Original Article

Candesartan Ameliorates Cardiac Dysfunction Observed in Angiotensin-Converting Enzyme 2–Deficient Mice

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The renin-angiotensin (Ang) system plays a critical role in the regulation of blood pressure, body fluid, electrolyte homeostasis, and organ remodeling under physiological and pathological conditions. The carboxypeptidase ACE2 is a homologue of angiotensin-converting enzyme (ACE). It has been reported that ACE2-deficient mice develop cardiac dysfunction with increased plasma levels of Ang II. However, the molecular mechanism by which genetic disruption of ACE2 results in heart dysfunction is not fully understood. Here, we generated mice with targeted disruption of the *Ace2* gene and compared the cardiovascular function of ACE2$^{-/y}$ mice with that of their wild-type littermates. ACE2-deficient mice were viable and fertile and lacked any gross structural abnormalities. Echocardiographic study detected no functional difference between ACE2$^{-/y}$ and wild-type mice at 12 weeks of age. Twenty-four-week-old ACE2$^{-/y}$ mice displayed significantly enlarged hearts with impaired systolic and diastolic function. The Ang II level was elevated in the plasma and heart of ACE2$^{-/y}$ mice. Pharmacological blockade of Ang II type 1 receptor (AT1) with candesartan attenuated the development of cardiac dysfunction in ACE2$^{-/y}$ mice. These results suggest that enhanced stimulation of AT1 may play a role in the development of cardiac dysfunction observed in ACE2-deficient mice. (*Hypertens Res* 2008; 31: 1953–1961)

***Key Words*: angiotensin-converting enzyme 2, angiotensin II, angiotensin II type 1 receptor, angiotensin-(1–7), cardiac dysfunction**

Introduction

The renin-angiotensin system plays a critical role in the regulation of blood pressure, body fluid, electrolyte homeostasis, and remodeling of various organs under physiological and pathological conditions (*1*–*3*). The carboxypeptidase ACE2 was isolated from the human failing heart as a homologue of angiotensin (Ang)-converting enzyme (ACE) (*4*, *5*). ACE2 cleaves Ang II and Ang I into Ang-(1–7) and Ang-(1–9), respectively. Although ACE transcripts can be detected ubiquitously, ACE2 is mainly expressed in the heart, kidneys, and testes under physiological conditions. The physiological role of ACE2 remained unknown until Crackower *et al*. provided

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Table 1. Characteristics of 24-Week-Old Wild-Type and ACE2-Deficient Mice

	WT (*n*)		ACE2$^{-/y}$ (*n*)	
	Can (−)	Can (+)	Can (−)	Can (+)
Body weight, g	29.1±0.7 (6)	28.0±2.0 (4)	28.8±1.0 (10)	29.4±1.4 (4)
Pulse rate, bpm	753.5±12.0 (6)	674.8±35.2 (4)	733.3±12.1 (10)	756.6±9.9 (4)
Systolic BP, mmHg	103.7±2.4 (6)	107.9±6.1 (4)	102.8±1.8 (10)	96.4±2.3 (4)
Urine volume, mL/24 h	1.6±0.3 (4)	n.d.	1.4±0.3 (4)	n.d.
Cross-sectional area, μm^2	188.9±5.9 (9)	n.d.	208.1±6.3 (5)	203±6.3 (6)
Collagen volume fraction, %	2.9±0.2 (7)	n.d.	2.6±0.4 (5)	2.8±0.1(6)

WT, wild-type mice; Can, candesartan (0.1 mg/kg/d); ACE2, angiotensin-converting enzyme 2; bpm, beats per minute; BP, blood pressure; n.d., not determined. The number of analyzed mice is reported in parentheses.

the mouse genetic evidence that ACE2 is essential in the maintenance of cardiac homeostasis (*6*). Targeted disruption of ACE2 in mice results in a severe cardiac contractility defect with increased Ang II levels and up-regulation of hypoxia-inducible genes in the heart.

It is well known that the Ang II level increases in the heart under pathological conditions (*7*). Pharmacological blockade of the Ang II type1 (AT1) receptor has been shown to protect several organs from damage associated with metabolic diseases (*8–11*). It has been reported that ACE2 and Ang-(1–7) are also up-regulated in the diseased heart (*12–14*). Recent reports indicate that Ang-(1–7) has antagonistic effects against Ang II (*15–18*). These findings suggest that Ang-(1–7) and ACE2 can exert protective roles in the cardiovascular system in opposition to Ang II and ACE axis (*18*, *19*). However, the molecular mechanism by which the Ang-(1–7) and ACE2 system plays a protective role in the cardiovascular system is not fully understood.

