MAP kinase/phosphatase pathway mediates the regulation of ACE2 by angiotensin peptides

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Gallagher PE, Ferrario CM, Tallant EA. MAP kinase/phosphatase pathway mediates the regulation of ACE2 by angiotensin peptides. Am J Physiol Cell Physiol 295: C1169–C1174, 2008. First published September 3, 2008; doi:10.1152/ajpcell.00145.2008.—Angiotensin-converting enzyme 2 (ACE2) catalyzes the conversion of the vasoconstrictor angiotensin II (ANG II) to the vasodilatory peptide angiotensin-(1-7) [ANG-(1-7)]. We showed that treatment of hypertensive rats with the AT1 receptor antagonist olmesartan increased ACE2 mRNA and protein in the thoracic aorta, suggesting that endogenous ANG II tonically reduces the enzyme. We now report that ANG II downregulates ACE2 activity and mRNA in rat aortic vascular smooth muscle cells (VSMCs) to reduce the conversion of ANG II to ANG-(1-7). Although ANG-(1-7) alone had no effect on the regulation of ACE2 mRNA, the heptapeptide prevented the ANG II-mediated reduction in ACE2 mRNA, an effect blocked by the selective ANG-(1-7) receptor antagonist [D-Ala7]-ANG-(1-7). The reduction in ACE2 mRNA by ANG II was also prevented by the mitogen-activated protein (MAP) kinase kinase inhibitor PD98059. Treatment of VSMCs with ANG II increased ERK1/ERK2 activity, which was significantly reduced by pretreatment with ANG-(1-7). Blockade of the ANG II-mediated reduction in ACE2 mRNA and increase in MAP kinase activity by ANG-(1-7) was prevented by pretreatment with sodium vanadate, a tyrosine phosphatase inhibitor, or okadaic acid, a serine-threonine phosphatase inhibitor, suggesting that the heptapeptide activates a MAP kinase phosphatase. This study is the first to show that the MAP kinase/phosphatase pathway is a primary molecular mechanism for regulating ACE2 to maintain the balance between ANG II and ANG-(1-7). The modulatory role of ANG-(1-7) in the regulation of ACE2 by ANG II suggests a complex interplay between the two peptides that is mediated by specific receptors to activate distinct signaling pathways.

angiotensin-converting enzyme 2; angiotensin II; angiotensin-(1-7), mitogen-activated protein kinase; vascular smooth muscle cells

THE SYSTEMIC renin-angiotensin system (RAS) is a primary regulator in the vasculature, controlling blood pressure and fluid homeostasis, whereas local tissue RAS are involved in a variety of autocrine, intracrine, and paracrine functions. The octapeptide angiotensin II (ANG II), a major effector hormone of the system, serves as a potent vasoconstrictor as well as a stimulator of thirst, aldosterone release, and cell proliferation. Conversely, ANG-(1-7) produces unique physiological responses that are often opposite to those of the well-characterized ANG II (9). ANG-(1-7), present in the circulation and tissues at concentrations similar to ANG II (16), is a vasodilator, alters renal fluid absorption, and has anti-proliferative properties (8). Both peptide hormones mediate their biological effects through interaction with distinct, high-affinity angiotensin receptors to activate a myriad of signaling pathways. Alterations in the concentration and/or ratio of ANG II and ANG-(1-7) can result in dysregulation of the system contributing to a plethora of pathological processes, including hypertension, myocardial infarction, heart failure, atherosclerosis, and inflammation.

Angiotensin-converting enzyme 2 (ACE2), the first reported ACE homolog, is a key component of the RAS. It is produced and secreted from a wide variety of cells and tissues, including the heart, kidney, gastrointestinal tissues, and the brain (5–7, 26, 27). The characterization of ACE2 knockout mice provided the first evidence of an important role for ACE2 in cardiovascular function. In the model of ablated ACE2 described by Crackower et al. (1), mice with reduced ACE2 had severely impaired cardiac function, including mild thinning of the left ventricle and a severe reduction in cardiac contractility. However, studies by Gurley et al. (12) suggest that the impairment of cardiac function by deletion of ACE2 is dependent on the strain of mice used for genetic manipulation. Transverse aortic constriction following ACE2 deletion resulted in the development of cardiac hypertrophy and dilatation in association with decreased cardiac contractility and increased perivascular and interstitial fibrosis, suggesting that the absence of ACE2 contributes to cardiovascular dysfunction following aortic constriction. Overexpression of ACE2 using a lentiviral vector was associated with an improvement in cardiac function following coronary artery occlusion (3) and improved cardiac function and reduced fibrosis in the spontaneously hypertensive rat (4) and following ANG II infusion (13). These studies suggest a role for ACE2 in cardiovascular function.

