TRANSLATIONAL PHYSIOLOGY

Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis

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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, Verma S. Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis. *Am J Physiol Heart Circ Physiol* 295: H1377–H1384, 2008. First published July 25, 2008; doi:10.1152/ajpheart.00331.2008.—The endothelium plays a central role in the maintenance of vascular homeostasis. One of the main effectors of endothelial dysfunction is ANG II, and pharmacological approaches to limit ANG II bioactivity remain the cornerstone of cardiovascular therapeutics. Angiotensin converting enzyme-2 (ACE2) has been identified as a critical negative modulator of ANG II bioactivity, counterbalancing the effects of ACE in determining net tissue ANG II levels; however, the role of ACE2 in the vasculature remains unknown. In the present study, we hypothesized that ACE2 is a novel target to limit endothelial dysfunction and atherosclerosis. To this aim, we performed in vitro gain and loss of function experiments in endothelial cells and evaluated in vivo angiogenesis and atherosclerosis in apolipoprotein E-knockout mice treated with AdACE2. ACE2-deficient mice exhibited impaired endothelium-dependent relaxation. Overexpression of ACE2 in human endothelial cells stimulated endothelial cell migration and tube formation, and limited monocyte and cellular adhesion molecule expression; effects that were reversed in ACE2 gene silenced and endothelial cells isolated from ACE2-deficient animals. ACE2 attenuated ANG II-induced reactive oxygen species production in part through decreasing the expression of p22phox. The effects of ACE2 on endothelial activation were attenuated by pharmacological blockade of ANG-(1-7) with A779. ACE2 promoted capillary formation and neovessel maturation in vivo and reduced atherosclerosis in apolipoprotein E-knockout mice These data indicate that ACE2, in an ANG-(1-7)-dependent fashion, functions to improve endothelial homeostasis via a mechanism that may involve attenuation of NADPHox-induced reactive oxygen species production. ACE2-based treatment approaches may be a novel approach to limit aberrant vascular responses and atherothrombosis.

endothelium; atherogenesis; angiogenesis

THE ENDOTHELIUM FUNCTIONS as a protective biocompatible barrier between all tissues and the circulating blood (17). Endothelial cells also function as a selective sieve to facilitate bidirectional passage of macromolecules and blood gases to and from tissues and blood (17). The strategic location of the endothelium allows it to "sense" changes in hemodynamic forces and blood-borne signals and "respond" by releasing a number of autocrine and paracrine substances. A balanced release of these bioactive factors facilitates vascular homeostasis. Endothelial cell dysfunction disrupts this balance, thereby predisposing the vessel wall to vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, prooxidation, thrombosis, impaired coagulation, vascular inflammation, and atherosclerosis. Improving endothelial function is a major goal of cardiovascular risk reduction.

The renin-angiotensin system (RAS) is a pivotal transducer of cardiovascular function and plays a causal role in the development and progression of endothelial dysfunction and atherosclerosis (7, 15, 17). RAS supports a series of complex enzymatic reactions that culminate in the generation of ANG II, the main proinflammatory effector of adverse cardiovascular phenotypes. Recently, a homologue of angiotensin converting enzyme (ACE), namely ACE2 has been identified, which serves as a negative regulator of the RAS (5, 6, 11). Unlike ACE, which processes ANG I to ANG II, ACE2 functions to promote the degradation of ANG II into the anti-inflammatory, vasodilator, and antitrophic heptapeptide, ANG-(1-7) (5, 6, 11). ACE2 plays a critical role in regulating ANG II production by ACE, either by stimulating an alternative pathway for ANG I degradation or by facilitating the degradation of ANG II into ANG-(1-7). Through counterbalancing the function of ACE, ACE2 may determine net ANG II bioavailability in tissues. We and others (3, 12, 18) have recently demonstrated that ACE2 expression was upregulated in failing hearts and that ACE2-deficient mice exhibited progressive impairment of heart contractility and renal disease. Importantly, this phenotype could be rescued by loss of ACE as evidenced in ACE/ACE2 double knockout (KO) mice, implicating a fundamental role of ACE2 in regulating ANG II bioactivity.

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ACE2 is highly expressed in vascular endothelial cells (as opposed to vascular smooth muscle cells or cardiomyocytes; Refs. 3, 4, 6, 11), where it may potentially regulate endothelial cell responses to inflammation by limiting the effects of ACE-mediated ANG II production. Improving endothelial function may be a central mechanism through which ACE2 exerts cardiovascular and renal protection; however, the role of ACE2 in endothelial homeostasis remains unknown. Using gain and loss of function strategies respectively, we systematically evaluated the role of ACE2 in endothelial regulation. Furthermore, since atherosclerosis represents a clinically relevant endpoint of progressive endothelial dysfunction we examined the potential of ACE2 to limit experimental atherosclerosis.

MATERIALS AND METHODS

Materials and reagents. Unless otherwise stated, materials were obtained from Sigma-Aldrich. A recombinant ACE2 adenovirus (AdACE2) encoding full-length human ACE2 and coexpressing the GFP protein and a control recombinant GFP adenovirus (AdGFP) were generated by Vector Biolabs.

Mice. Animal procedures were in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Institutional Animal Care Committee. Male ACE2-KO mice have been described previously (3), and male wild-type (WT) littermates acted as controls (10–12 wk old). Baseline measurements of systolic blood pressure were similar between male ACE2-KO mice (131 ± 4 mmHg) and male WT littermate controls (136 ± 4 mmHg).

Cells. Endothelial cells were isolated from the lungs of 6-wk-old ACE2-KO mice and WT littermates as described previously (8). Human umbilical vein endothelial cells (HUVEC; Clonetics) and THP-1 cells (ATCC) were grown respectively in MCDB-131 complete medium and in RPMI 1640 (ATCC), both supplemented with 10% FBS.