Here, we compared the cardiac function of ACE2-deficient mice with that of their wild-type littermates. At 12 weeks of age, no functional difference was detected between ACE2-deficient and wild-type mice by echocardiography. At 24 weeks, ACE2-deficient mice displayed significantly enlarged left ventricles (LVs) with impaired systolic and diastolic function. Ang II levels in the plasma and heart were elevated in ACE2-deficient mice. Pharmacological blockade of the AT1 receptor with candesartan attenuated the development of cardiac dysfunction. These results suggest that enhanced stimulation of AT1 by Ang II may play a role in the development of cardiac dysfunction caused by ACE2 disruption.

Methods

Animals and Reagents

ACE2-deficient mice were generated as previously described (*20*) and maintained in our animal facility. Female ACE2$^{-/+}$ mice were intercrossed with male C57BL/6J mice (SLC, Shizuoka, Japan), and the resulting littermates were used for this study. Candesartan cilexetil was kindly provided by TAKEDA Pharmaceutical Ltd. (Osaka, Japan). The mice received either vehicle (0.5% carboxymethyl cellulose) or candesartan (0.1 mg/kg/d) every day by gavage, starting at 10 weeks of age. Blood pressures and pulse rates were measured with a tail cuff and a pneumatic pulse transducer (BP-98AW, Vers.12; Softron, Tokyo, Japan). Urine was collected using metabolic cages for mice (Natsume, Co. Ltd, Tokyo, Japan). Mice were housed in the metabolic cage for 24 h before the urine sampling. All experimental procedures and protocols were approved by the Animal Care and Use Committee of the University of Tokyo and complied with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, revised 1985).

Echocardiography

Ultrasound studies were performed using an echocardiography system (EnVisor M2540A; Phillips, Tokyo, Japan) equipped with a 15-MHz linear array transducer. Two-dimensional images and M-mode tracings were recorded from the short-axis view at the high papillary muscle level. Left ventricular end-diastolic dimension (LVEDD) and end-systolic dimension (LVESD) were measured in a blinded fashion. Fractional shortening (FS) was calculated using the following equation.

$$FS\ (\%) = (LVEDD - LVESD)/LVEDD \times 100.$$

Hemodynamic Study

Hemodynamic studies were performed under general anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneal injection). LV pressure was monitored with a Millar tip transducer catheter (model SPR-671, 1.4F; Millar Instruments, Inc., Houston, USA) cannulated from the right carotid artery. Data were analyzed using the MacLab/400 data acquisition system (AD Instruments, Bella Vista, Australia).

Histological Analysis

Mice were sacrificed after measurement of hemodynamic parameters. The heart was carefully removed after perfusion