ACE2 catalyzes with high efficiency the conversion of ANG II to ANG-(1-7) (28). Zisman et al. (29) reported that production of ANG-(1-7) from ANG II in the human heart was inhibited by a selective ACE2 inhibitor (C16, MLN-4760), in agreement with studies by Trask et al. (26), showing that ANG-(1-7) was generated from ANG II in the hearts of hypertensive rats, a process inhibited by the ACE2 inhibitor MLN-4760. ACE2 is also present in the brain, in both the hypothalamus and brainstem, where it converts ANG II to ANG-(1-7) (5, 7), and injection of the ACE2 inhibitor MLN-4760 reduces baroreceptor reflex sensitivity for control of the heart rate (5). We observed a marked upregulation of ACE2 mRNA in cardiac tissue of normotensive Lewis rats or rats with a myocardial infarction following treatment with the angiotensin type 1 (AT1) receptor blockers losartan or olme-
Angiotensin II for 28 days, suggesting a feedback regulation to favor ANG II production (15). This increase in ACE2 correlated directly with plasma levels of ANG-(1-7) and inversely with plasma levels of ANG II. The plasma ANG-(1-7)-to-ANG II ratios were significantly augmented in the losartan-treated group compared with untreated control animals, demonstrating increased formation of ANG-(1-7) from ANG II. Taken together, these studies suggest that ACE2 serves as a critical enzymatic regulator to directly balance the levels of ANG II and ANG-(1-7), thereby modulating the pressor/mitogenic and depressor/growth inhibitory arms of the RAS.

The regulatory role for ACE2 is supported further by our studies in the vasculature. We showed a marked increase in ACE2 mRNA and protein as well as ANG-(1-7) in the thoracic aorta of spontaneously hypertensive rats following chronic administration with the AT1 receptor antagonist olmesartan (14). Olmesartan treatment of the rats resulted in a significant decrease in the media-to-lumen ratio and medial thickness of the thoracic aorta, which was independent of blood pressure. This study suggests that ANG II, acting through the AT1 receptor, negatively regulates ACE2 to favor vasoconstrictive and growth-promoting responses, whereas ANG-(1-7) modulates the ANG II effect and reduces vascular growth. ACE2, by mediating the angiotensin peptide balance, may be a critical regulator of pressure-independent vascular remodeling. The aim of the present study was to identify the signaling pathways involved in the regulation of ACE2 by angiotensin peptides in vascular smooth muscle cells (VSMCs).

METHODS

Preparation of VSMCs. VSMCs were isolated by explant culture from the thoracic aorta of 12- to 14-wk-old Sprague-Dawley rats, as described previously (10). Cells were used between passage 4 and 10 and made quiescent by a 48-h treatment with defined serum-free media containing DMEM-F12, penicillin, streptomycin, 3 μg/ml insulin, and 5 μg/ml transferrin. All experimental procedures were performed in accordance with guidelines set forth by the Institutional Animal Care and Use Committee.

Preparation of cell lysates and Western blot hybridization. Cultured VSMCs were solubilized in Triton-lysis buffer containing 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 50 mM Tris·HCl, pH 7.4, with 0.01 mM NaVO₄, 0.1 mM phenylmethylsulfonylfluoride, and 0.6 μM leupeptin. The supernatant was clarified by centrifugation (10,000 g for 10 min, 4°C), and protein concentration was quantified by the Lowry method (19).

ACE2 assay. An ACE2 fluorescence assay was performed according to Vickers et al. (28) with modifications. Reaction mixtures containing the substrate 50 μM 7-methoxycoumarin-4-acetyl-alanine-proline-lysine-(2,4-dinitrophenyl)-OH, cell or tissue homogenate, and 10 mM Tris·HCl, pH 7.4, with 0.01 mM NaVO₄, 0.1 mM phenylmethylsulfonylfluoride, and 0.6 μM leupeptin. The supernatant was clarified by centrifugation (10,000 g for 10 min, 4°C), and protein concentration was quantified as the ratio of target/control product is first detected and expressed as the ratio of target/control product (relative gene expression).

Statistics. All data are presented as means ± SE. Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc tests. For Fig. 6, potential differences in the control versus incubations with each of the phosphatase inhibitors were evaluated by ANOVA. However, because okadaic acid reduced the control values, difference between treatment with ANG II versus ANG II and ANG-(1-7) alone or in the presence of each phosphatase inhibitor were compared by Student’s t-test. The criterion for statistical significance was set at P < 0.05.

