

ORIGINAL RESEARCH COMMUNICATION

Upregulation of Angiotensin (1-7)-Mediated Signaling Preserves Endothelial Function Through Reducing Oxidative Stress in Diabetes

Yang Zhang,^{1,2} Jian Liu,^{1,2} Jiang-Yun Luo,^{1,2} Xiao Yu Tian,¹ Wai San Cheang,^{1,2} Jian Xu,^{1,3} Chi Wai Lau,¹ Li Wang,^{1,2} Wing Tak Wong,^{1,4} Chi Ming Wong,^{1,2} Hui Yao Lan,⁵ Xiaoqiang Yao,¹ Mohan K. Raizada,⁶ and Yu Huang^{1,2}

Abstract

Aims: Angiotensin-converting enzyme 2 (ACE2)–angiotensin (1-7) [Ang (1-7)]–Mas constitutes the vasoprotective axis and is demonstrated to antagonize the vascular pathophysiological effects of the classical renin–angiotensin system. We sought to study the hypothesis that upregulation of ACE2–Ang (1-7) signaling protects endothelial function through reducing oxidative stress that would result in beneficial outcome in diabetes. **Results:** *Ex vivo* treatment with Ang (1-7) enhanced endothelium-dependent relaxation (EDR) in renal arteries from diabetic patients. Both Ang (1-7) infusion *via* osmotic pump (500 ng/kg/min) for 2 weeks and exogenous ACE2 overexpression mediated by adenoviral ACE2 *via* tail vein injection (10^9 pfu/mouse) rescued the impaired EDR and flow-mediated dilatation (FMD) in *db/db* mice. Diminazene aceturate treatment (15 mg/kg/day) activated ACE2, increased the circulating Ang (1-7) level, and augmented EDR and FMD in *db/db* mouse arteries. In addition, activation of the ACE2–Ang (1-7) axis reduced reactive oxygen species (ROS) overproduction determined by dihydroethidium staining, CM-H₂DCFDA fluorescence imaging, and chemiluminescence assay in *db/db* mouse aortas and also in high-glucose-treated endothelial cells. Pharmacological benefits of ACE2–Ang (1-7) upregulation on endothelial function were confirmed in ACE2 knockout (ACE2 KO) mice both *ex vivo* and *in vitro*. **Innovation:** We elucidate that the ACE2–Ang (1-7)–Mas axis serves as an important signal pathway in endothelial cell protection in diabetic mice, especially in diabetic human arteries. **Conclusion:** Endogenous ACE2–Ang (1-7) activation or ACE2 overexpression preserves endothelial function in diabetic mice through increasing nitric oxide bioavailability and inhibiting oxidative stress, suggesting the therapeutic potential of ACE2–Ang(1-7) axis activation against diabetic vasculopathy. *Antioxid. Redox Signal.* 23, 880–892.

Introduction

DIABETES MELLITUS ACCELERATES atherosclerosis and increases the risks for cardiovascular complications. Although the underlying mechanisms are not fully understood, endothelial dysfunction is implicated in the pathogenesis of diabetic vascular complications (6). The overproduction of reactive oxygen species (ROS) or in-

creased oxidative stress contributes to the impairment of endothelial function in diabetes (16). Angiotensin-converting enzyme 2 (ACE2), a homolog of ACE, is the monocarboxypeptidase in the renin–angiotensin system (RAS). It negatively regulates the RAS by cleaving angiotensin II (Ang II) to generate angiotensin (1-7) [Ang (1-7)], which acts as a vasodilator and vasoprotective peptide through stimulation of its receptor Mas (48, 50, 51, 53). Thus, the ACE2–Ang (1-7)-

¹Institute of Vascular Medicine, ²Li Ka Shing Institute of Health Sciences, and ³School of Life Sciences, Chinese University of Hong Kong, Hong Kong SAR, China.

⁴Department of Cardiovascular Sciences, Houston Methodist Research Institute, Houston, Texas.

⁵Department of Medicine and Therapeutics, Chinese University of Hong Kong, Hong Kong SAR, China.

⁶Department of Physiology and Functional Genomics, University of Florida, Gainesville, Florida.

Innovation

The present study provides novel evidence that angiotensin (1-7) [Ang (1-7)] protects endothelial function in renal arteries of diabetic patients and reveals that the upregulation of the angiotensin-converting enzyme 2 (ACE2)-Ang (1-7) axis of the renin-angiotensin system (RAS) by genetic overexpression or pharmacological activation preserves endothelial function through normalizing overproduction of reactive oxygen species in diabetic *db/db* mice. Thus, the vascular benefits of the ACE2-Ang (1-7)-Mas axis established in the present study highlight the therapeutic potential of drugs that activate this healthy arm of the local RAS in the treatment of diabetic vasculopathy if their safety profile is proven.

Mas axis is proposed as a counter-regulator of the ACE-Ang II arm in the cardiovascular system (7) and kidney (37). The balance between Ang II and Ang (1-7) regulates the overall RAS activity, which in turn controls blood pressure, sodium-water homeostasis, and renal function.

Animal studies show that ACE2 overexpression improves endothelial function in hypertension (43), attenuates the progression of atherosclerosis (29, 59), and suppresses the adverse myocardial remodeling (8, 61). In diabetic *db/db* mice, ACE2-Ang (1-7) upregulation prevents pancreatic beta-cell dysfunction and apoptosis (2) and improves renal function (37). By contrast, ACE2 deficiency leads to or aggravates vascular dysfunction (39, 55), diabetic cardiomyopathy, and nephropathy (38, 47). These studies indicate that the ACE2-Ang (1-7)-Mas axis of the RAS is vasoprotective, probably through reducing Ang II production or acting against Ang II-stimulated oxidative stress and vascular inflammation.

The heart and blood vessels are the main sites for the formation of Ang (1-7) and also the major targets for the action of Ang (1-7). Ang (1-7) infusion preserves cardiac function, coronary perfusion, and aortic endothelial function in rats with coronary ligation-induced heart failure and in diabetic hypertensive rats (1, 28). Ang (1-7) also prevents cardiac remodeling through a direct effect in Ang II-induced hypertensive mice (32) and in 5/6 nephrectomy mice (24). As a vasodilator, Ang (1-7) directly relaxes several vascular beds (3, 41, 42). Ang (1-7)-induced vasodilatation involves an increased production of endothelium-derived relaxing factors (3, 15, 34). Through stimulation of Ang (1-7) receptor Mas, Ang (1-7) exerts counter-regulatory effects in the vasculature and kidney in response to RAS activation or Ang II by suppressing oxidative stress and inflammation (36).

However, the exact role of the local ACE2-Ang (1-7) in the initiation and maintenance of endothelial dysfunction in diabetes, especially in human subjects, is only partially understood. Although adenovirus transduction or human recombinant protein have been employed to overexpress ACE2, targeting to elevate ACE2 activity has rarely been considered in previous studies as an effective therapeutic alternative in the treatment of vascular disease. In the present study, we hypothesize that the upregulation of the ACE2-Ang (1-7) axis by both genetic overexpression and pharmacological activation protects endothelial function in type 2 diabetes.

Results

Ang (1-7) preserves endothelial function in ex vivo human renal arteries

Acetylcholine (ACh)-induced endothelium-dependent relaxation (EDR) was significantly less in renal arteries from diabetic patients than those from nondiabetic subjects (Fig. 1A). By contrast, the arteries from both groups showed identical relaxations induced by the endothelium-independent dilator, sodium nitroprusside (SNP) (Fig. 1B). Renal arteries from diabetic patients were cultured in DMEM containing 1 μ M Ang (1-7) for 24 h and this treatment markedly augmented EDR without affecting SNP-induced relaxations (Fig. 1A, B).

In human umbilical vein endothelial cells (HUVECs), stimulation with Ca^{2+} ionophore, A23187 (0.1 μ M), increased DAF-FM diacetate fluorescence intensity, which reflects the level of NO production in normal glucose (NG, 5 mM) condition. Exposure (48 h) to high glucose (HG, 30 mM) inhibited A23187-stimulated NO production, while cotreatment with Ang (1-7) (1 μ M) reversed the effect of HG (Fig. 1C, D). These results demonstrate a vasoprotective benefit of Ang (1-7) in human arteries and endothelial cells.

Compared with nondiabetic subjects, diabetic patients had increased Ang II level, decreased Ang (1-7) level, and a lower Ang (1-7)/Ang II ratio in their plasma (Supplementary Fig. S1A–C; Supplementary Data are available online at www.liebertpub.com/ars). However, the plasma ACE2 activity was slightly but insignificantly higher in diabetics (Supplementary Fig. S1D).

Ang (1-7) restores endothelial function in diabetic mice

ACh-induced EDR in *db/db* mouse aortas was significantly less compared with relaxations in *db/m*⁺ mouse aortas (Fig. 2A and Supplementary Fig. S2B). Ang (1-7) infusion (500 ng·kg⁻¹·min⁻¹, 2 weeks) *via* the osmotic minipump increased the plasma Ang (1-7) concentration in *db/db* mice (Supplementary Fig. S2A) and restored the impaired EDR in aortas from *db/db* mice without affecting EDR in *db/m*⁺ mice (Fig. 2A). SNP-induced relaxations were similar among different groups (Fig. 2B). In addition, flow-mediated dilatation (FMD) in the 2nd-order mesenteric resistance arteries was evoked by creating a pressure difference of 20 mm Hg that equals an initial shear stress of \approx 15 dynes/cm². FMD was markedly impaired in *db/db* mice compared with *db/m*⁺ mice and it was restored in arteries from Ang (1-7)-infused *db/db* mice (Fig. 2C and 2D). Ang (1-7) infusion also normalized the diminished phosphorylations of Akt at Thr³⁰⁸ and Ser⁴⁷³ and of endothelial nitric oxide synthases (eNOS) at Ser¹¹⁷⁷, but suppressed the phosphorylation of eNOS at the inhibitory site Thr⁴⁹⁵ in *db/db* mouse aortas (Fig. 3A–E).