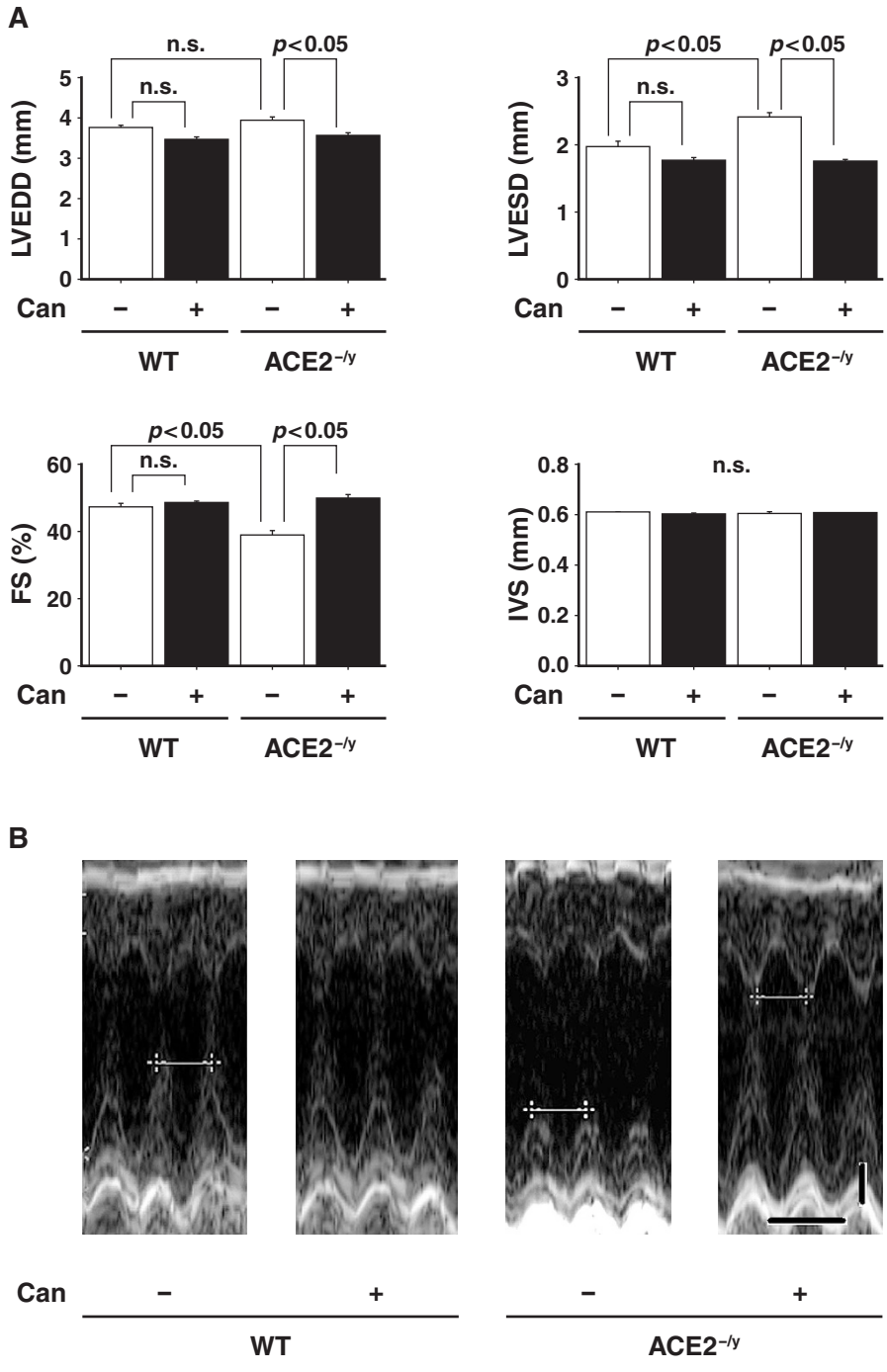

Fig. 1. *Echocardiogram of 24-week-old wild-type and ACE2$^{-/y}$ mice. A: Echocardiographic study was performed every 4 weeks from 12 weeks to 24 weeks. Vehicle or candesartan (Can, 0.1 mg/kg/d) was administered every day. Echocardiograms were performed on wild-type mice (WT, n = 12), WT mice treated with Can (n = 4), ACE2$^{-/y}$ mice (n = 13), and ACE2$^{-/y}$ mice treated with Can (n = 6). Results are expressed as mean ± SEM. n.s., not significant. LVEDD, end-diastolic dimension; LVESD, LV end-systolic dimension; FS, fractional shortening; IVS, interventricular septal wall. B: Representative M-mode echocardiograms. Horizontal scale bar: 200 ms; vertical scale bar: 1.0 mm.*

Table 2. Hemodynamic Analysis of 24-Week-Old Wild-Type and ACE2-Deficient Mice

	WT		ACE2$^{-/y}$	
	Can (−) (n=10)	Can (+) (n=3)	Can (−) (n=8)	Can (+) (n=6)
Heart rate, bpm	429±16	414±9	489±21	467±37
LVEDP, mmHg	0.90±0.28	1.12±0.30	3.68±0.62*	1.27±0.26
LV +dP/dt, mmHg/s	12,780±563	11,302±544	9,981±860*	11,844±1,323
LV −dP/dt, mmHg/s	8,122±477	7,731±505	7,326±727	8,227±563

At 24 weeks of age, hemodynamic study was performed under general anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneal injection). Left ventricular (LV) pressure was monitored with a Millar tip transducer catheter (model SPR-671, 1.4F; Millar Instruments, Inc.) cannulated from the right carotid artery. Data were analyzed with the MacLab/400 data acquisition system (AD Instruments). + (−) dP/dt, maximum (minimum) first derivative of the change in LV pressure with time; LVEDP, LV end-diastolic pressure; WT, wild-type mice; ACE2, angiotensin-converting enzyme 2; Can, candesartan; bpm, beats per minute. Results are expressed as mean±SEM. *p<0.05 *vs*. WT.

