ACE2 gene transfer attenuates hypertension-linked pathophysiological changes in the SHR

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Díez-Freire, Carlos, Jorge Váquez, María F. Correa de Adjounian, Merari F. R. Ferrari, Lihui Yuan, Xeve Silver, Raquel Torres, and Mohan K. Raizada. ACE2 gene transfer attenuates hypertension-linked pathophysiological changes in the SHR. *Physiol Genomics* 27: 12–19, 2006. First published June 20, 2006; doi:10.1152/physiolgenomics.00312.2005.—Recently discovered, angiotensin-converting enzyme-2 (ACE2) is an important therapeutic target in the control of cardiovascular diseases as a result of its proposed central role in the control of angiotensin peptides. Thus our objective in the present study was to determine whether ACE2 gene transfer could decrease high blood pressure (BP) and would improve cardiac dysfunctions induced by hypertension in the spontaneously hypertensive rat (SHR) model. Five-day-old SHR and normotensive WKY rats received a single intracardiac bolus injection of lentiviral vector containing either murine ACE2 (ACE2) or control enhanced green fluorescent protein (EGFP) genes. Systolic BP, cardiac functions, and perivascular fibrosis were evaluated 4 mo after ACE2 gene transduction. ACE2 gene transfer resulted in a significant attenuation of high BP in the SHR (149 ± 2 mmHg in lenti-ACE2 vs. 180 ± 9 mmHg in lenti-EGFP, P < 0.01). In contrast, no significant effect of lenti-ACE2 on BP of WKY rats was observed. Lenti-ACE2-treated SHR showed an 18% reduction in left ventricular wall thickness (1.52 ± 0.04 vs. 1.86 ± 0.04 mm in lenti-EGFP, P < 0.01). In addition, there was a 12% increase in left ventricular end diastolic and a 21% increase in end systolic diameters in lenti-ACE2-treated SHR. Finally, lenti-ACE2 treatment resulted in a significant attenuation of perivascular fibrosis in the SHR. In contrast, ACE2 gene transfer did not influence any of these parameters in WKY rats. These observations demonstrate that ACE2 overexpression exerts protective effects on high BP and cardiac pathophysiology induced by hypertension in the SHR.

angiotensin-converting enzyme-2; heart; blood pressure; lentiviral vector; hypertrophy; spontaneously hypertensive rat model

ANGIOTENSIN-CONVERTING ENZYME-2 (ACE2) is the newest member of the renin-angiotensin system (RAS) and shares ~40% similarity with the somatic form of ACE (10, 11, 25). In spite of this similarity, ACE2 is distinct in both substrate specificity and functions (10, 11, 25). ACE2 catalyzes the formation of angiotensin-(1–7) [ANG-(1–7)] from ANG II and ANG-(1–9) and is resistant to ACE inhibitors (2, 25). Thus it has been proposed that ACE2 is a critical enzyme of the RAS cascade that is potentially important in countering the vasoconstrictor and proliferative effects of ANG II with the vasodilatory and anti-proliferative effects of ANG-(1–7) and other related peptides (14, 25). As a result, ACE2 has been implicated to play a central role in the development and establishment of cardiovascular diseases including hypertension (2, 23, 28). Further support for this view is provided by the following evidence. 1) ACE2 gene maps to a defined quantitative trait locus (QTL) associated with hypertension (5, 9). 2) ACE2 expression is increased in infarcted area after coronary artery ligation. In addition, blockade of angiotensin type I (AT1) receptor after occlusion of coronary artery results in a significant increase in cardiac ACE2 (4, 16). 3) ACE2 expression and its activity are increased in failing hearts (19, 30, 37). 4) Treatment of spontaneously hypertensive rats (SHR) with all-trans retinoic acid upregulates ACE2 in heart and kidney and reduces blood pressure (BP) (36). 5) Our recent observations demonstrate that ACE2 overexpression prevents cardiac hypertrophy in ANG II infusion rat model of hypertension (22). 6) ACE2 levels are decreased in animal models of hypertension (6, 33). 7) ANG-(1–7), a major product of ACE2, acts as a potent vasodilator and is involved in the prevention of cardiac hypertrophy, fibrosis, and hypertension (3, 18). It has also been shown to improve endothelial function and coronary perfusion and increase cardiac output and stroke volume (17, 31). Finally, transgenic rats overexpressing ANG-(1–7) exhibit a reduced induction of cardiac hypertrophy and improved postischemic function in an isolated perfused heart preparation (35). Collectively, these observations led us to hypothesize that ACE2 inhibition would be associated with an increase in BP and would result in cardiovascular pathologies, whereas its overexpression would lead to beneficial outcomes in the cardiovascular system. Thus the objective of our present investigation was to test this hypothesis by overexpressing ACE2 by lentiviral-mediated gene transfer of ACE2. We have chosen the SHR as an animal model for this study, because the development of high BP and cardiovascular pathophysiology is comparable with human primary hypertension (1, 24). In addition, the RAS in general and ANG-(1–7) in particular have been shown to be important players in the expression and control of hypertensive state in this rat model (2, 15). Our observations indicate that long-term overexpression of ACE2 attenuates high BP and cardiac pathophysiology in the SHR.