RESULTS

ANG II regulation of ACE2. Cultured rat aortic VSMCs were treated with 100 nmol/l ANG II to determine whether the peptide regulates ACE2. A marked reduction in ACE2 activity was observed following a 12-h incubation period of cultured VSMCs with ANG II (Fig. 1A). ETDA (0.5 mM) was added to the cultured cells to prevent the degradation of ANG II by blocking the activity of shed metalloproteases. Addition of the AT1 receptor antagonist losartan (1.0 μmol/l) effectively blocked the ANG II-mediated reduction of ACE2 activity, resulting in enzyme activity comparable to the control level. Losartan alone had no effect on ACE2 activity.

The mode of regulatory control was determined by examining the effect of ANG II treatment on ACE2 mRNA. ACE2 mRNA was decreased significantly following a 12-h incubation of cultured VSMCs with 100 nmol/l ANG II (Fig. 1B). The ANG II-mediated reduction of ACE2 mRNA was blocked by 1.0 μmol/l losartan, resulting in ACE2 mRNA concentrations equivalent to the untreated control, whereas losartan alone had no effect. This study demonstrates that ANG II acting through the AT1 receptor significantly reduces VSMC ACE2 by regulation of transcription or mRNA stability.

Regulation of ACE2 mRNA by angiotensin peptides. The regulation of ACE2 mRNA by both ANG II, a substrate for ACE2, as well as by ANG-(1-7), the product of ACE2 metabolism of ANG II, was measured at increasing doses of the peptides and with increasing times of incubation. As shown in Fig. 2B, ANG II caused a dose-dependent decrease in ACE2 mRNA, with a maximal effect between 10 and 100 nmol/l. The ANG II-mediated reduction in ACE2 mRNA was also time dependent, with a maximal effect following 12 h of incubation with the peptide (Fig. 2B). ANG-(1-7) alone had no effect on ACE2 mRNA.
ACE2 mRNA, at concentrations of the peptide up to 1 μmol/l or times of incubation up to 24 h.

Blockade of ANG II-mediated ACE2 downregulation by ANG-(1-7). Cultured VSMCs were incubated with ANG-(1-7) to determine whether the heptapeptide also regulated ACE2 at transcription. As shown in Fig. 2, treatment of the cells with 1.0 μmol/l ANG-(1-7) alone did not alter the ACE2 mRNA. However, the ANG II-mediated downregulation of ACE2 was attenuated completely by the addition of ANG-(1-7), as shown in Fig. 3. The counter regulation of the ANG II decrease in ACE2 mRNA by ANG-(1-7) was prevented by coinubcation with 1.0 μmol/l [d-Ala^7]-ANG-(1-7), an ANG-(1-7) receptor antagonist (Fig. 3B). [d-Ala^7]-ANG-(1-7) had no effect on the reduction of ACE2 mRNA by ANG II. This study demonstrates that the heptapeptide inhibition of the ANG II down-regulation of ACE2 is mediated by a specific ANG-(1-7) receptor.

Signaling mechanism for the ANG II downregulation of ACE2. Cultured rat VSMCs were incubated with 100 nmol/l ANG II for 12 h in the presence or absence of a MAP kinase inhibitor to investigate the cellular signaling pathways involved in the reduction of ACE2 mRNA by ANG II. As shown in Fig. 4, the MAP/ERK kinase (MEK) inhibitor PD98059 blocked the ANG II-mediated decrease in ACE2 mRNA.

Treatment of the VSMCs with the MEK inhibitor alone had no effect. These results suggest that downregulation of ACE2 by ANG II is mediated by a MAP kinase signaling pathway.

DISCUSSION

In the present study, we showed that ANG II markedly reduced ACE2 in cultured rat VSMCs through a regulatory process mediated by the angiotensin type 1 (AT1) receptor. Treatment of VSMCs with ANG-(1-7), the product of ACE2 hydrolysis of ANG II, did not affect ACE2 mRNA; however, ANG-(1-7) prevented the ANG II-mediated reduction in ACE2 mRNA. Addition of [d-Ala^7]-ANG-(1-7), a selective AT1 receptor antagonist, blocked the inhibitory actions of ANG-(1-7). These data are the first to demonstrate opposing transcriptional regulation of ACE2 by ANG II and ANG-(1-7) in VSMCs and suggest a complex interplay between these two peptides that is mediated by distinct receptor pathways. ANG-(1-7) prevented the ANG II-mediated reduction in VSMC ACE2 mRNA, and the inhibitory action of the heptapeptide was blocked by the addition of [d-Ala^7]-ANG-(1-7). Since ACE2 preferentially converts ANG II to ANG-(1-7), downregulation of the enzyme by ANG II constitutes a positive feedback system that may favor ANG II-mediated responses, whereas an increase in ANG-(1-7) serves as a negative regulator to counterbalance the ANG II-initiated signaling pathway. These results are in agreement with our previous in vitro studies demonstrating that treatment of cultured astrocytes with ANG II caused a marked
reduction in neural ACE2 mRNA and protein, a response mediated by the AT1 receptor (11). The ANG II-mediated effect in medullary astrocytes was blocked by coadministration with the heptapeptide, an effect prevented by an ANG-(1-7) antagonist. Thus the distinct differences in the regulation of ACE2 by the two peptides may prove to be a general mechanism for controlling the levels of the enzyme to alter the ratio of ANG II/ANG-(1-7) in response to physiological stimuli.