In *ex vivo* cultured *db/db* mouse aortas, a 24-h exposure to Ang (1-7) (1 μ M) improved the impaired EDR, and the effect of Ang (1-7) was reversed by the Ang (1-7) antagonist, A779 (1 μ M) (Fig. 2E). The restored EDR by Ang (1-7) was abolished by L-NAME or in rings without the endothelium (Supplementary Fig. S3A, B). In *db/m*⁺ mouse aortas, HG (30 mM, 48 h)-induced impairment of EDR was reversed by cotreatment with Ang (1-7) and this vascular benefit of Ang (1-7) was antagonized by Mas receptor antagonist, A779 (Supplementary Fig. S3C). A779 alone did not affect EDR in *db/m*⁺ mouse aortas (Supplementary Fig. S3D).

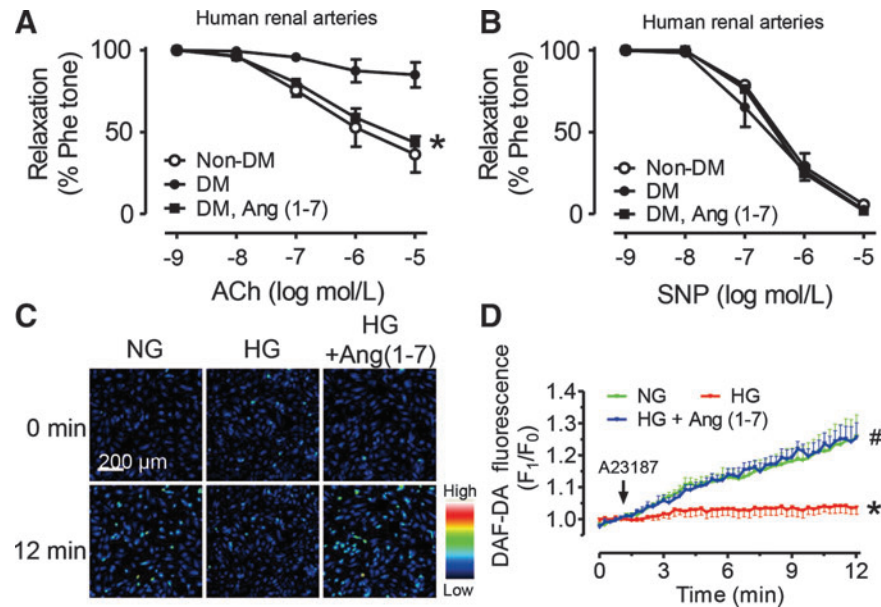


FIG. 1. Angiotensin (1-7) [Ang (1-7)] improves endothelial function in renal arteries from diabetic patients. (A) Acetylcholine (ACh)-induced endothelium-dependent relaxations (EDRs) and (B) sodium nitroprusside (SNP)-induced endothelium-independent relaxations in renal arteries from nondiabetic (Non-DM) subjects and diabetic patients (DM). The effect of *ex vivo* 24-h exposure to Ang (1-7) (1 μ M) on EDR in renal arteries from diabetic patients. Data are mean \pm SEM from 4 separate human arteries, * p < 0.05, versus DM. Nitric oxide (NO) production stimulated by A23187 in human umbilical vein endothelial cells (HUVECs) after different treatments *in vitro* for 48 h. (C) Representative images and (D) summarized data showing the real-time effect of Ang (1-7) (1 μ M) to restore NO production that was suppressed by high glucose (HG: 30 mM) in HUVECs. Data are mean \pm SEM from five experiments. * p < 0.05 versus NG, # p < 0.05 versus HG. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Exogenous ACE2 overexpression rescues endothelial function in diabetic mice

ACE2 catalyzes formation of Ang (1-7). ACE2 overexpression by adenoviral transduction (Ad-ACE2) increased ACE2 content in *db/db* mouse aortas as shown by qPCR, Western blotting, and endothelium *en face* immunofluorescence staining (Fig. 4A, Supplementary Figs. S4 and S5A), while the relative activity of aortic ACE2 per unit mass and plasma ACE2 in *db/db* mice was unaffected by adenoviral transduction (Supplementary Fig. S5B, C). However, the plasma Ang(1-7) level was elevated by Ad-ACE2 transduction in *db/db* mice (Fig. 4B). *In vivo* treatment with ACE2 adenovirus, but not vector control, improved EDR in *db/db* mouse aortas (Fig. 4C). Likewise, FMD in mesenteric arteries was also augmented by ACE2 overexpression (Fig. 4D). In addition, in *ex vivo* culture of *db/db* mouse aortas, Ad-ACE2 transduction also enhanced EDR compared with Ad-GFP transduction and nonadenovirus control (Fig. 4E) without affecting SNP-induced relaxations (Fig. 4F).

Diminazene aceturate increases Ang (1-7) production and protects endothelial function in *db/db* mice

Both expression and activity of aortic ACE2 in the *db/db* mouse were slightly but insignificantly lower than those in *db/m*⁺ mouse aortas (Supplementary Fig. S6A–C). The plasma Ang (1-7) level was smaller in *db/db* compared with *db/m*⁺ mice (Supplementary Fig. S6D). Diminazene aceturate (DIZE) elevated ACE2 activity in HUVECs in a concentration-dependent manner (Supplementary Fig. S7A). Two-week

treatment with putative ACE2 activator, DIZE (15 mg/kg/day), to *db/db* mice increased ACE2 activity in their aortas (Fig. 5A) and plasma (Supplementary Fig. S7B). By contrast, aortic ACE2 expression was unchanged (Fig. 5B). DIZE treatment also significantly elevated the plasma Ang (1-7) level in *db/db* mice (Fig. 5C) without affecting either glucose tolerance or insulin tolerance (Supplementary Fig. S8).

Chronic DIZE treatment restored both the impaired EDR (Fig. 5D) and FMD in *db/db* mice (Fig. 5E) without affecting SNP-induced relaxations (Fig. 5F). *Ex vivo* 24-h treatment with DIZE (100 μ M) also augmented EDR in *db/db* mouse aortas (Fig. 5G) and HG (30 mM)-treated aortas of *db/m*⁺ mice (Supplementary Fig. S3E). The *ex vivo* beneficial effect of DIZE was reversed by either ACE2 inhibitor, DX600 (1 μ M), or Ang(1-7) antagonist, A779 (1 μ M) (Fig. 5G and Supplementary Fig. S3E).

Next, ACE2-deficient mice were employed to confirm the ACE2-mediated effect of DIZE. EDR and plasma Ang (1-7) level were similar between ACE2 wild-type (ACE2 WT) and ACE2 knockout mice (ACE2 KO) (Supplementary Fig. S3F and S6E). In aortas of ACE2 WT mice, HG-impaired EDR was rescued by *ex vivo* treatment with either DIZE (100 μ M) or Ang (1-7) (1 μ M) (Fig. 5H). However, in aortas from ACE2 KO mice, HG-induced impairment of EDR was rescued only by Ang (1-7), but not by DIZE (Fig. 5I).

ACE2-Ang (1-7) activation normalizes endothelial ROS overproduction in *db/db* mice

The ROS level indicated by dihydroethidium (DHE) staining was higher in the *en face* endothelial layer or the vascular wall

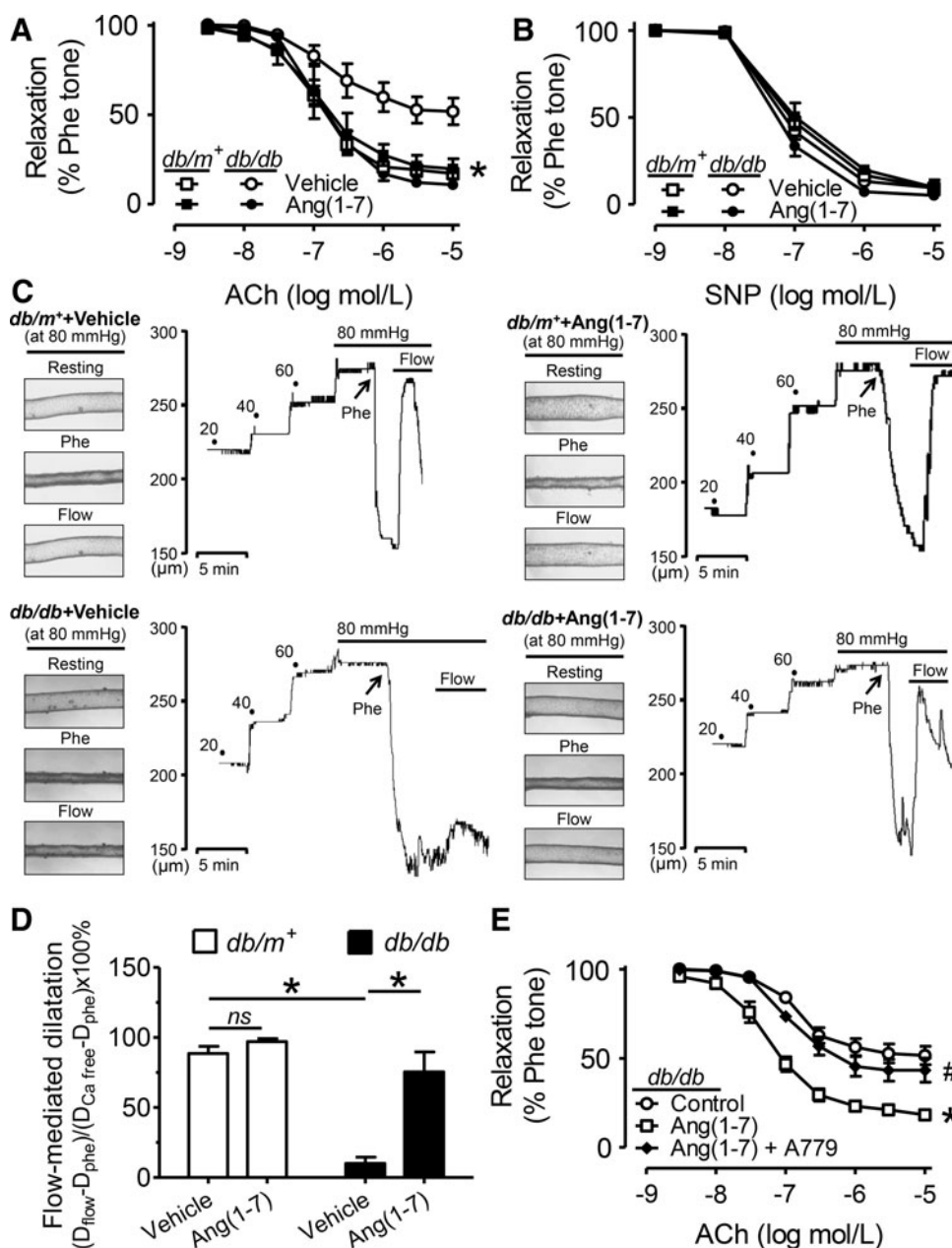


FIG. 2. Ang (1-7) improves endothelial function in *db/db* mice. (A) ACh-induced EDRs and (B) SNP-induced endothelium-independent relaxations in aortas from *db/m⁺* and *db/db* mice with and without Ang (1-7) infusion (500 ng·kg⁻¹·min⁻¹, 2 weeks). (C) Representative traces and (D) summarized data for flow-mediated dilatation (FMD) in the 2nd-order of mesenteric arteries from the four groups of mice. Data are mean ± SEM of four to six mice. **p* < 0.05 versus *db/db* vehicle. ns, non-significant. (E) EDRs in *db/db* aortas following a 24-h exposure to Ang (1-7) (1 μM) and Ang (1-7) plus Mas receptor antagonist, A779 (1 μM). Data are mean ± SEM of five mice. **p* < 0.05 versus control, #*p* < 0.05 versus Ang (1-7).