MATERIALS AND METHODS

Cloning of murine ACE2 in lentiviral vector and production of lenti-mACE2 viral particles. We have cloned the membrane-bound form of murine ACE2 (ACE2). Complementary DNA encoding Mus musculus ACE2 (21, 26) was used as a template for a PCR amplification reaction with the following primers: ACE2 NehI, sense 5’-AAGCTAGCATAAGGCTCTTGAAGGCTCTTTC-3’; ACE2

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SαI, antisense 5'-AAGTCGACCTAAAAGGAAGTCTGAGCATCATTACGTG-3'. PCR amplification product was cloned into PCR-Blunt TOPO vector (Invitrogen, Carlsbad, CA) and then subcloned from this vector into pTY-EF1-IRE-EGFP using the NheI and SαI sites.

Lentiviral particles containing enhanced green fluorescent protein (EGFP; EF1α-IRE-EGFP, lenti-EGFP) or ACE2 (EF1α-ACE2-IRE-EGFP, lenti-ACE2) were prepared as previously described (7, 21). Viral medium containing EGFP or ACE2 was collected, concentrated, and titered. Concentration of viral particles was determined with the use of HIV-1 p24 antigen ELISA assay (Beckman Coulter, Fullerton, CA), following the manufacturer’s instructions.

**Measurements of efficacy of lenti-ACE2.** Rat cardiac myoblasts (H9C2) from American Type Culture Collection (Manassas, VA) were used to determine the efficacy of lenti-ACE2 by measuring the ACE2 activity. The cardiac myoblasts were maintained in DMEM with 4 mM L-glutamine and 10% fetal bovine serum according to the ACE2 activity. The cardiac myoblasts were maintained in DMEM were used to determine the efficacy of lenti-ACE2 by measuring the (H9C2) from American Type Culture Collection (Manassas, VA)

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animal and receive coil were inserted into an 8.8-cm-diameter transmit-only quadrature volume coil. Dorsal and sagittal images were acquired using cardiac-gated cinetographic gradient echo sequence with the following parameters: dorsal field of view (FOV) = 70 × 30 mm, and sagittal FOV was 81.4 × 40.7 mm, matrix = 256 × 128, repetition time (TR) = 12 ms, echo time (ET) = 2.2 ms, n = 4, slice thickness = 2.0 mm, 10 frames with 1 frame/12 ms. On the basis of the sagittal and dorsal views, short-axis images were prescribed from

Fig. 2. Lentiviral vector-mediated transduction of placental alkaline phosphatase (PLAP) in the spontaneously hypertensive rat (SHR). A: lenti-PLAP (3 × 10¹⁸ TU in 30 μl) was injected in the ventricular space of 5-day-old SHR. One hundred twenty days after viral administration, whole hearts were removed, fixed, and stained for PLAP (29). a and b: whole hearts. c: myocardium at 2.5× magnification. d: transverse section (10 μm) stained with PLAP at 5.0× magnification. B: co-staining of PLAP-transduced SHR heart with anti-myosin antibody. Ten-micrometer sections of ventricular tissue were subjected to immunohistochemistry with the use of a monoclonal antibody to myosin after PLAP staining, as described in MATERIALS AND METHODS. a: PLAP staining (arrows represent PLAP-transduced cells). b: staining with anti-myosin antibody (arrows represent myosin-positive cells). c: double staining (arrows reflect myocytes that express PLAP). Bar = 100 μm. C: lenti-PLAP transduction in the kidney. Kidney from a lenti-PLAP-transduced SHR was removed, fixed, and subjected to PLAP staining protocol, as described in MATERIALS AND METHODS. A significant but random staining (b, arrows) was observed in lenti-PLAP SHR kidney compared with lenti-EGFP control kidney (a).
Intracardiac administration of 3 expresses functional ACE2 activity. Next, we determined the blasts. These data established that lenti-ACE2 is active and although they exhibited green fluorescence in blasts infected with the same concentration of lenti-EGFP, (Fig. 1). No significant ACE2 activity was observed in myo-lenti-ACE2 resulted in a robust expression of ACE2 activity lenti-ACE2. Infection of cardiac myoblasts with 10 MOI of