Functional studies. Mice were killed by cervical dislocation, and 2-mm segments of thoracic aortas were mounted in a wire myograph for measurement of isometric tension. Vessel segments were equilibrated in aerated Krebs-Henseleit solution for 1 h at 37°C under a resting tension of 1,000 mg. Aortic segments were preconstricted with 1 µM phenylephrine and relaxation-response curves to acetycholine or SNP were generated. Vasorelaxation was calculated as a percentage of the phenylephrine-induced maximal response.

ACE2 mRNA overexpression and silencing. For overexpression experiments, HUVEC culture medium was replaced with that containing AdACE2- or AdGFP (100 multiplicity of infection) for 6 h. Silencing experiments were performed in the presence of 25 nM small-interfering RNA (siRNA) ACE2 (Ambion) in OptiMEM (Invitrogen) using siSPORT NeoFX (Ambion). Cell lysates were collected after 48 h.

Studies with ANG-(1-7) inhibition. AdACE2- and AdGFP-transfected HUVEC were incubated for 30 min with vehicle or the ANG-(1-7) inhibitor A779 (10 µM, Bachem) before being exposed to ANG II (0.1 µM) for 18 h.

RNA analyses. Real-time PCR was performed with the following primers: 5′-CATTGGAGCAAGTGTTGGATCTT-3′ and 5′-GAGCTA-ATGCATGCCATTCTCA-3′ for ACE2, 5′-GAGCGGCATCTAC-CTACTGG-3′ and 5′-TGATGGTGCCTCCGATCT-3′ for p22phox, 5′-CACCAGGGCTGCTTTTAACTCTGGTA-3′ and 5′-CCTTGACGGT-GCCATGGAATTGC-3′ for GAPDH. Monocyte chemoattractant protein 1 (MCP-1) primers were obtained from SuperArray. mRNA expression was analyzed using the method of Pfaffl (13). The relative abundance of RNA expression in untreated control cells was expressed as 100% for comparative purposes.

Protein analyses. HUVEC protein lysates (30 µg) were resolved on 10% SDS-PAGE and electrotransferred onto nitrocellulose. Blots were blocked in 5% milk-TBS-Tween before incubation with mouse

anti-ACE2 (R&D Systems), rabbit anti-p22phox (Santa Cruz Biotechnology), or mouse anti-actin (Chemicon International) antibodies. Immunoblots were probed with secondary antibodies and visualized by enhanced chemiluminescence (Amersham). Band intensity was quantified by densitometry.

ACE2 activity. ACE2 enzymatic activity in the culture medium was assayed using 7-methoxycoumarin-Tyr-Val-Ala-Asp-Ala-Pro-Lys-(2,4-dinitrophenyl)-OH (R&D Systems).

Tube formation assay. Angiogenic activities were monitored in AdACE2-transfected HUVEC, siRNA ACE2-transfected HUVEC and murine lung endothelial cells. Where required, endothelial cells were treated with 1 µM losartan for AT_1 receptor blockade. Cells (5×10^5) were cultured in Matrigel basement membrane matrix (BD Bioscience) coated 12-well plates. Cumulative tube length was quantified after 18 h with a NIH Image analysis software (ImageJ) and evaluated in 5 random fields.

Migration assay. HUVEC treated with AdACE2 or siRNA ACE2 were placed in the upper chamber of a modified Boyden chamber (5×10^4 cells per chamber; pore size = 8 µm; BD Bioscience). Endothelial cell migration in response to 24 h of VEGF (50 ng/ml) exposure was assessed in three random fields after staining with a Diff-Quik Stain kit (Dade Behring).

Proliferation. HUVEC were seeded at a density of 1×10^4 cells/well in 96-well plates, treated with Ad-ACE2 or ACE2 siRNA and incubated for 24 h with bromodeoxyuridine (BrdU) that was added to a final concentration of 10 µM. BrdU incorporation into newly synthesized cellular DNA acting as a surrogate for cell proliferation was determined with an enzyme-linked immunosorbent assay (Roche Diagnostics).

Static adhesion. AdACE2-transfected HUVEC and murine lung endothelial cells were plated at a density of 2×10^4 cells/well, stimulated for 6 h with TNF-α (10 ng/ml) and overlaid with PKH26-labeled THP-1 monocytes (5×10^5) for 30 min. Non-adherent THP-1 monocytes were washed off and the percentage of adherent monocytes correlated to the fluorescence intensity measured with a 544 nm/590 nm filter set.

Reactive oxygen species formation. After various treatments, HUVEC were incubated for 45 min with 10 µM 5(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA, Molecular Probes). Intra-cellular reactive oxygen species
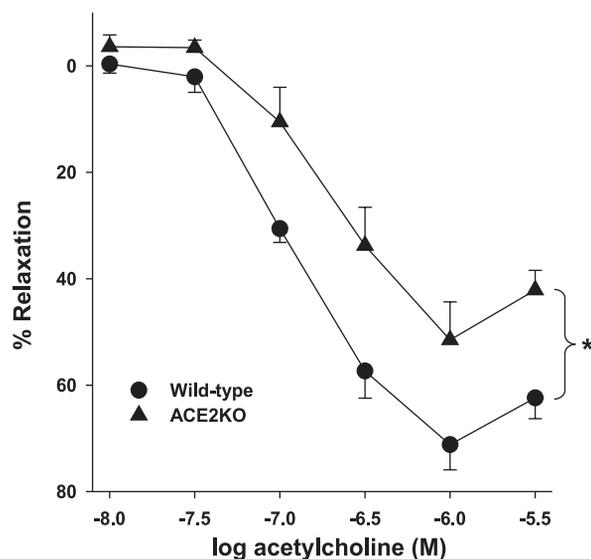

Fig. 1. Angiotensin converting enzyme-2 (ACE2) is critical in maintaining endothelial function. Phenylephrine-preconstricted thoracic aortic segments from ACE2-knockout (KO) mice demonstrate impaired endothelium-dependent acetylcholine-elicited responses compared with those from littermate wild-type (WT) controls. Data are means ± SE; $n = 5$; *$P < 0.05$.

(ROS) generation was assessed by measuring fluorescence at excitation and emission wavelengths of 485 nm and 535 nm respectively.