of aortas from *db/db* mice compared with those from *db/m⁺* mice (Fig. 6A, B and Supplementary Fig. S9). Two-week Ang (1-7) infusion suppressed the endothelial ROS overgeneration in *db/db* mouse aortas without affecting the basal ROS level in the endothelium of *db/m⁺* mouse aortas (Fig. 6A, B). Both *in vivo* Ad-ACE2 transduction and DIZE treatment reduced DHE fluorescence intensity in *db/db* mouse aortic endothelial cells (MAECs) (Fig. 6C, D and Supplementary Fig. S10).

Ex vivo 24-h treatment with either Ang (1-7) (1 μM) or DIZE (100 μM) inhibited the basal content of superoxide anions in *db/db* mouse aortas (Fig. 6E). In addition, a 48-h exposure to Ang (1-7) or DIZE reversed HG-stimulated superoxide elevation in C57BL/6 mouse aortas (Fig. 6F). Finally, in primary cultured MAECs, HG-triggered ROS overgeneration was inhibited by Ang (1-7) or tiron plus DETCA, while DIZE inhibited ROS overproduction in cells only from ACE2 WT mice, but not those from ACE2 KO

mice (Fig. 6G, H). Likewise, HG-induced ROS rise in HUVECs reflected by elevated CM-H₂DCFDA fluorescence intensity was inhibited by Ang (1-7) and DIZE, while ACE2 inhibitor, DX600, reversed the effect of DIZE, but not Ang (1-7). Ang (1-7) antagonist, A779, counteracted the ROS-lowering effects of both DIZE and Ang (1-7) in HG-treated HUVECs (Supplementary Fig. S11).

Upregulation of the ACE2-Ang (1-7) axis suppresses HG-induced MAPK phosphorylation and NADPH oxidase expression, while it increases UCP2 expression

The HG-elevated NOX2 expression was reversed by a 48-h cotreatment with DIZE (100 μM), while the expression of p67, p47, and NOX4 was unchanged in HG-exposed HUVECs with or without DIZE treatment (Supplementary Fig. S12). Exposure

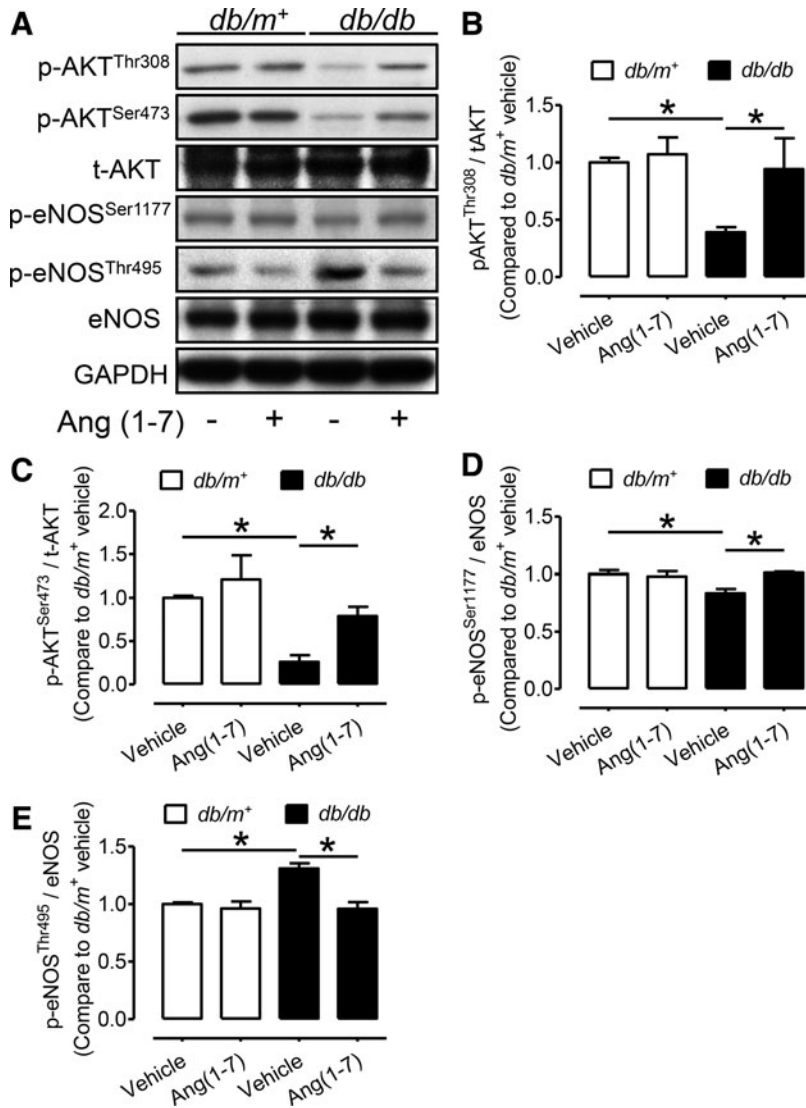


FIG. 3. The effect of Ang (1-7) on phosphorylation of Akt and eNOS in mouse aortas. The phosphorylation levels of Akt and eNOS in the aortas from *db/m⁺* and *db/db* mice with or without Ang(1-7) infusion shown by (A) representative blots and (B–E) summarized data. Data are mean \pm SEM from four individual experiments. * $p < 0.05$.

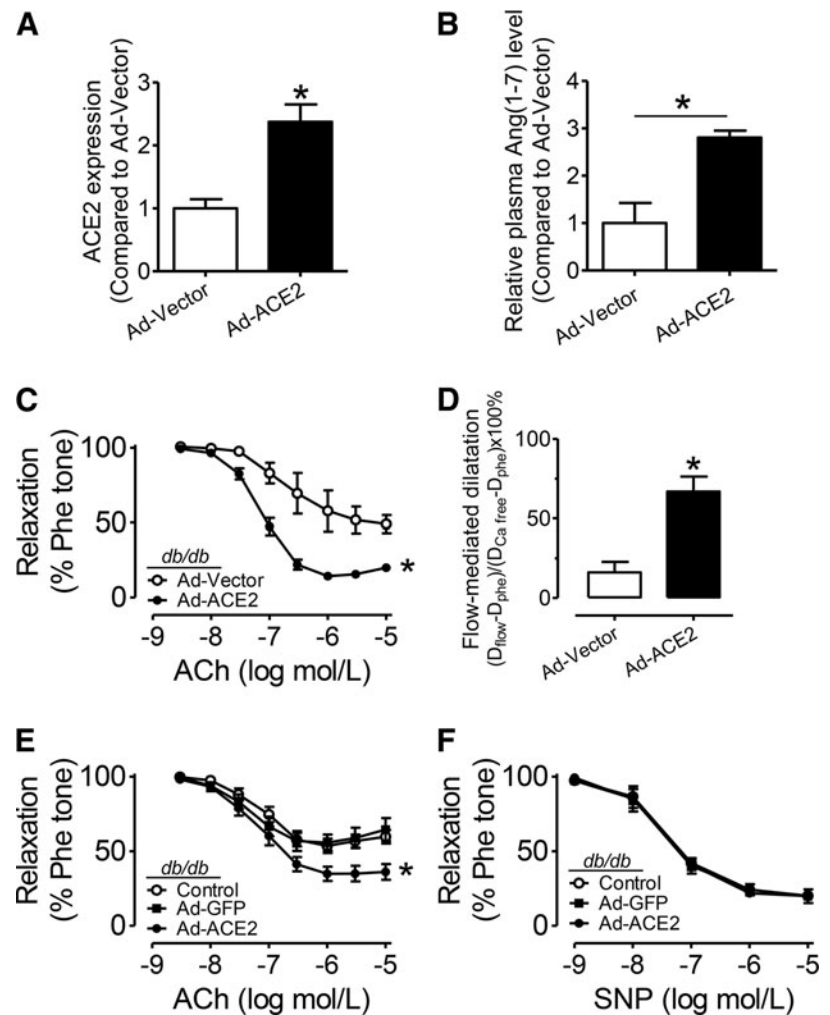
to HG also increased the phosphorylation of ERK1/2, p38, and JNK, which was reversed by Ang (1-7) (1 μ M) in HUVECs (Supplementary Fig. S13). Expression of several antioxidant genes was measured in human aortic endothelial cells (HAECs) transfected for 24 h with Ad-vector or Ad-ACE2. Uncoupling protein-2 (UCP2) was upregulated by ACE2 overexpression (Supplementary Fig. S14A). UCP2 knockdown by adenoviral UCP2 shRNA (Ad-UCP2 shRNA), which was proved by qPCR and Western blot (Supplementary Fig. S14B, C), diminished the inhibitory effect of Ang (1-7) on ROS production in HG-treated HAECs (Supplementary Fig. S15).

Discussion

The present study demonstrates a critical role of activation of the ACE2-Ang (1-7)-Mas axis in protecting endothelial function of *db/db* diabetic mice. The upregulation of ACE2-Ang (1-7) attenuates oxidative stress and increases the NO bioavailability, thus counteracting the deleterious effect of the local RAS in arteries in diabetic mice. Importantly, *ex vivo* Ang (1-7) treatment preserves endothelial function in arteries of diabetic patients.

