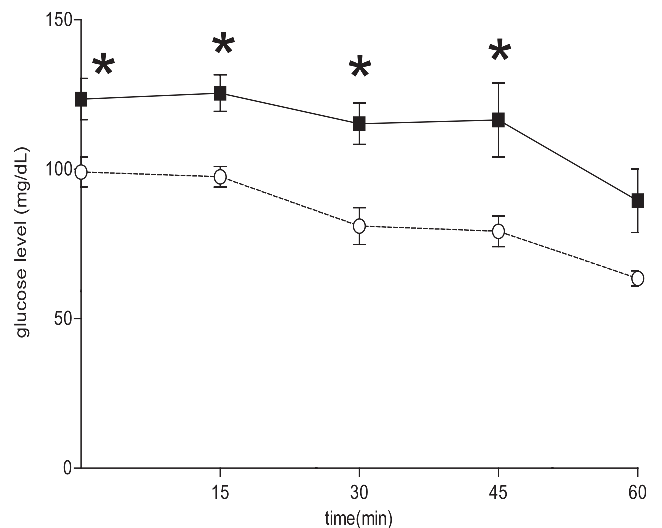


FIG. 2. Profiles of IPGTT and IPITT in standard diet–fed mice. IPGTT (*left panel*) and IPITT (*right panel*) testing of overnight-fasted, standard diet–fed mice that received 2-week infusions of vehicle (0.9% saline) (*A*) or angiotensin II (100 ng/kg/min) (*B*). Data are presented as the mean ± SEM (mg/dL), $n = 4$ in each group. *$P < 0.05$ vs. WT mice by Student *t* test.

IPGTT: 22,065 ± 1,810 vs. 21,435 ± 125 for WT vs. ACE2KO mice, respectively). The importance of angiotensin 1–7 signaling in the antihyperglycemic effect of ACE2 is further supported by the result showing that the dual inhibition of angiotensin II and angiotensin 1–7 (with losartan and A779, a selective angiotensin 1–7 inhibitor) in the WT mice fed the HFHS diet decreased glucose tolerance compared with the mice treated with only losartan (Fig. 4*A*). The AUC for IPGTT in the HFHS diet–fed WT mice treated with losartan and A779 was equivalent to that in the ACE2KO mice that received the same treatment (AUC of IPGTT: 28,633 ± 1,385 vs. 26,837 ± 2,066 for WT vs. ACE2KO mice, respectively).

Cellular signaling that is related to glucose transport in skeletal muscle in ACE2KO mice. Because skeletal muscle plays a major role in insulin-mediated glucose disposal, we observed the activation of the signal proteins, AKT and AMPK, which are related to glucose transport and the translocation of glucose transporters in the soleus muscle. Insulin phosphorylated AKT in the soleus muscle equally in the standard diet–fed ACE2KO mice compared with the WT mice (Fig. 6*A*). Angiotensin II infusion and the administration of the HFHS diet did not lead to differences in the insulin-induced phosphorylation of AKT in either type of mouse (data not shown). Similarly, the phosphorylation of AMPK was not altered by either genotype (Fig.