Vascular cell adhesion molecule-1 secretion. Levels of soluble Vascular cell adhesion molecule-1 (VCAM-1) protein in HUVEC culture media were measured with an ELISA kit (Biosource).

In vivo matrigel plug assay. C57Bl/6 mice were injected with Matrigel HC (0.5 ml; BD Bioscience) complexed with PBS, VEGF (200 ng), AdGFP [2.5×10^8 plaque-forming unit (PFU)] or AdACE2 (2.6×10^8 PFU). Blood vessel infiltration was quantified after 7 days by analysis of Cy3-labeled smooth muscle actin-staining.

Atherosclerotic lesions. Apolipoprotein (apo) E-KO mice (12 wk old) were placed on the Western diet for 4 wk and injected via the tail vein with AdACE2 or AdGFP ($1–1.1 \times 10^9$ PFU). Paraformaldehyde-fixed hearts and proximal aortas were excised for staining of aortic sinus sections (10 μm) with oil red O.

Immunohistochemistry. Macrophage infiltration was determined in the ascending aortas of adenovirus-treated apoE-KO mice by probing cryosections with a rat anti-mouse macrophage antibody (clone MOMA-2, Chemicon). Three sections from each mouse were examined.

Statistical analysis. Results are means $\pm$ SE for number of animals per samples with $P < 0.05$ representing significance. Where appropriate, means were compared by unpaired two-tailed Student's *t*-test. Differences among multiple means were evaluated by ANOVA.

RESULTS

ACE2-deficient mice exhibit impaired endothelium-dependent relaxation. We examined endothelial function in isolated thoracic aortic segments from ACE2-KO mice and littermate controls (WT). In endothelium-intact vessel segments from WT mice precontracted with phenylephrine (1 μM), acetylcholine induced concentration-dependent relaxation. Acetylcholine-mediated vasorelaxations were attenuated in aortic rings from ACE2-KO mice (E_{max}: 71.17 $\pm$ 4.71 vs. 51.40 $\pm$ 7.15% for segments from WT and ACE2-KO mice, respectively; $P < 0.05$; Fig. 1). Neither endothelium-independent vasorelaxation to sodium nitroprusside (E_{max}: 83.62 $\pm$ 12.73 vs. 77.88 $\pm$ 4.86% and EC_{50}: 30.50 $\pm$ 3.77 vs. 36.16 $\pm$ 4.39 nM) nor 1 μM
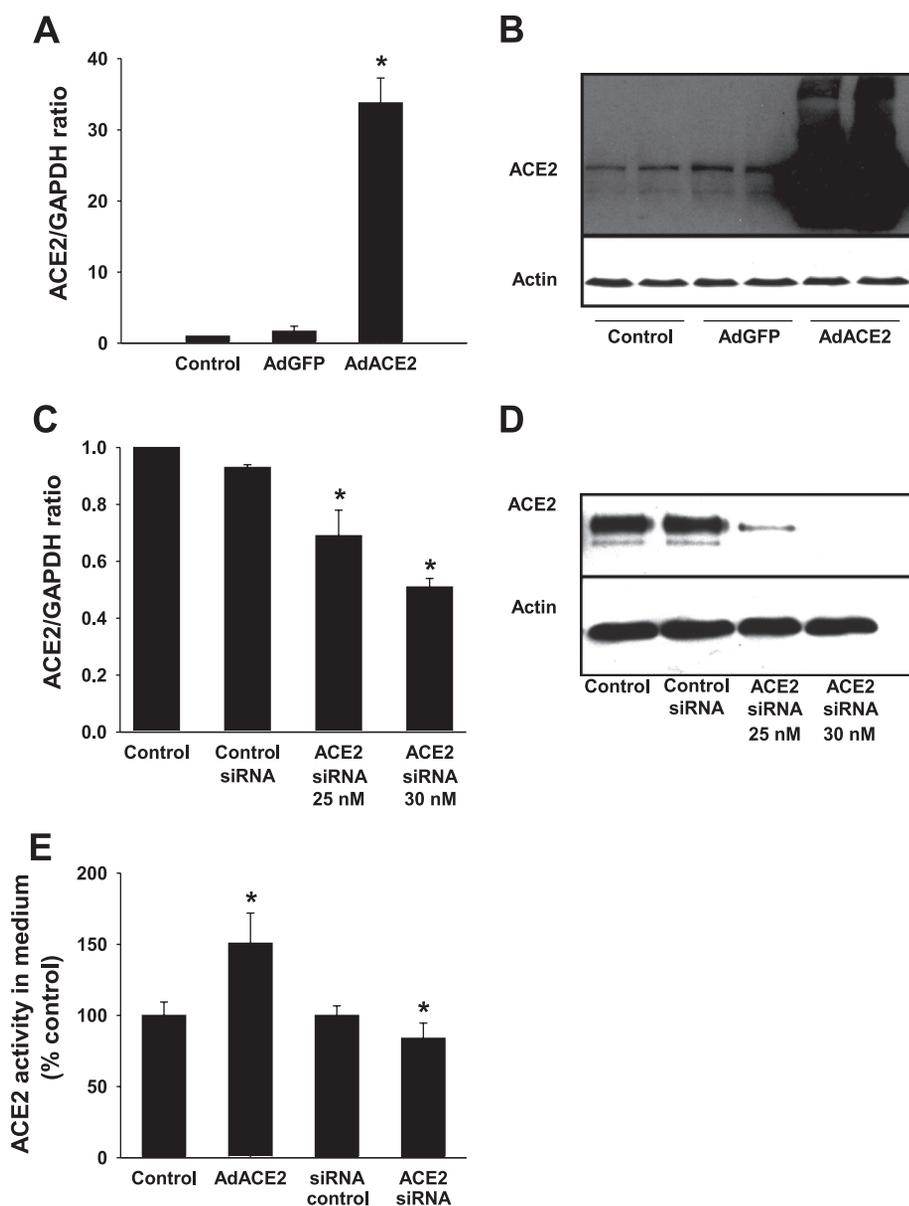