Ang (1-7), as an endogenous heptapeptide, is formed from precursors, Ang I or Ang II, under the catalytic reaction of several peptidases, including carboxypeptidases, ACE and ACE2 (17), and the conversion of Ang II to Ang (1-7) through ACE2 appears to represent the most preferred pathway for Ang (1-7) generation (51). Ang (1-7)-mediated activation of Mas receptor has been reported to counter-regulate Ang II-induced endothelial cell proliferation (44), to inhibit cardiac fibroblast proliferation and cardiac remodeling by negatively modulating extracellular signal-regulated kinase (ERK) (32), and to cause vasorelaxation by releasing NO and prostaglandins (4, 45). Although Ang (1-7) has been assessed for its acute pharmacological action on blood vessels, it is yet to be established whether Ang (1-7) can chronically ameliorate endothelial dysfunction in diabetes. The present study was probably among the first to demonstrate the beneficial effect of Ang (1-7) to augment EDR in *ex vivo* cultured renal arteries of diabetic patients. This observation in human arteries prompted us to reveal that chronic Ang (1-7) infusion restored the impaired endothelial function in *db/db* mice, as shown by both improved EDR in aortas and augmented FMD in resistance arteries. We further confirmed

FIG. 4. Angiotensin-converting enzyme 2 (ACE2) overexpression rescues endothelial function in *db/db* mice. (A) Summarized data showing *en face* immunofluorescence staining of ACE2 expression in endothelial cells of intact mouse aortas from *db/db* mice with adenoviral ACE2 (Ad-ACE2) or vector (Ad-vector) transduction. (B) Relative Ang(1-7) level in the plasma of *db/db* mice with Ad-ACE2 and Ad-vector transduction. (C) EDRs in aortas and (D) FMD in mesenteric arteries from Ad-ACE2 or Ad-vector cultured *db/db* mice. Data are mean \pm SEM of four to six mice. * $p < 0.05$ versus Ad-vector. (E) EDRs and (F) SNP-induced relaxations in *db/db* mouse aortas following a 48-h exposure to adenovirus. Data are mean \pm SEM of five to six mice. * $p < 0.05$ versus Ad-GFP.



the vascular benefit of *ex vivo* 24-h treatment with Ang (1-7) to rescue EDR in organ culture and also in HG-treated *db/m*⁺ mouse aortas. Moreover, the beneficial effect of Ang (1-7) was abolished by A779, a potent antagonist of Mas, thus suggesting that the vasoprotective benefit of Ang (1-7) is most likely through activation of Mas. The improved EDR by chronic Ang (1-7) treatment was also observed in apolipoprotein E-deficient mice (ApoE^{-/-}) (51) and salt-induced hypertensive rats (41). Ang (1-7)-mediated vasodilatation is reportedly mediated through stimulation of Mas, which is coupled to the downstream Akt/eNOS signaling (45). The present study shows that chronic Ang (1-7) treatment increased the basal phosphorylations of Akt and eNOS that were lower in aortas of *db/db* mice. Therefore, the vasoprotective effect of chronic Ang (1-7) infusion in *db/db* mice is likely mediated through a G-protein-coupled receptor Mas-mediated PI3K/Akt/eNOS cascade.

ACE2 gene therapy has been initiated experimentally against hypertension, cardiac dysfunction, and renal damage through suppressing the production and downstream signaling of Ang II (10, 26, 59, 61). The present study shows that ACE2 overexpression both *in vivo* and *ex vivo* by ACE2 adenovirus transduction rescued the impaired EDRs in aortas and FMD in resistance mesenteric arteries from diabetic mice, which was consistent with earlier studies on ApoE^{-/-}

mice and spontaneously hypertensive stroke-prone rats (29, 43). Recently, ACE2 priming by the pharmacological activator, xanthenone (XNT), has been shown to protect the function of endothelial progenitor cells in hypertension and diabetes (5, 11, 18, 23). In the present study, we have used a newly discovered ACE2 activator, DIZE (20, 40, 46), and shown that it enhances ACE2 activity *in vitro*. Chronic DIZE treatment elevated the activity of local vascular ACE2, subsequently increased the production of Ang (1-7), and augmented both EDRs in conduit aortas and FMD in resistance arteries from *db/db* mice. Likewise, *ex vivo* exposure of *db/db* mouse aortas to DIZE also enhanced EDRs. These effects are probably specific to the ACE2 and Ang (1-7) since the ACE2 inhibitor, DX600, and Ang (1-7) antagonist, A779, reversed the effect. Furthermore, the lack of vasoprotective effect in ACE2 knockout mice confirmed the selectivity of DIZE to activate ACE2.

Endothelial dysfunction results from a decreased bioavailability of NO or/and an increased production of ROS in the vascular wall. The antioxidant defense of the ACE2-Ang (1-7) axis is being just recently examined in several types of cells (14, 25, 33). The present study shows that ROS overgeneration in endothelial cells and aortic vascular wall of *db/db* mice was suppressed by Ang (1-7) infusion as well as ACE2 activation and overexpression. The elevated NO

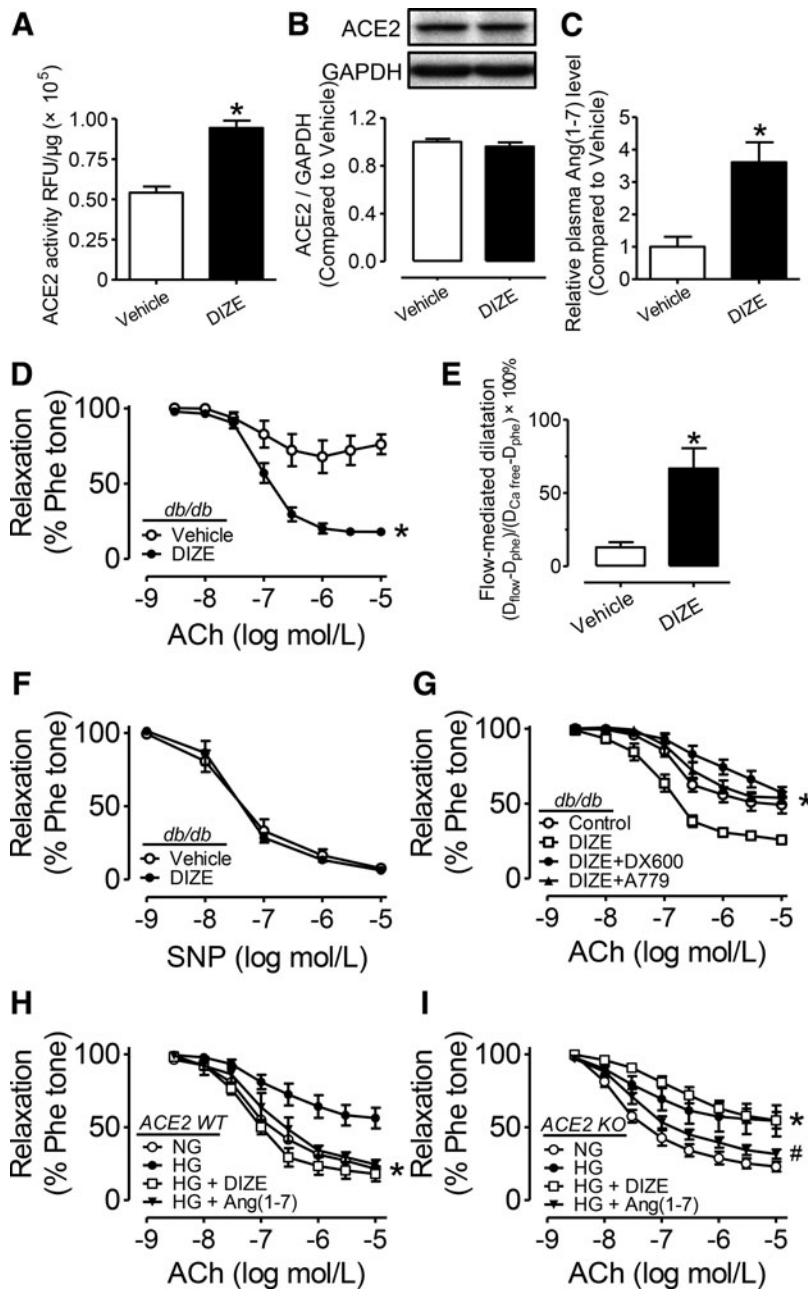


FIG. 5. ACE2 activation restores endothelial function in *db/db* mice. The effect of chronic treatment with diminazene aceturate (DIZE, 15 mg/kg/day, 14 days) on the (A) activity and (B) expression of ACE2 in *db/db* mouse aortas. RFU, relative fluorescence unit. (C) Plasma Ang (1-7) level in *db/db* mice with and without DIZE treatment. Data are mean \pm SEM of five to six mice. * $p < 0.05$ versus vehicle. (D) ACh-induced EDRs and (E) SNP-induced relaxations in aortas and (F) FMD in mesenteric arteries from *db/db* mice. Data are mean \pm SEM of six mice. * $p < 0.05$ versus vehicle. (G) EDRs in *db/db* mouse aortas following a 24-h exposure to DIZE (100 μ M), or DIZE plus ACE2 inhibitor, DX600 (1 μ M), or DIZE plus Mas receptor antagonist, A779 (1 μ M). Data are mean \pm SEM of five to six mice. * $p < 0.05$ versus DIZE. EDRs in aortas from (H) ACE2 WT or (I) ACE2 KO mice following a 48-h exposure to normal glucose (NG, 5 mM) high glucose (HG, 30 mM), HG plus DIZE (100 μ M), and HG plus Ang (1-7) (1 μ M). Data are mean \pm SEM of five mice. (H) * $p < 0.05$ versus HG, (I) * $p < 0.05$ versus NG, # $p < 0.05$ versus HG.