fixation with 10% neutralized formalin, cut into 3 transverse sections, and embedded in paraffin. Thin sections were deparaffinized and stained with hematoxylin and eosin (H&E) or Masson trichrome. Collagen fiber accumulation (collagen volume fraction [CVF]) was evaluated with image analysis software (Image J; Research Service Branch, NIH, USA) after Masson trichrome staining. Three different fields were selected from three different levels randomly. To measure the cross-sectional area (CSA) of cardiomyocytes, 90–120 randomly selected cardiomyocytes were analyzed.

In Situ Hybridization

Sections were made at 8 μm thickness. *In situ* hybridization was performed as described previously (*21*). Digoxigenin-labeled riboprobes were generated using cDNAs encoding murine AT1 (nucleotides 1382 to 2248; Genbank no. NM_177322) and Mas (nucleotides 635 to 2365; Genbank no. NM_008552).

Measurement of Plasma and Heart Ang II Levels

Plasma was collected in tubes containing EDTA (final concentration, 1 mmol/L). After adequate perfusion with heparinized saline, the LV was quickly removed and then stored at −80°C until measurement. Each heart was homogenized on ice in 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 2 mmol/L EDTA, 1% NP-40, 1% deoxycholate, 50 mmol/L NaF, and 1 mmol/L Na_3VO_4 containing protease inhibitor cocktail (Sigma, St. Louis, USA). Peptide extraction was performed as described previously (*22*). Ang II concentration was measured by radioimmunoassay using two antibodies specific for Ang II (SRL Co., Tokyo, Japan). This antibody does not have cross-reactivity to Ang-(1–7). The concentration of Ang-(1–7) was measured with a nanoLC/ESI-MS/MS system (cap-LC & Q-Tof micro; Waters Corporation, Milford, USA) using synthetic Ang-(1–7) (WAKO Chemicals, Osaka, Japan) as a standard.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was prepared from the LV using a commercially available isolation kit (RNA easy mini kit; QIAGEN, Tokyo, Japan) and DNase (QIAGEN). Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed as described elsewhere (*23*). First strand cDNA was synthesized from 1 μg of total RNA, using oligo-dT primer and MMLV-derived reverse transcriptase (ReverTra Ace, TOYOBO, Osaka, Japan). One-twentieth of the reaction mixture was used as a template for PCR amplification. PCR was performed using following primers: brain natriuretic peptide (BNP), 5′-ATG GATCTCCTGAAGGTGCTGTCCCAGATGAT-3′, and 5′-CTACAACAACTTCAGTGCGTTACAGCCCA-3′; AT1, 5′-TCACCTGCATCATCATCTGG-3′, and 5′-AGCTGG TAAGAATGATTAGG-3′; Mas, 5′-CACCAGTCAGCA TTCGTCTGT-3′, and 5′-CCAGAAGTGAGGCGAGTA CAA-3′; and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5′-ACCACAGTCCATGCCATCAC-3′, and 5′-TCCACC ACCCTGTTGCTGTA-3′. GAPDH was used as an internal control.

Statistics

All data are expressed as means±SEM. Statistical analyses were performed with a commercially available software program (StatView 5.0; SAS Institute Inc., Cary, USA). Multiple comparisons were performed by ANOVA or non-parametric Mann-Whitney's U test. A p-value of <0.05 was considered to be significant.

Results

ACE2-Deficient Mice Show Age-Associated Cardiac Dysfunction

ACE2$^{-/y}$ mice are fertile and develop normally (Table 1).