Fig. 2. ACE2 overexpression and silencing in HUVEC. *A*: ACE2 mRNA. *B*: ACE2 protein levels in HUVEC 48 h after infection with 100 multiplicity of infection AdACE2 for 6 h. Data are means $\pm$ SE; $n = 5$; *$P < 0.05$ vs. control and AdGFP groups. *C*: ACE2 mRNA levels at 24 h. *D*: ACE2 protein levels at 48 h after transfection with small-interfering RNA (siRNA) ACE2. Data are means $\pm$ SE; $n = 5$; *$P < 0.05$ vs. control and control siRNA groups. *E*: ACE2 activity in the media of HUVEC transfected with AdACE2 or siRNA. Data are means $\pm$ SE; $n = 5$; *$P < 0.05$ vs. control.

phenylephrine-induced vasoconstrictive responses (447.82 ± 59.58 vs. 397.78 ± 67.35 mg) were different between WT and ACE2-KO mice, respectively.

ACE2 modulates endothelial cell function in vitro: gain and loss of function approaches. To characterize the role of ACE2 in endothelial cell function, we used gain- and loss-of-function strategies in HUVEC and evaluated tube formation and cell migration. The efficacy of ACE2 overexpression (AdACE2) and silencing strategies (siRNA) was confirmed by real-time PCR and Western blotting (Fig. 2, *A* and *D*). ACE2 activity, as measured in the HUVEC media, increased after Ad-ACE2 treatment and decreased after ACE2 siRNA exposure. ACE2 overexpression led to an increase in tube formation activity (Fig. 3*A*) and promoted endothelial cell migration (Fig. 3*C*). Specific blockade of AT_1 receptors with losartan did not attenuate the effects of ACE2 on tube formation (Fig. 3*A*). Conversely, gene silencing of ACE2 attenuated basal tube formation activity and endothelial cell migration (Fig. 3, *B* and *C*). Proliferation in ACE2-overexpressing cells was not significantly different from control cells after 24 h incubation with BrdU (supplemental data for this article are available online at the *Am J Physiol Heart Circ Physiol* website; see Supplementarl Fig. 1), thus excluding any effect of proliferation on the evaluation of endothelial cell tube formation or migration.

Since endothelial cell dysfunction uncovers a proinflammatory milieu which promotes monocyte adhesion to endothelial cells, we evaluated the role of ACE2 on endothelial-monocyte interaction. ACE2-overexpressing HUVEC were less responsive to TNF-α-induced monocyte-endothelial adhesion whereas ACE2 silencing increased monocyte-endothelial interaction (Fig. 3*D*).

ACE2 modulates ANG II-induced ROS production in endothelial cells. The major source of ROS in endothelial cells is NADPH oxidase. ANG II stimulates membrane-bound NADPH oxidase which in turn generates ROS within endothelial cells(14). ANG II-induced ROS generation, as assessed by dihydroethidium fluorescence were attenuated by ACE2, and this effect was attenuated by inhibition of ANG-(1-7) with A779 (Fig. 4*A*). We extend these observations further by providing data that the effects of ANG II to upregulate the expression of p22phox (protein and transcript) are attenuated by ACE2. These effects are also inhibited by A779 (Fig. 4, *B* and *C*). ANG II-induced increases in MCP-1 and VCAM-1 in endothelial cells are likewise attenuated by ACE2, and these effects are also blunted during coincubation with A779 (Fig. 4, *D* and *E*). These data suggest that ACE2, in a ANG-(1-7)-dependent fashion, functions to improve endothelial homeostasis, via a mechanism that may involve attenuation of NADPHox-induced ROS production.

Endothelial cells from ACE2-deficient mice exhibit dysfunction. To further validate the role of ACE2 in endothelial function, we cultured lung endothelial cells from ACE2-KO
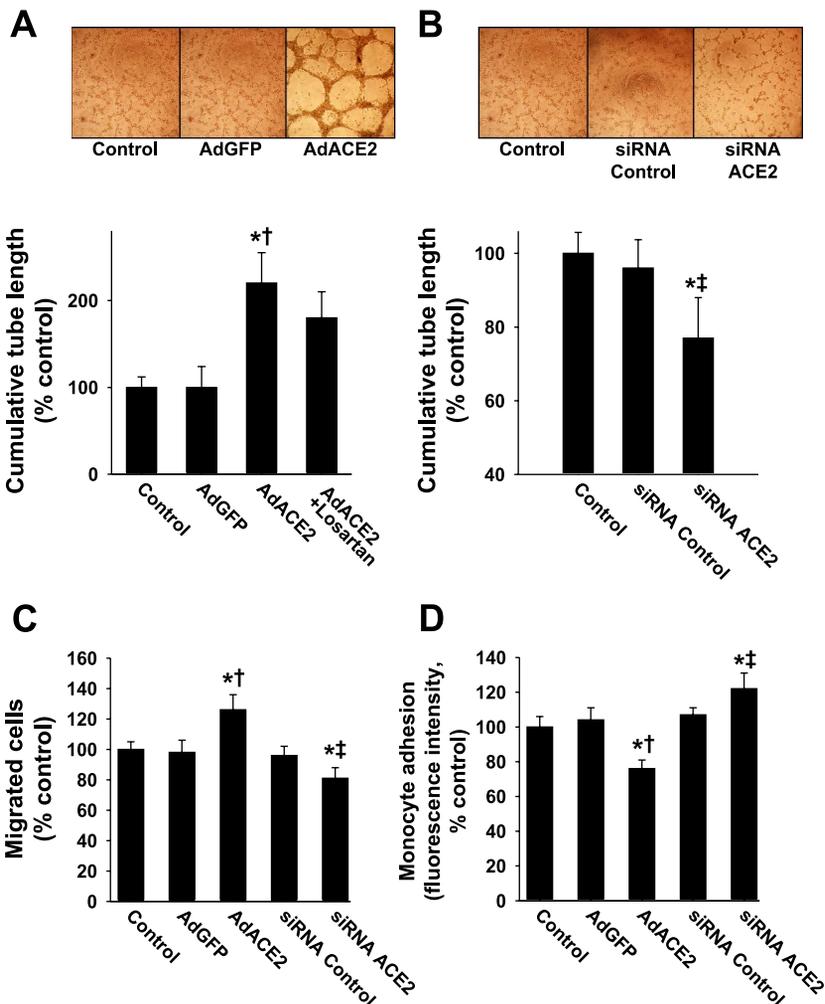
A Control AdGFP AdACE2