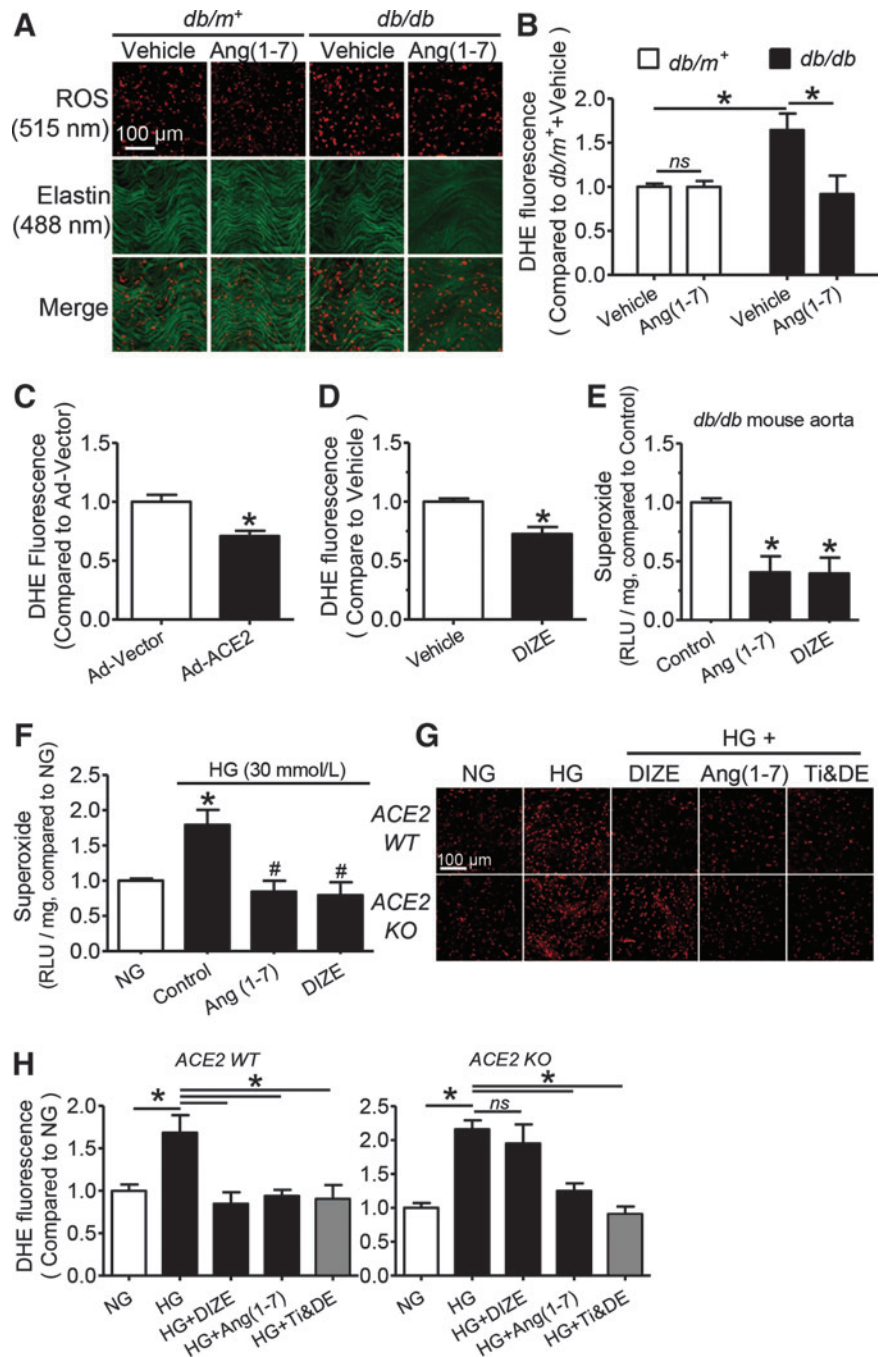
production is likely due to the antioxidative benefit of the upregulation of the ACE2-Ang (1-7) axis. This was further supported by the observation that DIZE fails to reduce ROS in endothelial cells from ACE2 KO mice. In ACE2 KO mice, Ang (1-7) was still able to inhibit ROS generation. The present results also indicate that ACE2 upregulation and activation may target NADPH oxidases to lower the vascular ROS level in diabetic mice. In fact, the *in vitro* results show that activation of the ACE2-Ang (1-7) axis suppressed NOX2 expression and attenuated MAPK activation under hyperglycemic conditions, which agrees with a previous report about its beneficial effects on renal proximal tubular cells (12, 52), endothelial progenitor cells (5), and endothelial function (11). Moreover, the downstream antioxidant molecular mechanism initiated by ACE2-Ang (1-7) was investigated. UCP2, which preserves endothelial function in diet-induced

obese mice and hypertensive rats (27, 57), can be upregulated by ACE2 overexpression in HAECs. Knockdown of UCP2 attenuated the inhibitory effect of Ang (1-7) on ROS overproduction in HG-treated HAECs, suggesting the possible involvement of UCP2 in Ang (1-7)-mediated antioxidant effects in the vasculature of diabetic mice.

A previous study showed that the ACE2 overexpression in the pancreas improves glucose metabolism through increasing the first-phase insulin secretion without affecting peripheral insulin sensitivity in the early stage of diabetes in *db/db* mice, while the beneficial effects of ACE2 were absent in 16-week-old *db/db* mice (2). The present study does not show a metabolic benefit as neither glucose metabolism nor insulin sensitivity was altered in adult *db/db* mice (>14-week) treated with ACE2 activator. The effect of ACE2 activation on improving glucose metabolism is likely to be related to

FIG. 6. Activation of the ACE2-Ang (1-7) axis reduces reactive oxygen species (ROS) overgeneration in endothelial cells of *db/db* mice.

(A) Representative images and (B) summarized data showing ROS production as detected by dihydroethidium (DHE, 5 μ M) staining in endothelium *en face* of aortas from *db/m⁺* and *db/db* mice with and without receiving Ang (1-7) infusion. Summarized data showing the relative ROS level in endothelium *en face* of aortas from *db/db* mice (C) with Ad-vector or Ad-ACE2 transduction and (D) chronic treatment with vehicle or DIZE. (A high-quality color representation of this figure is available in Supplementary Fig. S8). Data are mean \pm SEM of five to six mice. * p < 0.05. Relative chemiluminescence units (RLUs) showing the superoxide level in (E) *db/db* mouse aortas after a 24-h exposure to Ang (1-7) (1 μ M) or DIZE (100 μ M) and in (F) C57BL/6 mouse aortas after a 48-h treatment with high glucose (HG, 30 mM). (E) * p < 0.05 versus control, (F) * p < 0.05 versus NG, # p < 0.05 versus HG. (G) Representative images and (H) summarized data of the ROS level in primary cultured mouse aortic endothelial cells from ACE2 WT and ACE2 KO mice following a 48-h exposure to normal glucose (NG, 5 mM), high glucose (HG, 30 mM), HG+DIZE (100 μ M), HG+Ang (1-7) (1 μ M), and HG plus ROS scavengers, tiron and DETCA (Ti & DE, tiron:1 mM, DETCA: 100 μ M). Data are mean \pm SEM of six experiments, * p < 0.05. Red: DHE fluorescence (excitation: 515 nm) in the nucleus; Green: autofluorescence of elastin beneath the endothelium (excitation: 488 nm). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars



the pathological stage of diabetes. Nevertheless, whether ACE2 activation improves peripheral insulin resistance is still unknown.

Although ACE2 deficiency induces or exacerbates vascular inflammation, heart failure, atherosclerosis, and renal damage in pathological conditions (19, 35, 38, 39, 54, 55), the EDRs in aortas and the capacity of ROS production in primary cultured mouse endothelial cells and the plasma Ang (1-7) level are not different between ACE2 wild-type and deficient mice on normal chow. Moreover, the pharmacological inhibition of ACE2-Ang (1-7) did not impair EDRs in aortas from normal mice. In the physiological condition, the normal RAS might play a minor role in the control of the endothelial function even if the ACE2-Ang(1-7) axis is in-

hibited. On the other hand, overactivation of the ACE-Ang II arm of the RAS associated with diabetic situations results in the increased production of Ang II, but the decreased production of Ang (1-7), as shown in both diabetic patients and *db/db* mice. However, overexpression or activation of the ACE2-Ang (1-7) axis through either genetic or pharmacological approaches is effective to protect against endothelial dysfunction in diabetes through counteracting the exaggerated ACE-Ang II pathway. The post-translational modification or conformational change of ACE2 by pharmacological treatment can become a potential therapeutic strategy in addition to classic ACE2 gene therapy.

There are still some controversies over replenishing ACE2 as a potential therapeutic target. There are reports that show a

link between upregulation of ACE2 or Ang (1-7) to ventricular arrhythmias (9), cardiac fibrosis (31), and renal injury (22). In addition, the expression and activity of ACE2 are found to be higher in the kidney of diabetic mice (49, 58). Although a compensatory increase in ACE2 activity could be proposed under diabetic conditions, more studies are needed to understand the kidney-specific balance between ACE and ACE2 at the post-transcriptional and post-translational levels during the time course of diabetes. Our results on human plasma, however, suggest that the slightly increased ACE2 is probably inadequate to convert Ang II into Ang (1-7). It is probable that functional ACE2 might also depend on the post-translational modification, including conformational change, phosphorylation, and ubiquitination, which are still unclear. Although the present results suggest that pharmacological priming targeting the ACE2-Ang (1-7) axis may be a promising approach to preserve endothelial function in diabetes, the potential tissue-specific side effects certainly need further investigation.

Endothelial dysfunction in diabetes is a chronic pathological process involving not only elevated circulating glucose but also many other inter-related factors such as advanced glycation end products and angiotensin II. The use of 30 mM glucose in both *ex vivo* and *in vitro* experiments is simply aimed to support the *in vivo* vascular benefits of Ang (1-7) and ACE2 activator to preserve endothelial function in diabetic mice. It should be noted that this concentration of glucose is not translatable to the clinical settings in humans.

In summary, the present study establishes the beneficial effect of the ACE2-Ang (1-7)-Mas axis of the RAS to restore endothelial function in renal arteries from diabetic patients and in conduit as well as resistance arteries from diabetic mice. The upregulation and activation of ACE2-Ang (1-7) increase the NO bioavailability, probably as a result of the inhibition of ROS production in endothelial cells. The vascular benefit of the ACE2-Ang (1-7) axis elucidated in the present study highlights the therapeutic potential of drugs that activate this healthy arm of the local RAS in the treatment of diabetic vasculopathy if their safety profile is proven.