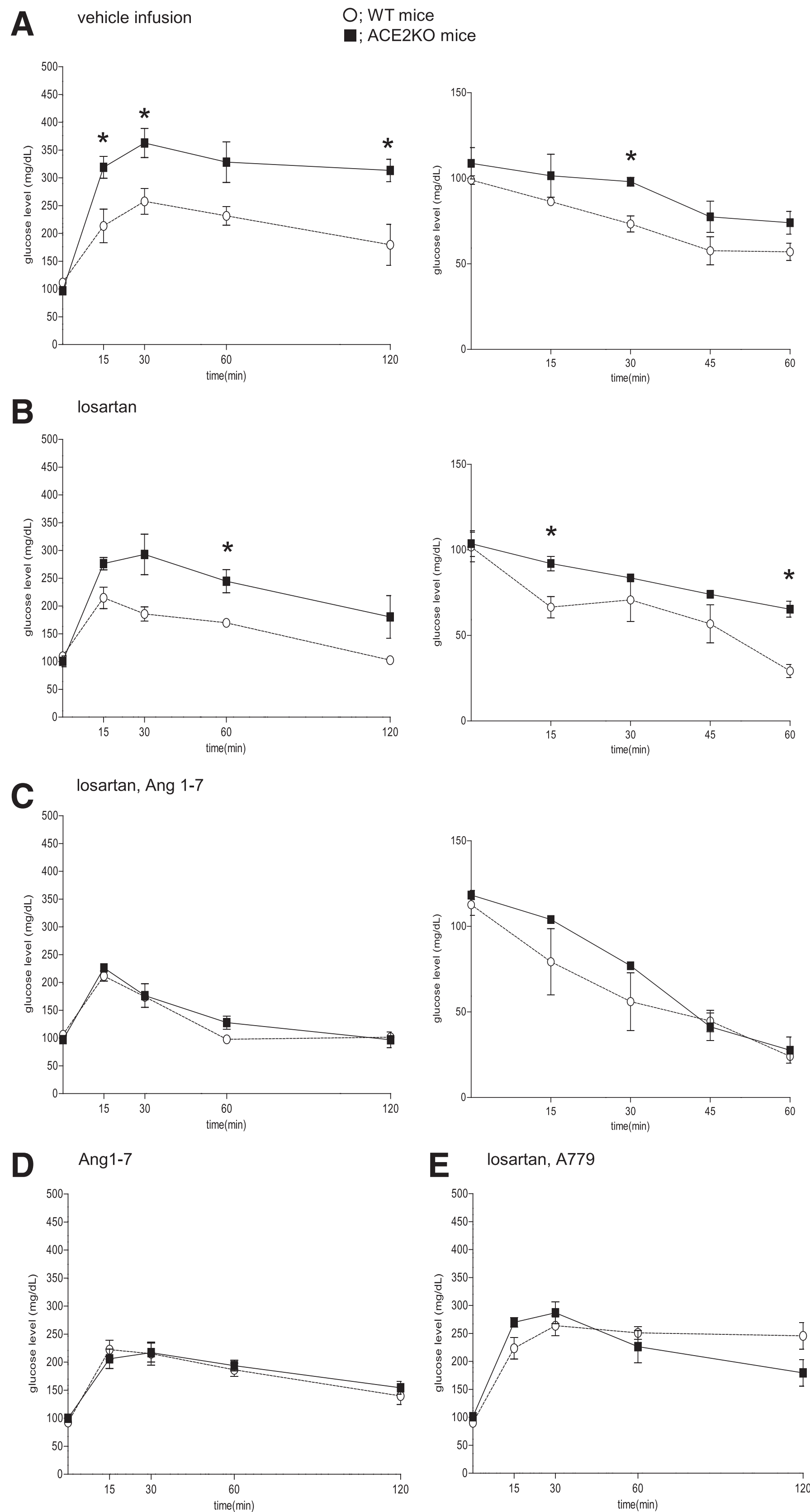


FIG. 3. Profiles of IPGTT and IPITT in HFHS diet–fed mice. IPGTT (*left panel*) and IPITT (*right panel*) testing of overnight-fasted, HFHS diet–fed mice with vehicle (0.9% saline) infusion (*A*) and vehicle infusion and 3 mg/kg/day losartan (*B*) or angiotensin 1–7 infusion (100 ng/kg/min) and losartan (*C*). IPGTT of overnight-fasted, HFHS diet–fed mice with angiotensin 1–7 infusion (*D*) or A779 infusion (300 ng/kg/min) and losartan (*E*). Data are presented as the mean ± SEM (mg/dL), *n* = 4 in each group. *P < 0.05 vs. WT mice by Student *t* test.