RESULTS

Measurement of ACE2 mRNA in lenti-ACE2-transduced heart. At the end of the physiological experiments, hearts were removed and total RNA was extracted with RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Semiquantitative RT-PCR was used to determine the transduction effectiveness. The assay was initially standardized for linearity with respect to PCR cycle numbers and for optical density. The following primers were used: sense, 5'-CTC-CGATCATCAACGCTCAACT-3'; antisense, 5'-TTGATCTTGGCGAAGGTACGGTCT-3'. The results were normalized to 18S rRNA (Applied Biosystems, Foster City, CA).

Assessment of perivascular fibrosis. Lenti-EGFP- and lenti-ACE2-transduced WKY rat and SHR hearts were postfixed in ice-cold PLP solution (2% paraformaldehyde, 75 mmol/l lysine, 37 mmol/l sodium phosphate, and 10 mmol/l sodium peroxide). Sections (10 μm) were processed for Masson’s trichrome staining to assess the extent of collagen deposition, and the extent of fibrosis was analyzed as described previously (13, 18).

Measurement of ACE2 activity in hearts of WKY rats and SHR. Ventricular tissues from 70-day- and 3-mo-old WKY rats and SHR were removed and homogenized in the reaction buffer, and homogenates were centrifuged at 20,800 g for min. Supernatants were used to measure ACE2 activity in the presence of 10 μmol/l captopril, as described above.

Statistical analysis. Six WKY rats and six SHR were used in lenti-EGFP control, and similarly six WKY rats and six SHR were used in lenti-ACE2 experimental groups. Results are expressed as means ± SE. Data were analyzed by one-way ANOVA with the use of StatView Statistical Package (SAS Institute, Cary, NC). Values of P ≤ 0.05 were considered statistically significant.

RESULTS

Our first objective was to establish the efficacy of the lenti-ACE2. Infection of cardiac myoblasts with 10 MOI of lenti-ACE2 resulted in a robust expression of ACE2 activity (Fig. 1). No significant ACE2 activity was observed in myoblasts infected with the same concentration of lenti-EGFP, although they exhibited green fluorescence in >95% of myoblasts. These data established that lenti-ACE2 is active and expresses functional ACE2 activity. Next, we determined the transduction efficiency of a similar lentiviral vector in vivo in the SHR with the use of the reporter gene human PLAP. Intracardiac administration of 3 × 10⁸ TU of lenti-PLAP caused a robust and random transduction throughout the myocardium (Fig. 2A, a and b). High-magnification analysis of the image indicated that predominantly transduced cells had myocyte morphology (Fig. 2A, c and d). This was further confirmed with the use of double staining with myosin-specific antibody (Fig. 2B). The number of PLAP-positive cells varied throughout the myocardium, from as high as >50% in some areas to <5% in others. In addition, there were regions in the myocar-

dium that did not show any PLAP staining. These data indicate that, although lentiviral vector predominantly transduces myocytes, this transduction was random. In addition to the heart, there was random but significant transduction in the kidney (Fig. 2C). In contrast, there was little or no noticeable transduction of pulmonary and vascular beds with lenti-PLAP by this protocol.

Five-day-old WKY rats and SHR were transduced with lenti-EGFP or the lenti-ACE2 virus, and indirect BP monitoring was carried out at 30, 70, and 120 days after gene delivery. There was no significant change in BP between lenti-EGFP and lenti-ACE2 SHR at 30 and 70 days. However, by 120 days, lenti-ACE2-treated SHR showed a significant attenuation in BP. The lenti-ACE2 SHR had a BP of 149 ± 2 mmHg compared with 180 ± 9 mmHg in the lenti-EGFP SHR group (P < 0.01, n = 6; Fig. 3). This attenuation in BP was exclusive for the SHR, since the WKY rats treated with lenti-ACE2 showed no difference in BP from their control group (Fig. 3).