Materials and Methods

Human artery specimen

The use of human renal arteries was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. Human renal arteries were obtained during surgery after informed consent from diabetic patients undergoing nephrectomy at ages between 49–76 years. The diabetic patients had a fasting plasma glucose level of over 7.0 mM (or 126 mg/dl) or 2-h plasma glucose \geq 11.1 mM (or 200 mg/dl). The background information of human subjects is presented in Supplementary Table S1.

Animals

All animal experiments were approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong (CUHK), and were conducted under institutional guidelines for the humane treatment of laboratory animals. Male C57BL/6J, leptin receptor-deficient *db/db* mice (homozygous) and *db/m*⁺ mice (heterozygous) were supplied by

the CUHK Laboratory Animal Center. ACE2 wild-type (ACE2 WT, ACE2^{+/+}) and knockout (ACE2 KO, ACE2^{-/-}) mice were generated as described (7). All mice were kept at 22–23°C and 55% \pm 5% humidity with a 12-h light/12-h dark cycle and free access to diet and water.

Metabolic parameters

The insulin tolerance test was performed by measuring blood glucose levels with a commercial glucometer (Ascensia) at 0, 15, 30, 60, and 120 min after injection of insulin (0.5 U/kg; Sigma) to each mouse after 2 h of fasting. For the oral glucose tolerance test, mice were loaded with 10% glucose solution (1.2 g/kg body weight) *via* oral gavage after 8 h of fasting before blood glucose measurement.

Osmotic minipump infusion

Vehicle or Ang (1-7) (5 mg/ml; Enzo Life Sciences, Inc.) dissolved in phosphate-buffered saline were infused into *db/db* and *db/m*⁺ mice using the osmotic pump (Alzet) with an infusion rate of 500 ng/kg/min for 2 weeks.

Adenoviral construction and transduction *in vivo* and *ex vivo*

Human ACE2 cDNA was PCR amplified from hACE2 plasmid (Addgene ID 1786) and ligated into adenoviral shuttle plasmid pAdTrack-CMV (Addgene ID 16405). The shRNA (5'-GCCTGTATGATTCTGTCAAAC-3') sequence targeting UCP2 mRNA was designed using BLOCK-iT™ RNAi Designer (Life Technologies) and cloned to the pAd-track-U6 vector. The procedures of plasmid construction, virus package, amplification, and purification followed the previous report (30). Adenovirus was packaged in HEK293A cells and titered with 1×10^9 pfu/ml. For the *in vivo* study, 100 μ l (1×10^9 pfu/ml) of adenoviral ACE2 (Ad-ACE2) or adenoviral vector (Ad-vector) was delivered to *db/db* mice *via* tail vein injection and experiments were carried out 7 days after injection. For *ex vivo* studies, aortas were transduced with Ad-ACE2 or Ad-GFP adenovirus ($\sim 10^7$ pfu) in 10% FBS DMEM for 48 h. UCP2 knockdown efficiency was confirmed by detection of UCP2 mRNA by qPCR and protein by Western blotting.

Drug administration

At the age of 14 weeks, male *db/db* mice were treated with DIZE, the ACE2 activator dissolved in water, *via* oral gavage at 15 mg \cdot kg⁻¹ day⁻¹ for 2 weeks (13).

Artery preparation and culture

After mice were sacrificed by CO₂ suffocation, thoracic aortas and mesenteric arteries (2nd-order) were dissected out and placed in oxygenated ice-cold Krebs solution containing (in mM) 119 NaCl, 4.7 KCl, 2.5 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, and 11 D-glucose. Aortas were cut into ring segments \sim 1.8 mm long in sterile Krebs solution and incubated in a 10% FBS DMEM supplemented with 1% antibiotic–antimycotic (Invitrogen). Arteries were exposed to HG (30 mM) or normal glucose (NG, 5 mM plus 25 mM mannitol) prepared in DMEM as osmotic control. The chemicals, including DX600 (62337; AnaSpec, Inc.), A779

(H2888; Bachem, Torrance, CA), tiron (D7389; Sigma), and diethyldithiocarbamate (DETCA, D3506; Sigma), were added individually into the culture medium at desired concentrations.

Functional study

Changes of isometric tension of aortas were recorded in a wire myograph (Danish Myo Technology), and FMD was determined in a pressure myograph (60). For FMD study, the vessel diameter was continuously monitored on a light-inverted microscope (Zeiss Axiovert 40 Microscope, model 110P) through a video camera operated on the Myo-View system (Danish Myo Technology). Phenylephrine ($5 \mu\text{M}$) was used to induce a steady constriction after the artery was stabilized at an intraluminal pressure of 80 mmHg. FMD was then triggered by pressure change that equals ~ 15 dynes/cm² shear stress. At the end of the experiment, a maximum passive dilation was induced by switching to Ca²⁺-free Krebs solution containing 2 mM EGTA. FMD was expressed as the percentage changes of diameter = [(flow-induced dilation – Phe tone)/(passive dilation – Phe tone)] \times 100%.

Endothelial cell culture

HUVECs (Lonza) were cultured in endothelial cell growth medium (EGM, CC3024; Lonza) with 10% FBS and antibiotics. HAECs from Invitrogen were cultured in medium 200 (Gibco, M-200-500) supplemented with 2% FBS. Cells from passage 4–8 were used for experiments. Twelve-hour post-transduction with adenoviral vectors, the culture medium was changed with fresh medium. Primary MAECs were isolated, cultured, and identified at passage 2 by positive immunostaining of anti-eNOS and anti-VE-cadherin (60). MAECs at passage 2–4 with 70% confluence were used.

ACE2 activity measurement

The ACE2 activity was determined using an assay kit (AnaSpec, SensoLyte[®] 390). Mca/Dnp fluorescence resonance energy transfer (FRET) test was performed under the Envision multiple fluorescent reader (Perkin Elmer). The FRET effect was monitored at the endpoint of reagents' reaction and recorded as the relative fluorescence unit (RFU), which was a ratio of fluorescence intensity at excitation/emission = 330 nm/390 nm. The ACE2 activity was expressed as the RFU *versus* protein amount (μg for tissue or cell sample).

Plasma Ang (1-7) and Ang II measurement

Plasma samples were extracted using the Extraction-Free Kit (EIAS) (S-5000; Bachem). Ang (1-7) and Ang II levels were measured by the high-sensitivity peptide enzyme immunoassay kit (Angiotensin I/II (1-7)-EIA Kit: S-1330; Bachem; Angiotensin II EIA Kit: 589301; Cayman). The absorbance was read at 450 nm for Ang (1-7) assay and 414 nm for Ang II assay in a 96-well microtiter plate reader (Bio-rad).

ROS production in arteries and endothelial cells

The amount of intracellular ROS production in the vascular wall and *en face* endothelium were determined using

DHE (Molecular Probes, Invitrogen) (60). For *en face* DHE staining, after *ex vivo* culture, aortic rings were incubated with DHE ($5 \mu\text{M}$) for 15 min in extracellular medium (ECM, in mM: 121 NaCl, 5 NaHCO₃, 10 Na-HEPES, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2 CaCl₂, 10 glucose; pH = 7.4). Rings were washed twice in ECM and cut open longitudinally. Subsequently, the endothelium was placed upside down between two coverslips (1#, Thermo). The fluorescence images were captured on a confocal microscope (FV1000; Olympus) (DHE: excitation: 535 nm, emission: 565–605 nm; autofluorescence of elastin excitation: 488 nm, emission 520–535 nm). Intracellular ROS in MAECs, HAECs, or HUVECs was assayed using DHE (Molecular Probes, Invitrogen, excitation: 535 nm, emission: 565–605 nm) or CM-H₂DCFDA (Invitrogen, excitation: 488 nm; emission: 505–525 nm). In addition, the amount of vascular superoxide anion was also determined using the lucigenin-enhanced chemiluminescence method (21).

Western blot analysis

Equal amounts of protein samples were electrophoresed on a 10% SDS-polyacrylamide gel, and then transferred onto an immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked by 2% BSA in 0.05% Tween-20 phosphate-buffered saline (PBST), then incubated overnight at 4°C with primary antibodies, including ACE2 (#3583-1; Epitomics), phospho-Akt^{Thr308} (#4056; Cell Signaling Technology), phospho-Akt^{Ser473} (#9271; Cell Signaling Technology), Akt1 (#2967, Cell Signaling Technology), phospho-MAPK family members (p-p38, p-Erk1/2, p-SAPK/JNK, #9910 antibody sampler kit; Cell Signaling Technology), Erk1/2 (#9107, Cell Signaling Technology), p38 (#9212; Cell Signaling Technology), SAPK/JNK (#9252; Cell Signaling Technology), phospho-eNOS^{Ser1177} (ab51038; Abcam), phospho-eNOS^{Thr495} (#9574; Cell Signaling Technology), eNOS (610297; BD Transduction Laboratories), NOX2/gp91^{phox} (ab31092, Abcam), NADPH oxidase 4 (NOX4, ab60940, Abcam), p47^{phox} (sc-14015; Santa Cruz), p67^{phox} (#3923, Cell Signaling Technology), and UCP2 (AF4739; R&D System). The membranes were developed with an enhanced chemiluminescence detection system (ECL reagents; Amersham Pharmacia) and finally exposed to X-ray films.

qPCR analysis

Total RNA was isolated with TRIZOL reagent (Invitrogen) from the aorta, heart, liver, or HAECs. cDNA was synthesized using 1.0 μg RNA as a template with high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was performed on the ViiATM7 real-time PCR system (Applied Biosystems) with GAPDH used as an internal control. Gene expression was analyzed by the comparative CT method and expressed compared with GAPDH. The sequences of primers used are presented in Supplementary Table S2.

NO detection in endothelial cells

Fluorometric NO measurements were performed on HUVECs as described before (56) using the Olympus FV1000 laser scanning confocal system (Olympus) mounted on an

inverted microscope (IX81; Olympus) equipped with 10X objective (NA 0.5). 4-Amino-5-methylamino-2',7'-difluoro-fluorescein diacetate (DAF-FM diacetate; Molecular Probes) was used as the NO indicator. Cells were incubated with 1 μ M DAF-FM DA in the dark for 15 min and then washed thrice, 5 min each. NO production stimulated by 1 μ M A23187 was evaluated by measuring fluorescence intensity excited at 495 nm and emitted at 515 nm. Changes in intracellular NO production were displayed as relative fluorescence intensity F1/F0 (F0=basal fluorescence, F1=fluorescence after A23187 administration).