B Control siRNA Control siRNA ACE2

Fig. 3. ACE2 is critical for angiogenesis and endothelial cell migration. *A*: AdACE2 transfection enhances HUVEC tube formation (angiogenesis) in the presence or absence of 1μM losartan, while ACE2 silencing by transfection with siRNA ACE2 (*B*) significantly attenuated tube formation activity. Magnification of representative micrographs is ×40. Data are means ± SE; n = 4; *P < 0.05 vs. control; †P < 0.05 vs. AdGFP; ‡P < 0.05 vs. siRNA control. *C*: AdACE2 and siRNA ACE2 transfection respectively augment and diminish HUVEC migration. Data are means ± SE; n = 3–7; *P < 0.05 vs. control; †P < 0.05 vs. AdGFP; ‡P < 0.05 vs. siRNA control. *D*: AdACE2 and siRNA ACE2 transfection respectively reduce and augment TNF-α-induced THP-1 monocyte adhesion to HUVEC. Data represent means ± SE; n = 3; *P < 0.05 vs. control; †P < 0.05 vs. AdGFP; ‡P < 0.05 vs. siRNA control.
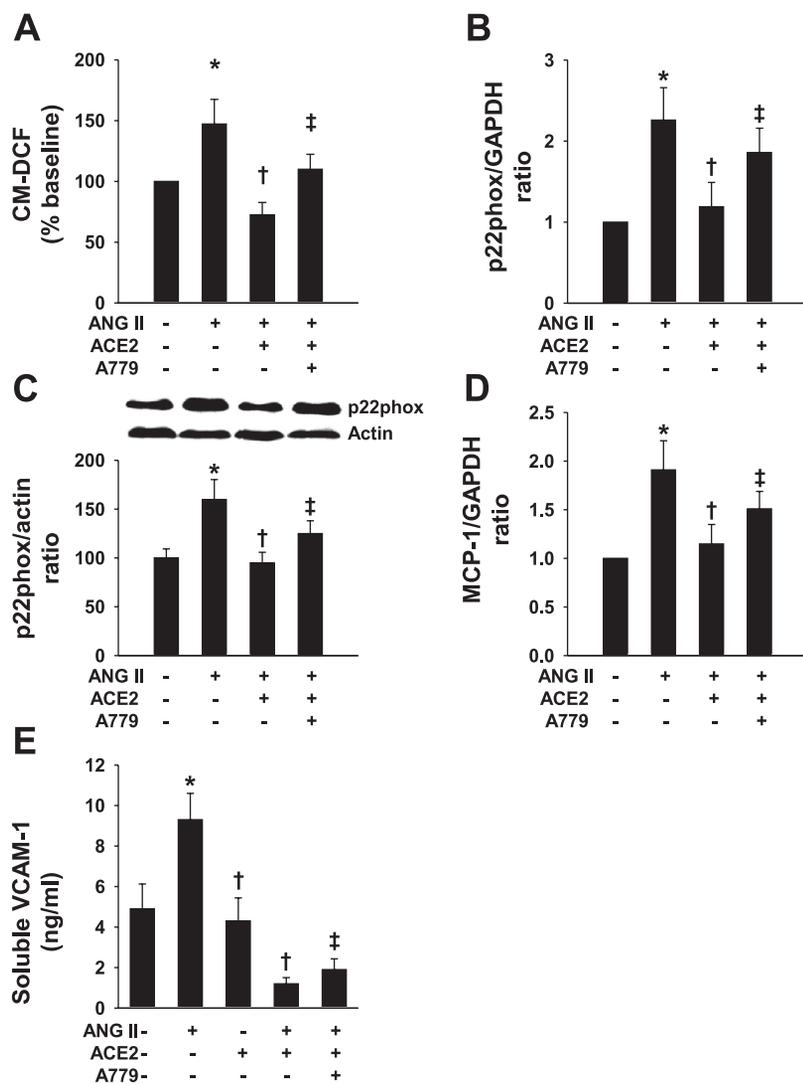
Fig. 4. ACE2 overexpression antagonizes ANG II effects in HUVEC, which is reversible by A799. *A*: ANG II-induced ROS generation is suppressed in AdACE2-transfected cells. Data are means ± SE; $n = 4$; *$P < 0.05$ vs. control; †$P < 0.05$ vs. ANG II; ‡$P < 0.05$ vs. ANG II + ACE2. *B* and *C*: ACE2 overexpression prevents ANG II-stimulated increase in p22phox RNA and protein expression. Data are means ± SE; $n = 6$; *$P < 0.05$ vs. control; †$P < 0.05$ vs. ANG II; ‡$P < 0.05$ vs. ANG II + ACE2. *D*: ANG II-mediated elevations of HUVEC MCP-1 mRNA expression is decreased by AdACE2 transfection. Data are means ± SE; $n = 4$; *$P < 0.05$ vs. control; †$P < 0.05$ vs. ANG II; ‡$P < 0.05$ vs. ANG II + ACE2. *E*: ANG II-mediated augmentation of soluble VCAM-1 protein levels in HUVEC culture media is reduced by AdACE2 transfection. Data are means ± SE; $n = 4$; *$P < 0.05$ vs. control; †$P < 0.05$ vs. ANG II; ‡$P < 0.05$ vs. ANG II + ACE2.

and WT mice. ACE2-KO endothelial cells exhibited diminished tube formation activity and augmented TNF-α-induced monocyte-endothelial cell adhesion (Fig. 5), consistent with the observations in ACE2-silenced HUVEC.