At 4 mo of age, the animals were subjected to cardiac MRI to determine left ventricular wall thickness and to evaluate cardiac function. Representative MR images of the hearts of animals of each study group and the corresponding left ventricular wall thickness are shown in Fig. 4. A and B, respectively. SHR treated with lenti-ACE2 showed an 18% reduction in left ventricular wall thickness compared with lenti-EGFP-treated animals (1.52 ± 0.04 vs. 1.86 ± 0.04 mm, respectively; P < 0.001). This reduction in left ventricular wall thickness brings these ACE2-treated SHR values to levels that are comparable with those of WKY rats. In contrast, there was no effect of lenti-ACE2 on left ventricular wall thickness in WKY rats. In addition, cardiac MR images were used to determine the effect of lenti-ACE2 on the left ventricular end diastolic (LVEDD) and end systolic diameters (LVESD) and fractional shortening (FS) of the left ventricular diameter. Figure 5 shows that treatment with lenti-ACE2 resulted in a 12% increase in the LVEDD (0.87 ± 0.009 cm lenti-ACE2 vs. 0.77 ± 0.002 cm lenti-EGFP).

Fig. 3. Effect of lenti-ACE2 on systolic blood pressure (BP) in WKY rats and SHR. Twelve 5-day-old WKY rats and 12 SHR were used for the study. Six animals from each group were used to deliver lenti-EGFP (control), and the remaining six were used for lenti-ACE2 (experimental), as described in MATERIALS AND METHODS. Indirect BPs were measured at indicated time periods by tail vein plethysmography. Data are presented as means ± SE (n = 6). *P < 0.01 vs. SHR treated with lenti-EGFP.
cm lenti-EGFP; \( P < 0.05 \) and a 21% increase in the LVEDD (0.46 ± 0.02 cm lenti-ACE2 vs. 0.38 ± 0.02 cm lenti-EGFP; \( P < 0.05 \)) in the SHR.

Hearts were removed after MRI, and ventricles were divided into three parts, one for measurement of ACE2 mRNA levels, a second for AT1 and AT2 receptors by Western blotting, and a third for examination of fibrosis. Cardiac delivery of lenti-ACE2 in the SHR resulted in an approximate threefold increase in ACE2 mRNA levels (Fig. 6). In contrast, Western blot analysis (32) showed that there was no significant change in the levels of AT1 receptor or AT2 receptor proteins between control and lenti-ACE2-treated SHR (data not shown).

The effect of ACE2 overexpression on cardiac perivascular fibrosis was determined with the use of Masson’s trichrome staining. There was a marked increase (~3 times) in perivascular fibrosis in the lenti-EGFP-treated SHR compared with that in lenti-EGFP WKY rats (Fig. 7, A and B). This perivascular fibrosis in the SHR was markedly attenuated in lenti-ACE2-treated SHR (Fig. 7C). We also observed some myocardial fibrosis in lenti-EGFP-treated SHR at 4 mo of age, although it was not significantly different from age-matched WKY rats.

Finally, endogenous ACE2 activity was compared in hearts of 10-day- and 3-mo-old WKY rats and SHR. ACE2 activity was significantly lower in hearts of 10-day-old WKY rats and SHR compared with 3 mo of age (Fig. 8). Although there was no difference in ACE2 activity between WKY rats and SHR at 10 days of age, there was a modest but significant decrease in cardiac ACE2 activity in 3-mo-old SHR compared with age-matched WKY rats.

DISCUSSION

The most significant observation of our study is that ACE2 overexpression after neonatal development provides a significant protection from high BP and cardiac pathophysiology in the SHR. Although we have previously demonstrated cardioprotective effects of ACE2 in the ANG II infusion model of
hypertension (22), this report presents evidence of beneficial effects of ACE2 overexpression on both BP and cardiac dysfunction and pathophysiology. The precise mechanism of this anti-hypertensive action remains to be fully elucidated. However, alterations in the renal RAS by lentivirus-mediated transduction of the kidney and increases in the plasma ACE2 by proteolytic release of this enzyme, individually or together, may contribute to this effect (27). Support for this view is based on the following. 1) Release of membrane-bound ACE2 into the circulation by actions of secretase and other proteases has been demonstrated (27). In fact, Donoghue et al. (12) have demonstrated the presence of soluble ACE2 after cardiac overexpression of membrane-bound ACE2. 2) Our present data show that intracardiac delivery of lenti-ACE2 causes modest but significant transduction of renal tissue.