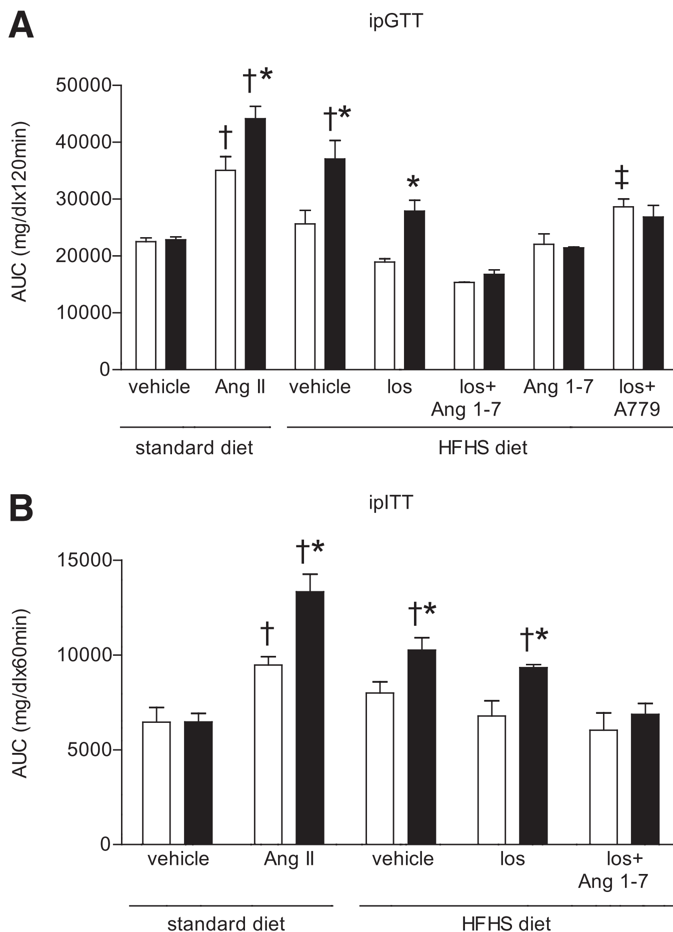


FIG. 4. Summary of the AUC of IPGTT and IPITT. AUCs of IPGTT test (*A*) and IPITT test (*B*) for WT mice (□) and ACE2KO mice (■). Data are presented as the mean ± SEM (mg/dL), n = 4 in each group. *P < 0.05 vs. WT mice that received the same treatment by Student t test. †P < 0.05 vs. genotype-matched, standard diet–fed mice with vehicle infusion by a one-way ANOVA. ‡P < 0.05 vs. HFHS diet–fed WT mice that received losartan alone by Student t test. Ang II, angiotensin II; los, losartan; Ang 1–7, angiotensin 1–7.

6*A*) or by angiotensin II infusion or the administration of the HFHS diet (data not shown) in the ACE2KO or WT mice.

Alteration in the expression of glucose transporters in ACE2KO mice. As shown in Fig. 6*B*, the protein expression of GLUT4 was dramatically reduced in the soleus muscles of the ACE2KO mice fed the standard diet. The expression of GLUT4 was also reduced in the white adipose tissues of the ACE2KO mice compared with the WT mice (Fig. 6*C*). In contrast, there was no difference in the expression of GLUT2 in the liver between the ACE2KO and WT mice (Fig. 6*D*). In the soleus muscle, ACE2 deficiency resulted in a marked reduction in the expression of MEF2A, which is a major transcriptional factor involved in GLUT4 transcription. To observe the effect of angiotensin 1–7 signaling on the transcription of GLUT4, angiotensin 1–7 or A779 was administered to mice fed the standard diet for 2 weeks (until age 12 weeks). As illustrated in Fig. 6*E* and *F*, angiotensin 1–7 increased GLUT4 and MEF2A in the soleus muscles of the ACE2KO mice. In contrast, A779 inhibitor decreased GLUT4 and MEF2A in the soleus muscles of the WT mice. These results suggest that angiotensin 1–7 signaling is a major determinant of the ACE2-mediated transcriptional regulation of GLUT4.

Regulation of MEF2A and GLUT4 by angiotensin 1–7 during differentiation of mice myoblast cells. To clarify whether GLUT4 expression is regulated by direct effect of angiotensin 1–7 on skeletal muscle, we observed temporal expression pattern of MEF2A and GLUT4 during differentiation of C2C12 myoblast cells to myotubes with or without the treatment of angiotensin 1–7. As shown in Fig. 7*A*, C2C12 cells treated with angiotensin 1–7 showed increased expression of MEF2A and GLUT4 6 h after induction of differentiation by low-serum medium, whereas vehicle treatment did not alter the expression of these genes at the time point. Although the difference in GLUT4 mRNA expression between vehicle and angiotensin 1–7 in C2C12 cells was abolished 24 h after differentiation (Fig. 7*A*), increased protein levels of GLUT4 and MEF2A by angiotensin 1–7 were observed at the same time point (Fig. 7*B*). A779 inhibited the upregulation of MEF2A and GLUT4 protein by angiotensin 1–7 in C2C12 cells (Fig. 7*B*).

Alteration of glucose uptake by angiotensin 1–7 in differentiating myoblast cells. As shown in Fig. 7*C*, angiotensin 1–7 treatment significantly enhanced insulin-mediated glucose uptake in C2C12 cells 24 h after the induction of differentiation.