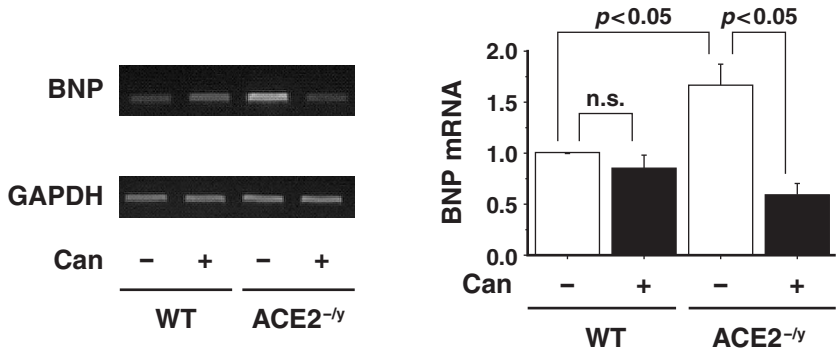

Fig. 2. *BNP expression in the left ventricle of WT and ACE2-deficient mice. RNA was extracted from LV 24-week-old mice. BNP expression was evaluated by semi-quantitative RT-PCR. Wild-type (WT, n = 4), WT treated with candesartan (WT+Can, n = 4), ACE2$^{-/y}$ (n = 9), and ACE2$^{-/y}$ mice treated with candesartan (ACE2$^{-/y}$+Can, n = 4) were analyzed. Results are expressed as mean ± SEM.*

There was no obvious morphological difference between wild-type and ACE2$^{-/y}$ mice in any organ. There was also no statistical difference in urinary secretion (Table 1). The blood pressure of ACE2$^{-/y}$ mice did not significantly differ from that of wild-type mice. Echocardiographic studies detected no significant difference between wild-type and ACE2$^{-/y}$ mice at 12 weeks of age. However, LVESD of 24-week-old ACE2$^{-/y}$ mice was significantly larger than that of wild-type mice, with depressed fractional shortening (FS) (Fig. 1). There was no significant difference in LV wall thickness between wild-type and ACE2$^{-/y}$ mice.

Candesartan Ameliorates Cardiac Dysfunction Observed in ACE2-Deficient Mice

Low-dose candesartan (0.1 mg/kg/d) was administered every day starting at 10 weeks of age. Candesartan affected neither blood pressure nor heart rate at this dose. However, deterioration of cardiac function in ACE2$^{-/y}$ mice was significantly attenuated by candesartan as determined by echocardiography (Fig. 1). Hemodynamic studies were also performed in 24-week-old wild-type and ACE2$^{-/y}$ mice (Table 2). The maximum rate of LV pressure change (LV $+dP/dt$) was significantly reduced in ACE2$^{-/y}$ mice. LV end-diastolic pressure (LVEDP) of ACE2$^{-/y}$ mice was higher than that of wild-type mice (Table 2). The minimum rate of LV pressure change (LV $-dP/dt$) also tended to be reduced in ACE2$^{-/y}$ mice. Consistent with the echocardiographic study, candesartan attenuated the impaired hemodynamic parameters of ACE2$^{-/y}$ mice. BNP expression was up-regulated in ACE2$^{-/y}$ mice (Fig. 2). Candesartan treatment significantly attenuated BNP elevation in ACE2$^{-/y}$ mice. Taken together, these results suggest that loss of ACE2 results in age-associated cardiac dysfunction, which could be ameliorated by blockade of the AT1 receptor.

Histological Changes of the LV

Next, we evaluated the histological changes of the LV in 24-week-old wild-type and ACE2-deficient mice. Cardiomyocyte hypertrophy was not observed in ACE2$^{-/y}$ mice as determined by myocyte CSA (Table 1). There was no statistical difference in interstitial fibrosis between wild-type mice and ACE2$^{-/y}$ mice as determined by CVF (Table 1). Candesartan showed no apparent effect on myocyte hypertrophy or interstitial fibrosis in ACE2$^{-/y}$ mice.

Expression of AT1 and Mas in ACE2-Deficient Mice

Localization of the AT1 receptor and Mas, the receptor of Ang-(1–7), in the LV was evaluated by *in situ* hybridization (Fig. 3A). Both AT1R and Mas were homogenously expressed in LV. Expression of AT1 and Mas was also evaluated by semi-quantitative RT-PCR. The AT1 expression level was similar between wild-type and ACE2$^{-/y}$ mice (Fig. 3B). Mas expression appeared to be down-regulated in ACE2$^{-/y}$ mice. Candesartan had no significant effect on the expression of AT1 or Mas in wild-type and ACE2$^{-/y}$ mice.

Increased Ang II Level in Plasma and Heart of ACE2-Deficinet Mice

To obtain clues to the mechanism by which ACE2$^{-/y}$ mice develop cardiac dysfunction, the Ang II concentration in the plasma and heart was measured (Fig. 4A). The Ang II level in plasma and heart was significantly higher in ACE2$^{-/y}$ mice than that in wild-type mice. Candesartan had no significant effect on the Ang II level. The concentration of plasma Ang-(1–7) tended to be decreased in ACE2$^{-/y}$ mice compared with that in wild-type mice (Fig. 4B).