Statistical analysis

Concentration–response curves were constructed using GraphPad Prism software (Version 5.0). Protein expression was quantified by Quantity One 1-D Analysis software (Version 4.6.2; Bio-Rad) and normalized to GAPDH, and then compared with control. Data are mean \pm SEM of n separate experiments. Student's *t*-test or one-way ANOVA, followed by Bonferroni *post hoc* tests for more than two treatments, were compared. $p < 0.05$ was regarded as significantly different.

Acknowledgments

This study is supported by the Hong Kong Research Grants Council (CUHK2/CRF/12G, 466110, T12/402/13N, CUHK3/CRF/12R), the NIH HL102033 grants, and the Natural Science Foundation of China (91339117, 2012CB517805).

Author Disclosure Statement

There are no competing financial interests.

References

- Benter IF, Yousif MH, Dhaunsi GS, Kaur J, Chappell MC, and Diz DI. Angiotensin-(1-7) prevents activation of NADPH oxidase and renal vascular dysfunction in diabetic hypertensive rats. *Am J Nephrol* 28: 25–33, 2008.
- Bindom SM, Hans CP, Xia H, Boulares AH, and Lazartigues E. Angiotensin I-converting enzyme type 2 (ACE2) gene therapy improves glycemic control in diabetic mice. *Diabetes* 59: 2540–2548, 2010.
- Brosnihan KB, Li P, and Ferrario CM. Angiotensin-(1-7) dilates canine coronary arteries through kinins and nitric oxide. *Hypertension* 27: 523–528, 1996.
- Castro CH, Santos RA, Ferreira AJ, Bader M, Alenina N, and Almeida AP. Evidence for a functional interaction of the angiotensin-(1-7) receptor Mas with AT1 and AT2 receptors in the mouse heart. *Hypertension* 46: 937–942, 2005.
- Chen J, Xiao X, Chen S, Zhang C, Chen J, Yi D, Shenoy V, Raizada MK, Zhao B, and Chen Y. Angiotensin-converting enzyme 2 priming enhances the function of endothelial progenitor cells and their therapeutic efficacy. *Hypertension* 61: 681–689, 2013.
- Cosentino F and Luscher TF. Endothelial dysfunction in diabetes mellitus. *J Cardiovasc Pharmacol* 32 Suppl 3: S54–S61, 1998.
- Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, Scholey J, Ferrario CM, Manoukian AS, Chappell MC, Backx PH, Yagil Y, and Penninger JM. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 417: 822–828, 2002.
- Dong B, Yu QT, Dai HY, Gao YY, Zhou ZL, Zhang L, Jiang H, Gao F, Li SY, Zhang YH, Bian HJ, Liu CX, Wang N, Xu H, Pan CM, Song HD, Zhang C, and Zhang Y. Angiotensin-converting enzyme-2 overexpression improves left ventricular remodeling and function in a rat model of diabetic cardiomyopathy. *J Am Coll Cardiol* 59: 739–747, 2012.
- Donoghue M, Wakimoto H, Maguire CT, Acton S, Hales P, Stagliano N, Fairchild-Huntress V, Xu J, Lorenz JN, Kadambi V, Berul CI, and Breitbart RE. Heart block, ventricular tachycardia, and sudden death in ACE2 transgenic mice with downregulated connexins. *J Mol Cell Cardiol* 35: 1043–1053, 2003.
- Feng Y, Yue X, Xia H, Bindom SM, Hickman PJ, Filipeanu CM, Wu G, and Lazartigues E. Angiotensin-converting enzyme 2 overexpression in the subfornical organ prevents the angiotensin II-mediated pressor and drinking responses and is associated with angiotensin II type 1 receptor downregulation. *Circ Res* 102: 729–736, 2008.
- Fraga-Silva RA, Costa-Fraga FP, Murca TM, Moraes PL, Martins Lima A, Lautner RQ, Castro CH, Soares CM, Borges CL, Nadu AP, Oliveira ML, Shenoy V, Katovich MJ, Santos RA, Raizada MK, and Ferreira AJ. Angiotensin-converting enzyme 2 activation improves endothelial function. *Hypertension* 61: 1233–1238, 2013.
- Gava E, Samad-Zadeh A, Zimpelmann J, Bahramifard N, Kitten GT, Santos RA, Touyz RM, and Burns KD. Angiotensin-(1-7) activates a tyrosine phosphatase and inhibits glucose-induced signalling in proximal tubular cells. *Nephrol Dial Transplant* 24: 1766–1773, 2009.
- Gjymishka A, Kulemina LV, Shenoy V, Katovich MJ, Ostrov DA, and Raizada MK. Diminazene Aceturate Is an ACE2 Activator and a Novel Antihypertensive Drug. *Faseb J* 24: 1032–1033, 2010.
- Gwathmey TM, Pendergrass KD, Reid SD, Rose JC, Diz DI, and Chappell MC. Angiotensin-(1-7)-angiotensin-converting enzyme 2 attenuates reactive oxygen species formation to angiotensin II within the cell nucleus. *Hypertension* 55: 166–171, 2010.
- Heitsch H, Brovkovich S, Malinski T, and Wiemer G. Angiotensin-(1-7)-stimulated nitric oxide and superoxide release from endothelial cells. *Hypertension* 37: 72–76, 2001.
- Higashi Y, Noma K, Yoshizumi M, and Kihara Y. Endothelial function and oxidative stress in cardiovascular diseases. *Circ J* 73: 411–418, 2009.
- Iusuf D, Henning RH, van Gilst WH, and Roks AJ. Angiotensin-(1-7): pharmacological properties and pharmacotherapeutic perspectives. *Eur J Pharmacol* 585: 303–312, 2008.
- Jarajapu YP, Bhatwadekar AD, Caballero S, Hazra S, Shenoy V, Medina R, Kent D, Stitt AW, Thut C, Finney EM, Raizada MK, and Grant MB. Activation of the ACE2/angiotensin-(1-7)/Mas receptor axis enhances the reparative function of dysfunctional diabetic endothelial progenitors. *Diabetes* 62: 1258–1269, 2013.
- Jin HY, Song B, Oudit GY, Davidge ST, Yu HM, Jiang YY, Gao PJ, Zhu DL, Ning G, Kassiri Z, Penninger JM, and Zhong JC. ACE2 deficiency enhances angiotensin II-mediated aortic profilin-1 expression, inflammation and peroxynitrite production. *PLoS One* 7: e38502, 2012.

20. Kulemina LV and Ostrov DA. Prediction of off-target effects on angiotensin-converting enzyme 2. *J Biomol Screen* 16: 878–885, 2011.
21. Lau YS, Tian XY, Mustafa MR, Murugan D, Liu J, Zhang Y, Lau CW, and Huang Y. Boldine improves endothelial function in diabetic db/db mice through inhibition of angiotensin II-mediated BMP4-oxidative stress cascade. *Br J Pharmacol* 170: 1190–1198, 2013.
22. Lely AT, Hamming I, van Goor H, and Navis GJ. Renal ACE2 expression in human kidney disease. *J Pathol* 204: 587–593, 2004.
23. Li G, Xu YL, Ling F, Liu AJ, Wang D, Wang Q, and Liu YL. Angiotensin-converting enzyme 2 activation protects against pulmonary arterial hypertension through improving early endothelial function and mediating cytokines levels. *Chin Med J (Engl)* 125: 1381–1388, 2012.
24. Li Y, Wu J, He Q, Shou Z, Zhang P, Pen W, Zhu Y, and Chen J. Angiotensin (1-7) prevent heart dysfunction and left ventricular remodeling caused by renal dysfunction in 5/6 nephrectomy mice. *Hypertens Res* 32: 369–374, 2009.
25. Liu C, Lv XH, Li HX, Cao X, Zhang F, Wang L, Yu M, and Yang JK. Angiotensin-(1-7) suppresses oxidative stress and improves glucose uptake via Mas receptor in adipocytes. *Acta Diabetol* 49: 291–299, 2012.
26. Liu CX, Hu Q, Wang Y, Zhang W, Ma ZY, Feng JB, Wang R, Wang XP, Dong B, Gao F, Zhang MX, and Zhang Y. Angiotensin-converting enzyme (ACE) 2 overexpression ameliorates glomerular injury in a rat model of diabetic nephropathy: a comparison with ACE inhibition. *Mol Med* 17: 59–69, 2011.
27. Liu L, Liu J, Tian XY, Wong WT, Lau CW, Xu A, Xu G, Ng CF, Yao X, Gao Y, and Huang Y. Uncoupling protein-2 mediates DPP-4 inhibitor-induced restoration of endothelial function in hypertension through reducing oxidative stress. *Antioxid Redox Signal* 21: 1571–1581, 2014.
28. Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AJ, Boomsma F, and van Gilst WH. Angiotensin-(1-7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation* 105: 1548–1550, 2002.
29. Lovren F, Pan Y, Quan A, Teoh H, Wang G, Shukla PC, Levitt KS, Oudit GY, Al-Omran M, Stewart DJ, Slutsky AS, Peterson MD, Backx PH, Penninger JM, and Verma S. Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis. *Am J Physiol Heart Circ Physiol* 295: H1377–H1384, 2008.
30. Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, and He TC. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat Protoc* 2: 1236–1247, 2007.
31. Masson R, Nicklin SA, Craig MA, McBride M, Gilday K, Gregorevic P, Allen JM, Chamberlain JS, Smith G, Graham D, Dominiczak AF, Napoli C, and Baker AH. Onset of experimental severe cardiac fibrosis is mediated by overexpression of Angiotensin-converting enzyme 2. *Hypertension* 53: 694–700, 2009.
32. Mercure C, Yogi A, Callera GE, Aranha AB, Bader M, Ferreira AJ, Santos RA, Walther T, Touyz RM, and Redelhuber TL. Angiotensin(1-7) blunts hypertensive cardiac remodeling by a direct effect on the heart. *Circ Res* 103: 1319–1326, 2008.
33. Moon JY, Tanimoto M, Gohda T, Hagiwara S, Yamazaki T, Ohara I, Murakoshi M, Aoki T, Ishikawa Y, Lee SH, Jeong KH, Lee TW, Ihm CG, Lim SJ, and Tomino Y. Attenuating effect of angiotensin-(1-7) on angiotensin II-mediated NAD(P)H oxidase activation in type 2 diabetic nephropathy of KK-A(y)/Ta mice. *Am J Physiol Renal Physiol* 300: F1271–F1282, 2011.
34. Muthalif MM, Benter IF, Uddin MR, Harper JL, and Malik KU. Signal transduction mechanisms involved in angiotensin-(1-7)-stimulated arachidonic acid release and prostanoic acid synthesis in rabbit aortic smooth muscle cells. *J Pharmacol Exp Ther* 284: 388–398, 1998.
35. Niu MJ, Yang JK, Lin SS, Ji XJ, and Guo LM. Loss of angiotensin-converting enzyme 2 leads to impaired glucose homeostasis in mice. *Endocrine* 34: 56–61, 2008.
36. Ohshima K, Mogi M, Nakaoka H, Iwanami J, Min LJ, Kanno H, Tsukuda K, Chisaka T, Bai HY, Wang XL, Ogimoto A, Higaki J, and Horiuchi M. Possible role of angiotensin-converting enzyme 2 and activation of angiotensin II type 2 receptor by angiotensin-(1-7) in improvement of vascular remodeling by angiotensin II type 1 receptor blockade. *Hypertension* 63: e53–e59, 2014.
37. Oudit GY, Liu GC, Zhong J, Basu R, Chow FL, Zhou J, Loibner H, Janzek E, Schuster M, Penninger JM, Herzenberg AM, Kassiri Z, and Scholey JW. Human recombinant ACE2 reduces the progression of diabetic nephropathy. *Diabetes* 59: 529–538, 2010.
38. Patel VB, Bodiga S, Basu R, Das SK, Wang W, Wang Z, Lo J, Grant MB, Zhong J, Kassiri Z, and Oudit GY. Loss of angiotensin-converting enzyme-2 exacerbates diabetic cardiovascular complications and leads to systolic and vascular dysfunction: a critical role of the angiotensin II/AT1 receptor axis. *Circ Res* 110: 1322–1335, 2012.
39. Pena Silva RA, Chu Y, Miller JD, Mitchell IJ, Penninger JM, Faraci FM, and Heistad DD. Impact of ACE2 deficiency and oxidative stress on cerebrovascular function with aging. *Stroke* 43: 3358–3363, 2012.
40. Qi Y, Zhang J, Cole-Jeffrey CT, Shenoy V, Espejo A, Hanna M, Song C, Pepine CJ, Katovich MJ, and Raizada MK. Diminazene aceturate enhances angiotensin-converting enzyme 2 activity and attenuates ischemia-induced cardiac pathophysiology. *Hypertension* 62: 746–752, 2013.
41. Raffai G, Durand MJ, and Lombard JH. Acute and chronic angiotensin-(1-7) restores vasodilation and reduces oxidative stress in mesenteric arteries of salt-fed rats. *Am J Physiol Heart Circ Physiol* 301: H1341–H1352, 2011.
42. Ren Y, Garvin JL, and Carretero OA. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension* 39: 799–802, 2002.
43. Rentzsch B, Todiras M, Iliescu R, Popova E, Campos LA, Oliveira ML, Baltatu OC, Santos RA, and Bader M. Transgenic angiotensin-converting enzyme 2 overexpression in vessels of SHRSP rats reduces blood pressure and improves endothelial function. *Hypertension* 52: 967–973, 2008.
44. Sampaio WO, Henrique de Castro C, Santos RA, Schiffrin EL, and Touyz RM. Angiotensin-(1-7) counterregulates angiotensin II signaling in human endothelial cells. *Hypertension* 50: 1093–1098, 2007.
45. Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, and Touyz RM. Angiotensin-(1-7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* 49: 185–192, 2007.
46. Shenoy V, Gjymishka A, Jarajapu YP, Qi Y, Afzal A, Rigatto K, Ferreira AJ, Fraga-Silva RA, Kearns P, Douglas JY, Agarwal D, Mubarak KK, Bradford C, Kennedy WR,