DISCUSSION

In this study, the ACE2KO mice showed normal glucose tolerances and insulin sensitivities when they were fed a standard diet. However, when they received a subpressor dose of angiotensin II, they exhibited an overt diabetic phenotype with increased fasting glucose and insulin concentrations, and their glucose tolerances and insulin sensitivities were much reduced compared with those of the WT mice. The protective role of ACE2 against insulin resistance was also tested under physiologic conditions by feeding the mice high-calorie diets. The absence of ACE2 resulted in exaggerated glucose intolerance with insulin resistance in the mice fed the HFHS diet. Our findings demonstrate the ability of endogenous ACE2 to act as a regulator of glucose homeostasis and insulin sensitivity after caloric overload.

By cleaving angiotensin II, ACE2 reduces angiotensin II and increases angiotensin 1–7. The negative effect of angiotensin II on insulin sensitivity has been extensively studied; for example, improved insulin resistance has been observed after exposure to an AT1 blocker or ACE inhibitor in insulin-resistant rats (14) and humans (1), and angiotensin II–induced insulin resistance has been observed in rats (15,16) and dogs (17). Angiotensin 1–7 was recently reported to improve insulin resistance in rodents (18,19). Notably, Mas receptor–deficient mice with a FVB/N background fed a standard diet exhibited insulin resistance without increasing body weight (9). In our study, the effect of ACE2 ablation on glucose homeostasis in mice fed a high-calorie diet was not eliminated by an AT1 blocker alone but was eradicated by angiotensin 1–7 or an AT1 blocker in combination with the angiotensin 1–7 inhibitor, A779, which specifically inhibited Mas signaling (9). Angiotensin 1–7 eliminated the strain difference in glucose tolerance by improving glucose metabolism more in the knockout mice than WT controls, whereas A779 eliminated the strain difference in glucose tolerance by impairing glucose metabolism more in the WT mice. These results imply that not only increased angiotensin II signaling but also the ablation of the ACE2/angiotensin

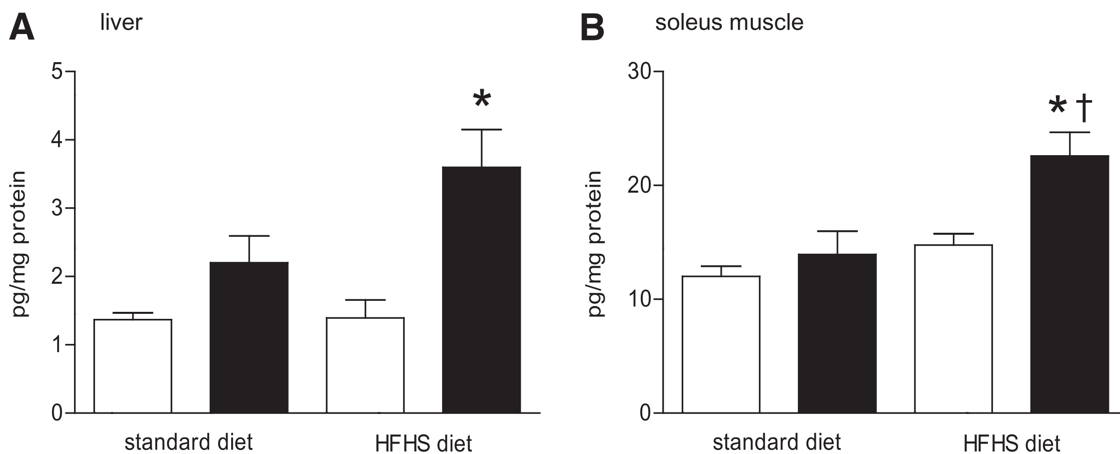


FIG. 5. Tissue angiotensin II concentrations. Angiotensin II concentration in liver (*A*) and soleus muscle (*B*) of WT mice (□) and ACE2KO mice (■). Data are presented as the mean ± SEM (mg/dL), n = 4–5 in each group. *P < 0.05 vs. WT mice with the same treatment by Student t test. †P < 0.05 vs. standard diet–fed ACE2KO mice by Student t test.

1–7/Mas axis was the important determinant of glucose intolerance in the ACE2KO mice.

The hyperglycemic phenotype of the HFHS diet–fed ACE2KO mice was accompanied by increased concentrations of angiotensin II in the liver and skeletal muscle. In the liver, insulin regulates glucose metabolism by the utilization and storage of glucose as lipids and glycogen, while the skeletal muscle has a primary role in insulin-mediated glucose disposal (13). As hepatocytes and skeletal muscle cells express endogenous ACE2 (3,10,12), the alteration of angiotensin II concentrations in these organs may reflect a lack of the tissue-specific conversion of angiotensin II to angiotensin 1–7 in the ACE2KO mice.