ACE2 promotes angiogenesis. Endothelial cells play a critical role in angiogenesis and via promoting neovascularization may serve to limit and/or resuscitate ischemic tissues. Compared with control, ACE2-impregnated Matrigel contained a greater number of infiltrating vessels (Fig. 6*A*), indicating that ACE2 may function in vivo to promote endothelial cell mediated neovascularization.

Anti-atherosclerotic effects of exogenous ACE2. Atherosclerosis is the endpoint of progressive endothelial cell dysfunction so we evaluated whether an ACE2-based strategy would limit atherosclerosis in apoE-KO mice. apoE-KO mice were placed on the Western diet and were injected with AdACE2 or AdGFP. Four weeks later, the aortic sinuses of mice receiving AdACE2 revealed significant less oil red-O-positive areas compared with that of mice injected with AdGFP (Fig. 6*B*). Additionally, the aortas of apoE-KO mice injected with AdACE2 demonstrated less prominent macrophage infiltration than the aortas from control aortas (Fig. 6*C*). These findings

suggest that ACE2 may function to limit aberrant endothelial dysfunction and the resultant development of atherosclerosis.

DISCUSSION

In the current study, we postulated that the endothelium is a key target of ACE2 activation, and that upon activation it limits endothelial dysfunction through counterbalancing the effects of ACE derived ANG II. Improving endothelial function may be a central mechanism of through which ACE2 exerts cardiovascular and renal protection; however, the role of ACE2 in endothelial homeostasis has not been previously examined. Our hypothesis was based on several key observations. First, ACE2 is highly expressed in coronary endothelial cells, as opposed to vascular smooth muscle cells or cardiomyocytes. Therefore, based on localization per se, it is reasonable to assume that ACE2 functions in the endothelium to regulate net tissue ANG II levels, via promoting ANG II degradation and augmenting the production of ANG-(1-7), a vasculoprotective molecule (2, 3). Second, our previous work (11, 12) in ACE2-KO mice provided indirect evidence that ACE2 may function to improve endothelial function and microvascular
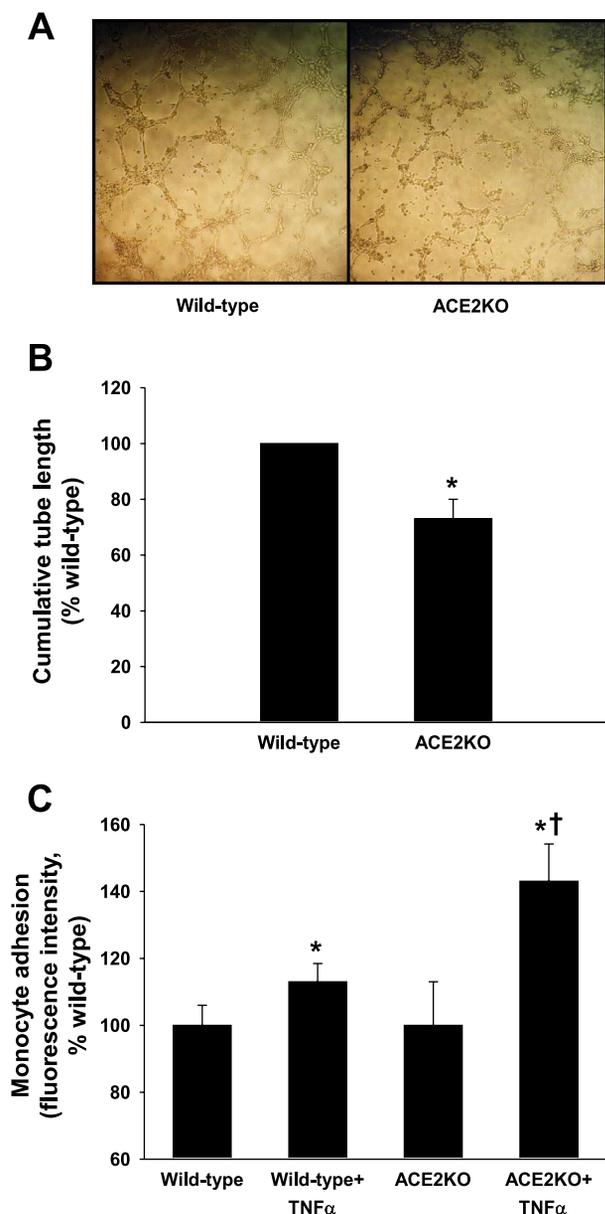
A

Wild-type   **ACE2KO**

B

C

Fig. 5. Endothelial cells from ACE2-KO mice demonstrate less angiogenesis and greater sensitivity to TNF-α than endothelial cells from wild-type littermate controls. *A*: representative micrographs of tube-forming activity in endothelial cells isolated from ACE2-KO mice (*right*) and wild-type littermates (*left*). Magnification = ×40. *B*: endothelial cells isolated from ACE2-KO mice demonstrated less angiogenic activity, as measured by cumulative tube length, than those originating from wild-type littermates. Data are means ± SE; $n = 3$; *$P < 0.05$ vs. control. *C*: compared with the endothelial cells isolated from wild-type mice, endothelial cells from ACE2-KO mice are more sensitive to TNF-α, thereby promoting THP-1 monocyte adhesion. Data are means ± SE; $n = 4$; *$P < 0.05$ vs. corresponding group of untreated cells; †$P < 0.05$ vs. TNF-α-stimulated cells from wild-type mice.

perfusion. Profound contractile dysfunction in ACE2-KO mice was associated with no hypertrophy, dilatation or fibrosis, and resembled a phenotype of cardiac stunning and/or hibernation, often ascribed to ischemia and impaired microvascular perfusion. Indeed, hypoxia inducible genes were upregulated in ACE2-KO mice, suggesting that impaired coronary perfusion, secondary to endothelial dysfunction may account for impaired contractile reserve.