- Jun JY, Rathinasabapathy A, Bruce E, Gupta D, Cardounel AJ, Mocco J, Patel JM, Francis J, Grant MB, Katovich MJ, and Raizada MK. Diminazene attenuates pulmonary hypertension and improves angiogenic progenitor cell functions in experimental models. *Am J Respir Crit Care Med* 187: 648–657, 2013.
47. Shiota A, Yamamoto K, Ohishi M, Tatara Y, Ohnishi M, Maekawa Y, Iwamoto Y, Takeda M, and Rakugi H. Loss of ACE2 accelerates time-dependent glomerular and tubulointerstitial damage in streptozotocin-induced diabetic mice. *Hypertens Res* 33: 298–307, 2010.
 48. Soares de Moura R, Resende AC, Emiliano AF, Tano T, Mendes-Ribeiro AC, Correia ML, and de Carvalho LC. The role of bradykinin, AT2 and angiotensin 1-7 receptors in the EDRF-dependent vasodilator effect of angiotensin II on the isolated mesenteric vascular bed of the rat. *Br J Pharmacol* 141: 860–866, 2004.
 49. Soler MJ, Wysocki J, Ye M, Lloveras J, Kanwar Y, and Batlle D. ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. *Kidney Int* 72: 614–623, 2007.
 50. Stegbauer J, Oberhauser V, Vonend O, and Rump LC. Angiotensin-(1-7) modulates vascular resistance and sympathetic neurotransmission in kidneys of spontaneously hypertensive rats. *Cardiovasc Res* 61: 352–359, 2004.
 51. Stegbauer J, Potthoff SA, Quack I, Mergia E, Clasen T, Friedrich S, Vonend O, Woznowski M, Konigshausen E, Sellin L, and Rump LC. Chronic treatment with angiotensin-(1-7) improves renal endothelial dysfunction in apolipoproteinE-deficient mice. *Br J Pharmacol* 163: 974–983, 2011.
 52. Su Z, Zimpelmann J, and Burns KD. Angiotensin-(1-7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. *Kidney Int* 69: 2212–2218, 2006.
 53. Tallant EA and Clark MA. Molecular mechanisms of inhibition of vascular growth by angiotensin-(1-7). *Hypertension* 42: 574–579, 2003.
 54. Thatcher SE, Zhang X, Howatt DA, Lu H, Gurley SB, Daugherty A, and Cassis LA. Angiotensin-converting enzyme 2 deficiency in whole body or bone marrow-derived cells increases atherosclerosis in low-density lipoprotein receptor-/- mice. *Arterioscler Thromb Vasc Biol* 31: 758–765, 2011.
 55. Thomas MC, Pickering RJ, Tsorotes D, Koitka A, Sheehy K, Bernardi S, Toffoli B, Nguyen-Huu TP, Head GA, Fu Y, Chin-Dusting J, Cooper ME, and Tikellis C. Genetic Ace2 deficiency accentuates vascular inflammation and atherosclerosis in the ApoE knockout mouse. *Circ Res* 107: 888–897, 2010.
 56. Tian XY, Wong WT, Wang N, Lu Y, Cheang WS, Liu J, Liu L, Liu Y, Lee SS, Chen ZY, Cooke JP, Yao X, and Huang Y. PPARdelta activation protects endothelial function in diabetic mice. *Diabetes* 61: 3285–3293, 2012.
 57. Tian XY, Wong WT, Xu A, Lu Y, Zhang Y, Wang L, Cheang WS, Wang Y, Yao X, and Huang Y. Uncoupling protein-2 protects endothelial function in diet-induced obese mice. *Circ Res* 110: 1211–1216, 2012.
 58. Wysocki J, Ye M, Soler MJ, Gurley SB, Xiao HD, Bernstein KE, Coffman TM, Chen S, and Batlle D. ACE and ACE2 activity in diabetic mice. *Diabetes* 55: 2132–2139, 2006.
 59. Zhang C, Zhao YX, Zhang YH, Zhu L, Deng BP, Zhou ZL, Li SY, Lu XT, Song LL, Lei XM, Tang WB, Wang N, Pan CM, Song HD, Liu CX, Dong B, Zhang Y, and Cao Y. Angiotensin-converting enzyme 2 attenuates atherosclerotic lesions by targeting vascular cells. *Proc Natl Acad Sci U S A* 107: 15886–15891, 2010.
 60. Zhang Y, Liu J, Tian XY, Wong WT, Chen Y, Wang L, Luo J, Cheang WS, Lau CW, Kwan KM, Wang N, Yao X, and Huang Y. Inhibition of bone morphogenic protein 4 restores endothelial function in db/db diabetic mice. *Arterioscler Thromb Vasc Biol* 34: 152–159, 2014.
 61. Zhong J, Basu R, Guo D, Chow FL, Byrns S, Schuster M, Loibner H, Wang XH, Penninger JM, Kassiri Z, and Oudit GY. Angiotensin-converting enzyme 2 suppresses pathological hypertrophy, myocardial fibrosis, and cardiac dysfunction. *Circulation* 122: 717–728, 18 p following 728, 2010.

Address correspondence to:

Dr. Yu Huang
School of Biomedical Sciences
and Shenzhen Research Institute
Lo Kwee-Seong Integrated Biomedical Sciences Building
Chinese University of Hong Kong
Hong Kong
China

E-mail: yu-huang@cuhk.edu.hk

Date of first submission to ARS Central, July 25, 2014; date of final revised submission, March 23, 2015; date of acceptance, April 9, 2015.

Abbreviations Used

ACE2 = angiotensin-converting enzyme 2
ACh = acetylcholine
Ad-ACE2 = adenovirus ACE2
Ang (1-7) = angiotensin (1-7)
Ang II = angiotensin II
DHE = dihydroethidium
DIZE = diminazene aceturate
DM = diabetes mellitus
EDR = endothelium-dependent relaxation
eNOS = endothelial nitric oxide synthases
FMD = flow-mediated dilatation
FRET = fluorescence resonance energy transfer
HAECs = human aortic endothelial cells
HG = high glucose
HUVECs = human umbilical vein endothelial cells
KO = knockout
MAECs = mouse aortic endothelial cells
NADPH = nicotinamide adenine dinucleotide phosphate
NO = nitric oxide
Phe = phenylephrine
RAS = renin-angiotensin system
RFU = relative fluorescence unit
RLU = relative chemiluminescence unit
ROS = reactive oxygen species
SNP = sodium nitroprusside
UCP2 = uncoupling protein-2
WT = wild-type