To identify the mechanism that is involved in the ACE2-mediated protection from insulin resistance, we first compared the activation of AKT and AMPK in the soleus muscles of the ACE2KO and WT mice because AKT and AMPK regulate insulin and the exercise-mediated translocation of GLUT4, respectively (20). However, there was no difference in the activation of AKT and AMPK between the ACE2KO and WT mice. Moreover, neither angiotensin II infusion nor the administration of a high-calorie diet altered the activation of these signaling proteins. This finding is partially consistent with a previous report that the chronic infusion of angiotensin II induced insulin resistance without decreasing AKT activation in rats (15).

However, we found a severe reduction in GLUT4 expression in the soleus muscles and white adipose tissues of standard diet–fed ACE2KO mice. In the soleus muscles of the ACE2KO mice, we also found the reduced expression of MEF2A, a transcriptional factor that enhances the gene expression of GLUT4 by binding to the GLUT4 promoter in combination with the GLUT4 enhancer factor (21). We could not detect MEF2A protein in the adipose tissue, but it has been reported that low levels of MEF2A can regulate the expression of GLUT4 in adipose tissue (21,22). Thus, the reduction of MEF2A-mediated transcription may be responsible for the reduced GLUT4 protein expression observed in the skeletal muscles and adipose tissues. In the soleus muscles, angiotensin 1–7 increased the protein levels of MEF2A and GLUT4 in the ACE2KO mice, whereas A779 decreased MEF2A and GLUT4 in the WT mice.

Consistent with the in vivo study, we also found that angiotensin 1–7 upregulated the mRNA and protein expression of MEF2A and GLUT4 in differentiating myoblast cells. The effect of angiotensin 1–7 was totally abolished by Mas inhibition with A779. We also found that angiotensin 1–7 increased glucose uptake in response to insulin in differentiating myoblast cells. These findings suggest that angiotensin 1–7/Mas signaling is a regulator of MEF2A and GLUT4, and thus enhances glucose uptake. These findings are consistent with a previous report that GLUT4 expression was reduced in the adipose tissues of standard diet–fed Mas-deficient mice with a FVB/N background (9). Thus, we propose that the ACE2/angiotensin 1–7/Mas axis plays a crucial role in the transcription of MEF2A and thus, of GLUT4. However, further studies will be required to elucidate the underlying mechanisms whereby angiotensin 1–7/Mas signaling regulates the transcription of MEF2A.

Given the finding of reduced expression of GLUT4 in the standard diet–fed ACE2KO mice, it is unclear why these mice showed normal glucose tolerances and insulin sensitivities. However, a previous study found that 53% of 2- to 4-month-old GLUT4 heterozygous knockout mice exhibited normal glucose and insulin levels with decreased GLUT4 expression in skeletal muscles and adipose tissues, although they developed a diabetic phenotype with aging (23). In addition, Zisman et al. (24) reported that the liver uptake of glucose was profoundly upregulated in insulin-resistant, muscle-specific GLUT4 knockout mice. Therefore, our ACE2KO mice likely had normal insulin sensitivities with decreased protein levels of GLUT4 due to compensatory mechanisms, which may have occurred in the liver, and that angiotensin II or a high-calorie diet disrupted the compensatory mechanisms and made the ACE2KO mice more insulin resistant than the WT mice. Further investigation is required to elucidate whether insulin-mediated glucose uptake is decreased in skeletal muscle and adipose tissue of standard diet–fed ACE2KO mice.

In addition to the aforementioned information, several known functions of ACE2 should be considered as factors influencing the observed phenotype of ACE2KO mice. First, ACE2 gene therapy in the pancreas was reported to improve the glycemic control of diabetic *db/db* mice by improving β-cell function and increasing insulin secretion (25). Previous reports have confirmed that the activation