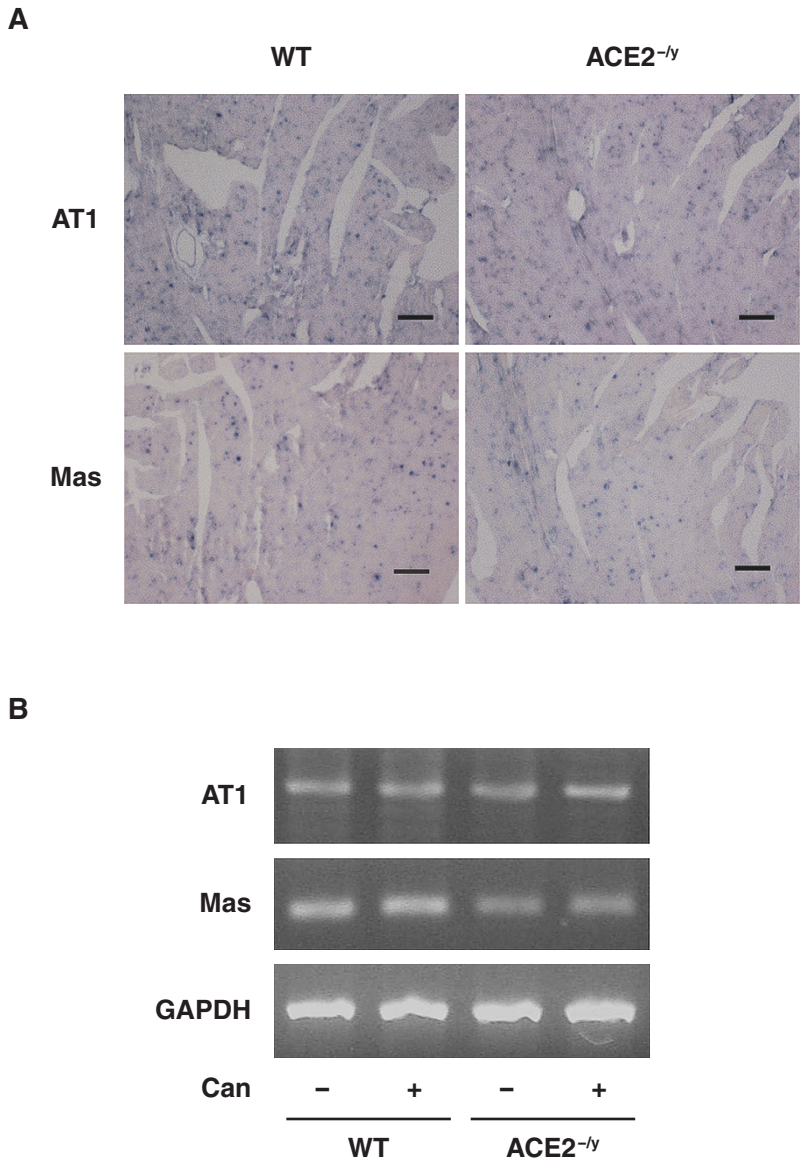

Fig. 3. *AT1 and Mas expression in the LV of WT and ACE2-deficient mice. The LV was obtained from 24-week-old wild-type and ACE2$^{-/y}$ mice. In situ hybridization for AT1 and Mas was performed for both wild-type (WT, n = 2) and ACE2$^{-/y}$ (n = 2) mice. A: Representative image of in situ hybridization. AT1 and Mas were homogeneously expressed in the LV. Scale bar: 100 μm. B: RT-PCR of AT1 and Mas mRNA expression.*

Discussion

In this study, ACE2-deficient mice displayed age-associated cardiac dysfunction. The LV was significantly enlarged with systolic and diastolic dysfunction. BNP expression was up-regulated at 24 weeks of age. The Ang II level in plasma and cardiac tissue was elevated. Pharmacological blockade of the AT1 receptor with candesartan rescued the cardiac dysfunction. These results suggest that ACE2 is a functional component of the renin-angiotensin system. ACE2 seems to attenuate Ang II–AT1 mediated signaling, thereby contributing to maintenance of cardiac homeostasis.