Using gain- and loss-of-function strategies respectively, we have carefully delineated the role of ACE2 in endothelial homeostasis, inflammation and atherosclerosis. We demonstrate that aortas from ACE2-deficient mice exhibit impaired endothelium-dependent vasodilation. Furthermore, silencing of the ACE2 transgene in isolated human endothelial cells results in decreased tube formation and migration, a phenotype that is also observed in endothelial cells isolated from ACE2-KO mice. Overexpression of ACE2 improves endothelial cell migration and tube formation. Endothelial cell dysfunction uncovers a proinflammatory milieu which promotes monocyte adhesion to endothelial cells, and an increase in cellular adhesion molecules, which in turn sets the stage for the development of fatty streaks and early atheroma. Therefore, we evaluated the role of ACE2 on endothelial-monocyte interaction. Endothelial overexpression of ACE2 inhibited TNF-α induced monocyte adhesion, whereas silencing of ACE2 increased monocyte-endothelial adhesion. Furthermore, ANG II-induced increases in cellular adhesion molecules, VCAM-1, and MCP-1 were attenuated in ACE2 overexpressed endothelial cells. These data suggest that ACE2 plays a permissive role in limiting endothelial cell inflammation, both under inflammatory and ANG II stimulated conditions.

One of the important effectors of ANG II is the generation of ROS. We demonstrate that endothelial ANG II-induced ROS generation, is attenuated by ACE2. To confirm if this effect is mediated, in part, through ANG-(1-7), we utilized A779, an antagonist to the ANG-(1-7) receptor. The effects of ACE2 to limit ANG II-induced ROS generation were attenuated by A779 confirming that ACE2 functions largely through an ANG-(1-7)-dependent pathway. We extend these observations further by demonstrating that the effects of ANG II to upregulate the expression of p22phox (protein and transcript) are attenuated by ACE2, and that these effects are also inhibited by A779. ANG II-induced increases in MCP-1 and VCAM-1 in endothelial cells are likewise attenuated by ACE2, and these effects are also blunted during coincubation with A779. Therefore, we believe that ACE2, in a ANG-(1-7)-dependent fashion, functions to improve endothelial homeostasis, via a mechanism that may involve attenuation of NADPHox-induced ROS production.

One of the important functions of endothelial cells is promoting angiogenesis, and hence we investigated the effects of ACE2 on neovascularization capacity. ACE2 impregnated Matrigel demonstrated an elevated number of infiltrating vessels indicating that ACE2 may function *in vivo* to promote endothelial cell mediated neovascularization.

Atherosclerosis is the cardiovascular endpoint of progressive endothelial cell dysfunction, and hence we evaluated whether an ACE2-based strategy would limit atherosclerosis in apoE-KO mice. Four wk treatment of atherosclerosis prone mice with AdACE2 revealed a significant decrease in oil red-O-positive areas compared with that of mice injected with AdGFP. Additionally, in apoE-KO mice injected with AdACE2 macrophage infiltration was less prominent than in control mice aorta, consistent with the notion that that ACE2 may function to limit aberrant endothelial function, which culminate into reduced atherosclerosis.

It is important to mention that ANG-(1-7) has been demonstrated to inhibit endothelial tube formation, and endothelial proliferation (1, 10) whereas other studies have demonstrated
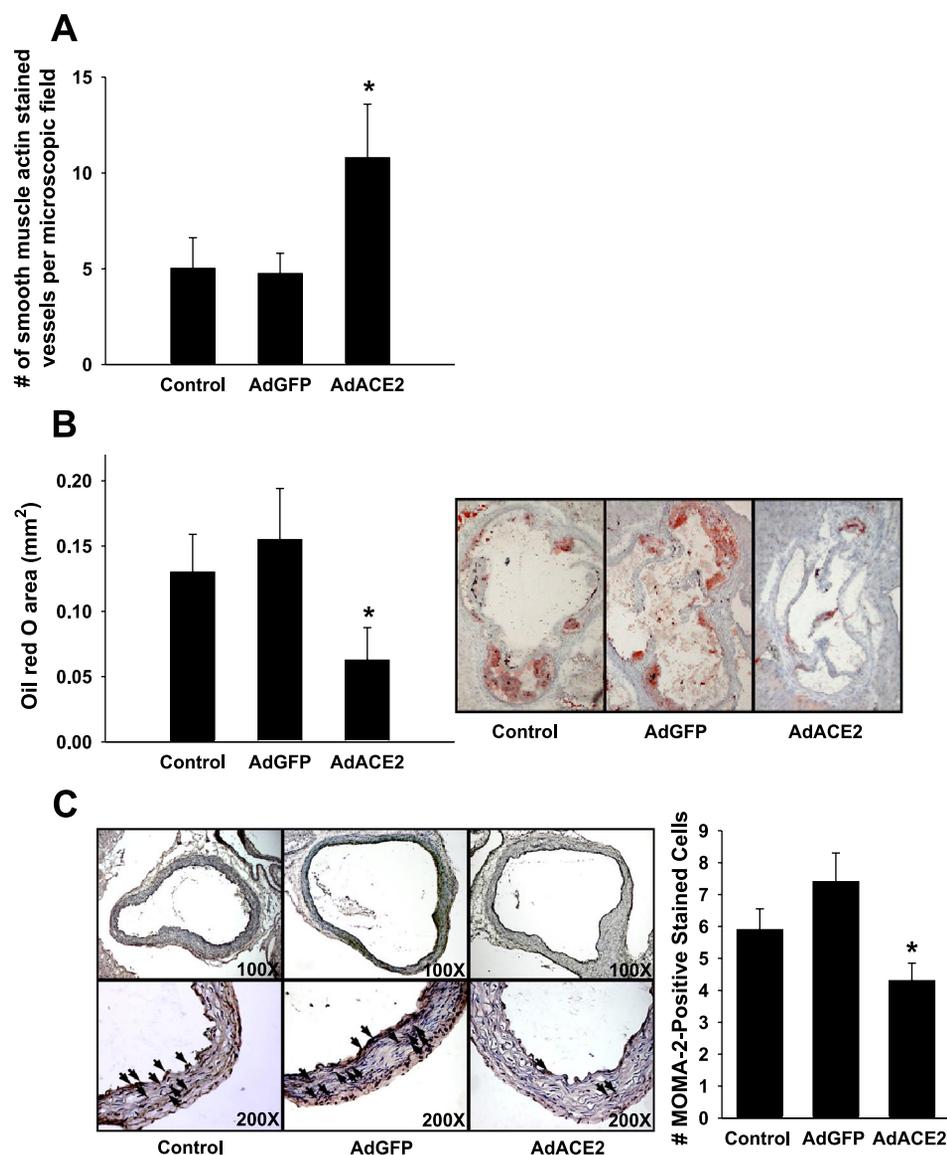
Fig. 6. ACE2 enhances neovascularization and protects against atherosclerosis. *A*: AdACE2, but not AdGFP, administration stimulated blood vessel infiltration *in vivo*. Data are means ± SE; $n = 4$; *$P < 0.05$ vs. control and AdGFP groups. *B*: AdACE2, but not AdGFP, administration reduces the generation of atherosclerotic aortic lesions in apolipoprotein E-KO mice fed the Western diet for 4 wk. Magnification of representative micrographs is ×40. Data are means ± SE; $n = 4$; *$P < 0.05$ vs. AdGFP group. *C*: thoracic aortic sections of AdACE2-treated apoE-KO mice fed the Western diet for 4 wk show less MOMA-2 (macrophage) staining compared with those harvested from apolipoprotein E-KO mice on the Western diet and administered AdGFP. Magnifications of representative micrographs are ×100 and ×200. Macrophages are indicated by arrows. Data are means ± SE; $n = 5$; *$P < 0.05$ vs. AdGFP group.