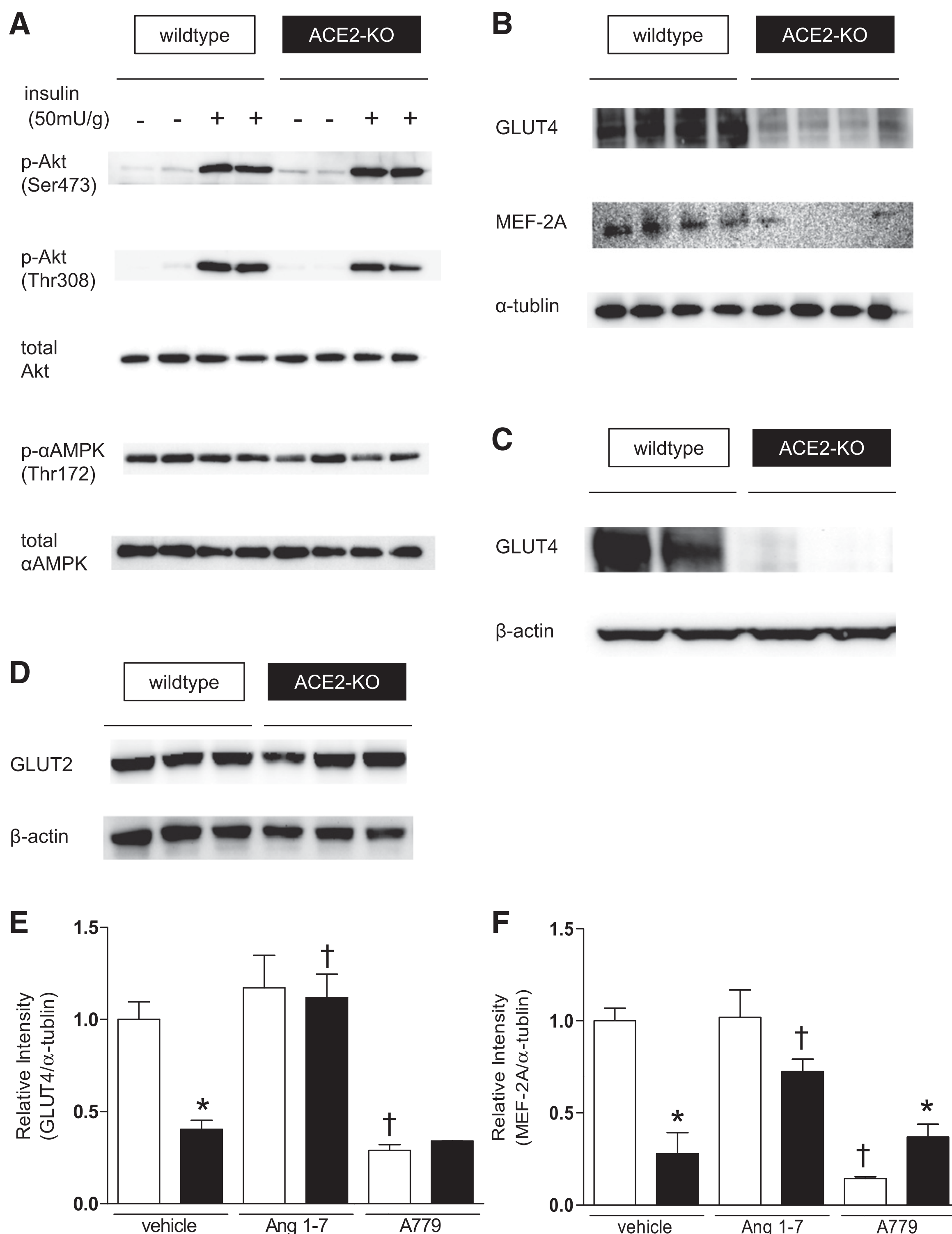


FIG. 6. Western blot analysis of soleus muscle, adipose tissue, and liver. ***A*****: Phosphorylation (p) of Akt at Ser473, Thr308, and of α-AMPK at Thr172, with or without intraperitoneal injection of insulin for 5 min in soleus muscle of standard diet–fed mice.** ***B*****: Total GLUT4 and MEF2A protein in soleus muscle of standard diet–fed mice (α-tubulin was used for internal control).** ***C*****: GLUT4 protein in adipose tissue of standard diet–fed mice.** ***D*****: GLUT2 protein in liver of standard diet–fed mice. Change in protein expression of MEF2A and GLUT4 in soleus muscle in standard diet–fed mice with administration of vehicle, angiotensin (Ang) 1–7, and Ang 1–7 inhibitor vehicle (0.9% saline), Ang 1–7 (100 ng/kg/min), and A779 (300 ng/kg/min) were administered via subcutaneously implanted osmotic pumps to 10-week-old, standard diet–fed mice. Soleus muscles were isolated at 12 weeks of age. Densitometric analyses were performed to calculate the relative intensities of GLUT4/α-tubulin (*E*) and MEF2A/α-tubulin (*F*) in WT (□) and ACE2KO mice (■). Data are presented as the mean ± SEM, n = 3–4 in each group. *P < 0.05 vs. WT mice that received the same treatment by Student t test. †P < 0.05 vs. genotype-matched, standard diet–fed mice with vehicle infusion by one-way ANOVA.**