Accumulating evidence indicates that Ang-(1–7), which is cleaved from Ang II mainly by ACE2, plays an essential role in the regulation of vascular tone, cardiac function, coronary perfusion, aortic endothelial function, blood pressure, and organ remodeling (*18*, *24–26*). It has been reported that Ang-(1–7) levels and ACE2 activity are up-regulated in the failing heart (*12*, *13*). It has therefore been hypothesized that increased Ang-(1–7) formation could serve a counter-regulatory, cardio-protective function in heart failure. The AT2

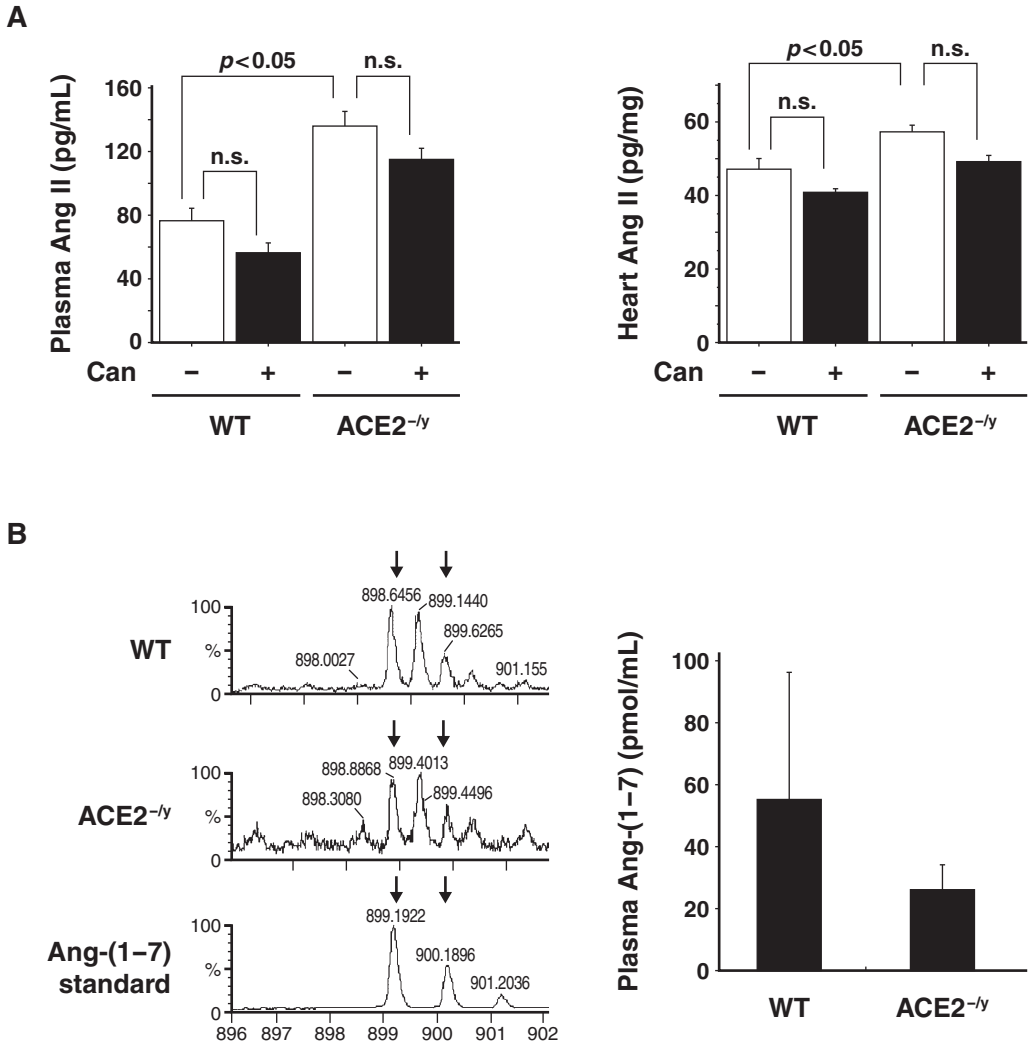

Fig. 4. *Ang II and Ang-(1–7) level in the plasma and heart of WT and ACE2-deficient mice. A: The plasma and LV were obtained from 24-week-old wild-type and ACE2-deficient mice before sacrifice. Ang II level was measured by radioimmunoassay in wild-type mice (WT, n = 4), WT mice treated with candesartan (WT+Can, n = 4), ACE2$^{-/y}$ mice (n = 5), and ACE2$^{-/y}$ mice treated with candesartan (ACE2$^{-/y}$+Can, n = 4). The Ang II level in the heart is expressed as a ratio to protein content. Data are expressed as mean ± SEM. B: Representative mass spectrum for plasma Ang-(1–7). Plasma was analyzed by mass spectrometry (nanoLC-ESI-MS, Waters CapLC system). Data were analyzed by Masslynx soft wear (Waters Co.). Plasma Ang-(1–7) concentration was measured in 24-week-old wild-type and ACE2-deficient mice using a synthetic Ang-(1–7) as a standard. Arrows indicate the peaks of Ang-(1–7).*

receptor has been assumed to mediate the biological actions of Ang-(1–7) (*27*). Santos *et al*. identified Mas, a G protein–coupled receptor encoded by the *Mas* protooncogene, as a functional receptor of Ang-(1–7) (*28*). It was demonstrated that Mas can hetero-oligomerize with the AT1 receptor and inhibit the actions of Ang II (*29*). Mice lacking the *Mas* gene showed enhanced Ang II–mediated vasoconstriction in mesenteric microvessels (*30*). Consistent with this, Crackower *et al*. demonstrated that genetic ablation of ACE on an ACE2 mutant background completely rescued the cardiac phenotype (*6*). Here, we found that ACE2$^{-/y}$ mice showed age-associated cardiac dysfunction, which could be attenuated by blockade of AT1 receptors. The Ang II levels in the plasma and heart were elevated in ACE2$^{-/y}$ mice, whereas there was no significant difference in plasma Ang-(1–7) level between ACE2$^{-/y}$ mice and wild-type mice. Our findings indicate that deficiency in ACE2 results in elevated Ang II levels in the plasma and heart, although Ang-(1–7) could be generated by miscellaneous enzymes other than ACE2 (*31*). Enhanced stimulation of AT1 by the increased Ang II appears to play a role in the development of cardiac dysfunction caused by ACE2 disruption. Consistent with these results,