that Ang1-7 inhibits vascular smooth muscle proliferation (16) and stimulates proliferation of endothelial progenitor cells (9). Although the effects of Ang1-7 on vascular biology are conflicting, our data set suggest that ACE2 may exert endothelial protection, in part via ANG-(1-7), however, they do not exclude the possibility that other non-ANG-(1-7) pathways may be involved in ACE2 mediated vasculoprotection.

Taken together, these data identify ACE2 as a novel target of endothelial cell function, and endothelial responses to injury. The endothelium is a key target of ACE2 activation, and serves to limit inflammation and ANG II-mediated vascular dysfunction. We postulate that via improving endothelial repair and regeneration, ACE2 plays a permissive role in reducing atherosclerosis. Pharmacological and cell based strategies aimed at increasing vascular ACE2 bioavailability may represent not only a new therapeutic avenue in the treatment of atherosclerotic cardiovascular disease, but as a target for various cardiometabolic, pulmonary, septic, and renal diseases where endothelial dysfunction is an etiological factor.

REFERENCES

1. **Anton L, Merrill DC, Neves LA, Brosnihan KB.** Angiotensin-(1-7) inhibits in vitro endothelial cell tube formation in human umbilical vein endothelial cells through the AT(1-7) receptor. *Endocrine* 32: 212–218, 2007.
2. **Chappel MC, Ferrario CM.** ACE and ACE2: their role to balance the expression of angiotensin II and angiotensin-(1-7). *Kidney Int* 70: 8–10, 2006.
3. **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, Penninger JM.** Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 417: 822–828, 2002.
4. **Danilczyk U, Eriksson U, Oudit GY, Penninger JM.** Physiological roles of angiotensin-converting enzyme 2. *Cell Mol Life Sci* 61: 2714–2719, 2004.
5. **Danilczyk U, Penninger JM.** Angiotensin-converting enzyme II in the heart and the kidney. *Circ Res* 98: 463–471, 2006.

6. **Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S.** A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* 87: E1–9, 2000.

7. **Dzau V.** The cardiovascular continuum and renin-angiotensin-aldosterone system blockade. *J Hypertens Suppl* 23: S9–17, 2005.

8. **Fleming I, Fisslthaler B, Dixit M, Busse R.** Role of PECAM-1 in the shear-stress-induced activation of Akt and the endothelial nitric oxide synthase (eNOS) in endothelial cells. *J Cell Sci* 118: 4103–4111, 2005.

9. **Iusuf D, Henning RH, van Gilst WH, Roks AJ.** Angiotensin-(1-7): pharmacological properties and pharmacotherapeutic perspectives. *Eur J Pharmacol* 585: 303–312, 2008.

10. **Machado RD, Santos RA, Andrade SP.** Mechanisms of angiotensin-(1-7)-induced inhibition of angiogenesis. *Am J Physiol Regul Integr Comp Physiol* 280: R994–R1000, 2001.

11. **Oudit GY, Crackower MA, Backx PH, Penninger JM.** The role of ACE2 in cardiovascular physiology. *Trends Cardiovasc Med* 13: 93–101, 2003.

12. **Oudit GY, Herzenberg AM, Kassiri Z, Wong D, Reich H, Khokha R, Crackower MA, Backx PH, Penninger JM, Scholey JW.** Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. *Am J Pathol* 168: 1808–1820, 2006.

13. **Pfaffl MW.** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.

14. **Rueckschloss U, Quinn MT, Holtz J, Morawietz H.** Dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells: protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 22: 1845–1851, 2002.

15. **Savoia C, Schiffrin EL.** Inhibition of the renin angiotensin system: implications for the endothelium. *Curr Diab Rep* 6: 274–278, 2006.

16. **Tallant EA, Clark MA.** Molecular mechanisms of inhibition of vascular growth by angiotensin-(1-7). *Hypertension* 42: 574–579, 2003.

17. **Verma S, Buchanan MR, Anderson TJ.** Endothelial function testing as a biomarker of vascular disease. *Circulation* 108: 2054–2059, 2003.

18. **Zisman LS, Keller RS, Weaver B, Lin Q, Speth R, Bristow MR, Canver CC.** Increased angiotensin-(1-7)-forming activity in failing human heart ventricles: evidence for upregulation of the angiotensin-converting enzyme homologue ACE2. *Circulation* 108: 1707–1712, 2003.