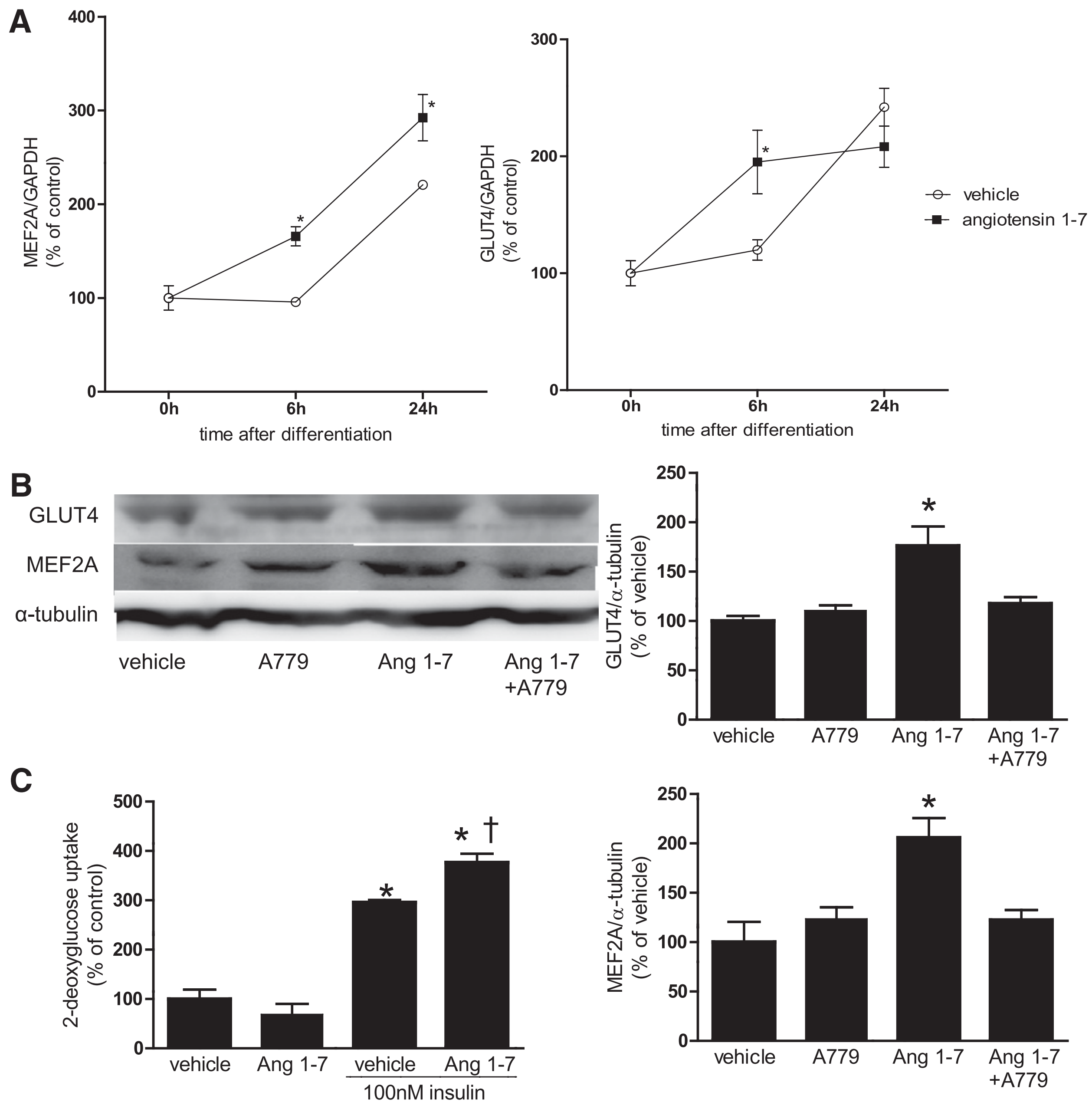


FIG. 7. In vitro assay using C2C12 cells. ***A*****: Real time PCR analysis of MEF2A and GLUT4 gene expression during differentiation of C2C12 cells from myoblast to myotubes with or without treatment of 10^{-8} mol/L angiotensin (Ang) 1–7; *n* = 4 in each group. *P < 0.05 vs. vehicle treatment control by a Student *t* test.** ***B*****: Immunoblot analysis of MEF2A and GLUT4 protein levels 24 h after induction of differentiation of C2C12 cells with vehicle, 10^{-5} mol/L A779, 10^{-8} mol/L Ang 1–7, or Ang 1–7 with A779. Representative blot (*left panel*) and the relative intensities of GLUT4/α-tubulin and MEF2A/α-tubulin (*right panel*) are shown; *n* = 3 in each group. *P < 0.05 vs. vehicle by one-way ANOVA.** ***C*****: Glucose uptake of C2C12 cells 24 h after induction of differentiation with or without treatment of 10^{-8} mol/L Ang 1–7; *n* = 3 in each group. *P < 0.05 vs. the same treatment without insulin by one-way ANOVA. †P < 0.05 vs. vehicle treatment with insulin by one-way ANOVA.**

of the RAS inhibits glucose-induced insulin secretion from pancreatic β-cells (26,27). Because endogenous ACE2 is expressed in the pancreas (25), it is conceivable that a loss of pancreatic ACE2 influenced the hyperglycemic phenotype of the ACE2KO mice. However, the effect of ACE2 deletion in the pancreas may have been reduced compared with that in the organs responsible for maintaining insulin sensitivity, considering the hyperinsulinemia observed in the angiotensin II–infused ACE2KO mice. Our findings are consistent with a previous report that the administration of the angiotensin 1–7 inhibitor worsened insulin sensitivities, but not insulin secretion, in vehicle-treated diabetic mice (25).

ACE2 can catalyze several types of humoral factors other than angiotensin II, including apelin-13, dynorphin A 1–13, and des-Arg-bradykinin (28). In particular, apelin-13 is closely associated with the maintenance of insulin sensitivity, as evidenced by the impaired insulin sensitivities in apelin knockout mice (29) and by improved insulin sensitivities after apelin-13 infusion in insulin-resistant mice (29,30). ACE2 may negatively affect insulin sensitivity by catalyzing and inactivating apelin-13 because ACE2

hydrolyzes apelin-13 with a similar potency as angiotensin II (31). However, our findings clearly demonstrate that the beneficial effect of ACE2 on the RAS supersedes this undesirable inactivation of apelin-13.