The anti-hypertensive effects of lenti-ACE2 in the SHR model are in contrast to our previous observations, where ACE2 overexpression failed to exert any significant effects on high BP in an ANG II infusion rat model of hypertension (22). This could be due to the differences in the characteristics of these two models. For example, the increase in BP is slow and steady in the SHR and takes months before hypertension is fully established, whereas it takes only 2–3 wk in the ANG II infusion model. In addition, there is little to no change in plasma ANG II in the former, whereas a rapid increase in its levels are seen in the latter model. Thus it is possible that a limited overexpression of ACE2 is unable to counteract a rapid rise in plasma ANG II in the ANG II infusion model. The anti-hypertensive effects are consistent with previous reports, where decreased levels of ACE2 were observed in both the SHR and stroke-prone SHR (5, 9). Moreover, the coding sequence of the ACE2 gene has been mapped to a QTL affecting susceptibility to hypertension in sabra- and stroke-prone SHR rat models of hypertension (5, 9).

In addition to alteration of high BP, ACE2 gene transfer induces cardioprotective effects in the SHR. The SHR exhibits an increase in the left ventricular wall thickness and reduced ventricular filling volume compared with its normotensive WKY rat strain, indicating that the SHR is progressing toward a decrease in cardiac functions leading to heart failure. Lenti-ACE2-treated SHR exhibited significant improvements in left ventricular wall thickness. However, there was no difference in the percent fractional shortening of left ventricular diameter between the lenti-ACE2-treated SHR and the WKY rat.

The study, although impressive in its outcome, raises many important questions that can be the subject matter of future inquiries. 1) For example, would improvements in left ventricular wall thickness and cardiac function lead to attenuation of cardiac remodeling? We do not have the data for the SHR, since it takes 1–1.5 yr for myocardial fibrosis to develop in this rat model of hypertension (8). However, our previous observation with the ANG II infusion rat model (22) and the significant improvement observed in perivascular fibrosis in this study lead us to anticipate a beneficial effect on myocardial fibrosis as well. 2) How is transduction of only a limited number of cardiac and renal cells by lentiviral vector able to attenuate cardiac pathology? Although the mechanism remains speculative at present, we have previously suggested that intra/intercellular communications directly, or through some yet unknown paracrine/endocrine factor, enable the propagation of signals from ACE2-transduced cells to the entire heart (22). 3) What is the mechanism of this anti-hypertensive effect of ACE2? ACE2 is a multifunctional enzyme that is central in balancing the levels of ANG II and AT1 receptor functions with vasoprotective peptides such as ANG-(1–7) (12, 25). Thus it is reasonable to assume that overexpression of ACE2 would tip the balance of cardiac and renal RAS toward the levels of...

Fig. 7. Effect of lenti-ACE2 transduction on perivascular fibrosis in SHR. Hearts were fixed in PLP solution, sectioned, and stained with Masson’s trichrome (13). Images are representatives of the extent of perivascular fibrosis in lenti-EGFP WKY (A), lenti-EGFP SHR (B), and lenti-ACE2 SHR (C). Bars = 50 μm. Arrows point out areas of fibrosis and collagen deposition.

Fig. 8. ACE2 activities in WKY rats and SHR hearts. Hearts from 10-day- and 3-mo-old WKY rats and SHR were removed, and ventricular tissue was excised and homogenized in the reaction buffer. ACE2 activity was measured as described in the MATERIALS AND METHODS. Data are means ± SE (n = 4; *P < 0.02).

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these vasoprotective peptides. The beneficial effects of ACE2 overexpression, however, are not likely to be mediated by changes in AT1 and AT2 receptors, since their levels do not change in lenti-ACE2-treated animals. Furthermore, our observation suggests that cardioprotective effects of ACE2 are BP independent. This review is supported by our previous study (22), demonstrating a similar protective effect of ACE2 overexpression in cardiac remodeling without influencing high BP.

Finally, our observations indicate that ACE2 gene transfer does not cause any adverse effects on the normal heart. This is contrary to the study of Donoghue et al. (12), which demonstrated that overexpression of ACE2 in transgenic mice resulted in a profound cardiac dysfunction. We believe that the differences in experimental protocols between the two studies may explain this disparity. In the transgenic study, ACE2 overexpression was maintained from the embryonic state, whereas in our study, lenti-ACE2 was delivered post-cardiac development. Thus we believe that ACE2 overexpression must be initiated after the completion and not before cardiac development to protect the heart from hypertension-induced pathophysiologies. However, because data are lacking on levels of ANG II and ANG-(1–7), noninvolvement of the RAS on these effects cannot be ruled out at the present time.

In conclusion, our observations provide proof of the concept that ACE2 produces beneficial effects and protects the cardiovascular system from hypertension-induced pathophysiologies. Thus they set the stage for future studies to explore the therapeutic potential of the ACE2 gene transfer strategy on long-term control of hypertension.

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