Yamamoto *et al*. demonstrated that LV hypertrophy and heart failure induced by trans-aortic constriction were markedly enhanced in ACE2$^{-/y}$ mice (*20*). The Ang II level is elevated in the pressure overloaded failing heart (*7*, *32*). Pharmacological blockade of AT1 attenuated the hypertrophy and heart failure (*20*). Taken together, these results suggest that the renin-angiotensin system is regulated by the balance between the "ACE–Ang II–AT1 receptor axis" and its counterpart, the "ACE2–Ang-(1–7)–Mas receptor axis" (*33–36*), although other oligo-peptides produced by ACE and/or ACE2 might also modulate cardiac function (*37*).

In contrast to the mild cardiac phenotype in our ACE2$^{-/y}$ mice, Crackower *et al*. reported that ACE2$^{-/y}$ mice had decreased cardiac contractility and exhibited LV dilatation as early as at 3 months of age (*6*). Our ACE2$^{-/y}$ mice have a mixed background of C57BL/6 and 129/SvEv. The mice of Crackower *et al*. were backcrossed to C57BL/6. It is plausible that the difference in background affected the severity of the phenotype. Consistent with this hypothesis, Gurley *et al*. reported that genetic background significantly influenced susceptibility to Ang II–induced hypertension in the absence of functional ACE2 (*38*). ACE2 deficiency was associated with a modest increase in blood pressure on the C57BL/6 background, whereas the absence of ACE2 had no effect on baseline blood pressures in 129/SvEv mice.

There was no significant difference between wild-type and ACE2$^{-/y}$ mice in cardiomyocyte hypertrophy or cardiac interstitial fibrosis, although ACE2$^{-/y}$ mice displayed significantly enlarged hearts with impaired systolic and diastolic function at 24 weeks of age. Low-dose candesartan had no effect on histological changes. It is plausible that the Ang II and AT1 receptor system—not only in the heart but also in other organs, including vascular trees—may play a role in the pathogenesis of cardiac dysfunction observed in ACE2-deficient mice.

In summary, our results support the idea that ACE2 is an essential regulator of heart function *in vivo* by counter-attacking the function of ACE. Our findings suggest that the balance between the ACE–Ang II–AT1 axis and the ACE2–Ang-(1–7)–Mas axis plays a critical role in maintenance of cardiac function.

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