In addition, recent studies have indicated that AT2 receptor–mediated angiotensin II signaling plays a crucial role in insulin resistance. The deletion of the AT2 receptor reduced insulin resistance in mice fed a high-fat diet (32). Mitsuishi et al. (33) reported that angiotensin II induced insulin resistance in mice with a reduction of muscle mitochondrial concentrations through AT1 receptor– and AT2 receptor–dependent actions. Further investigation will be required to elucidate the role of AT2 activation in the insulin sensitivity of ACE2KO mice.

One of the limitations of the current study is that we did not measure plasma and tissue concentrations of angiotensin 1–7. Because there are alternative enzymes to produce angiotensin 1–7 besides ACE2, including neprilysin, prolyl endopeptidase, and prolyl carboxypeptidase (34), the deletion of ACE2 should not be accompanied by complete elimination of angiotensin 1–7. The status of angiotensin 1–7 levels in ACE2 knockout mice has not been extensively studied. However, reduced concentrations of angiotensin 1–7 have been observed in renal cortical homogenates of ACE2KO mice (6). Thus, further investigation will be required to determine the extent to which insulin resistance in ACE2KO mice is a consequence of reductions of angiotensin 1–7 levels in specific tissues such as skeletal muscle and fat.

Finally, because the current study suggests that endogenous ACE2 expressed in insulin-sensitive organs has a crucial role in maintaining insulin sensitivity, ACE2 may be considered as a therapeutic target for improving insulin resistance in patients with type 2 diabetes. In addition to supporting the beneficial effects of ACE2 gene therapy on pancreatic insulin secretion (25) and diabetic nephropathy (35), our findings should motivate the development of pharmacologic or genetic approaches to increase ACE2 for the treatment of diabetic patients.

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M.T. performed experiments and wrote the manuscript. K.Y. conceived the idea of the study, designed experiments, and wrote and edited the manuscript. Yuk.T., K.K., M.O., and H.R. designed experiments. H.T., K.H., T.K., H.H.-Y., R.O., Yo.T., Yuj.T., Ya.T., and K.S. performed experiments. K.Y. is the guarantor of this work, and, as such, had full access to all the data in study and takes responsibility for the integrity of data and the accuracy of data analysis.

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