The molecular mechanism for the opposing regulation of ACE2 by ANG II and ANG-(1-7) in VSMCs resides at the level of the MAP kinases ERK1 and ERK2. Whereas ANG II stimulates MAP kinase activity to reduce ACE2, the addition of ANG-(1-7) blocks the MAP kinase activation. The ANG-(1-7)-mediated reduction in mitogen-stimulated ERK1/ERK2 activity in VSMCs was attenuated by either the tyrosine phosphatase inhibitor sodium vanadate or the serine-threonine phosphatase inhibitor okadaic acid, suggesting that ANG-(1-7) reduces MAP kinase activity by upregulating or activating a MAP kinase phosphatase. This study is the first to show that the MAP kinase-phosphatase pathway is a primary molecular mechanism for regulating ACE2 to maintain the balance between ANG II and ANG-(1-7). We previously showed that ANG-(1-7) inhibited the growth of cultured VSMCs with a significant dose-dependent reduction in ACE2 mRNA (Fig. 3). ANG-(1-7) prevented the downregulation of ACE2 mRNA by ANG II (Fig. 3A). VSMCs in defined serum-free media were incubated for 12 h with increasing concentrations of ANG II or ANG-(1-7) (A) or for increasing times in the presence of 100 nM ANG II or ANG-(1-7) (B). EDTA (0.5 mmol/l) was included to prevent the degradation of ANG II by ACE2, ACE, or nephrilysin. ACE2 mRNA was measured by reverse transcriptase, real-time PCR. n = 4.

Fig. 4. PD98059 (PD) blocks the ANG II-mediated downregulation of ACE2 mRNA in VSMCs. VSMCs in defined serum-free media were incubated for 12 h with either 100 nM ANG II, in the presence or absence of 30 μmol/l PD or with PD alone. EDTA (0.5 mmol/l) was included to prevent the degradation of ANG II by ACE2, ACE, or nephrilysin. ACE2 mRNA was measured by reverse transcriptase, real-time PCR. n = 5; *P < 0.01 compared with CTL in the absence of ANG II or PD.
concomitant decrease in MAP kinases ERK1/ERK2 activity, an effect blocked by the AT(1-7) receptor antagonist [d-Ala7]-ANG-(1-7) (10, 23, 24). Taken together, these results indicate that modulation of the MAP kinase activation is a major mechanism for the regulation of multiple physiological processes by ANG II/ANG-(1-7) in various cell types.

It is well-established that ANG II acting through the AT1 receptor contributes to VSMC hypertrophy, hyperplasia, and migration, leading to vascular remodeling (20, 25). We showed that ANG-(1-7) reduced the serum-stimulated growth of VSMCs isolated from rat thoracic aorta as well as mitogen-stimulated DNA synthesis (10). Attenuation of serum-stimulated thymidine incorporation by ANG-(1-7) was unaffected by antagonists selective for AT1 or AT2 receptors, whereas a selective AT(1-7) receptor antagonist blocked the growth inhibitory response to the heptapeptide. Tallant and Clark (23) showed that ANG-(1-7) treatment blocked the ANG II-stimulated phosphorylation and activation of ERK1 and ERK2. Inhibition of the growth of VSMCs was mediated by an increase in prostacyclin and the prostacyclin-induced increase in cAMP to activate the cAMP-dependent protein kinase. Furthermore, we showed that ANG-(1-7) reduced vascular growth stimulated by balloon catheter injury to the rat carotid artery (21, 22). An approximate threefold increase in the plasma ANG-(1-7), levels similar to the increases observed following treatment with an ACE inhibitor or AT1 receptor blockers was maintained by intravenous infusion of the heptapeptide into the carotid artery with a chronically implanted minipump (21). ANG-(1-7) infusion significantly reduced formation of a neointima compared with rats infused with saline controls but had no effect on the medial area of the injured or the contralateral uninjured artery, suggesting that only actively growing cells were inhibited by the heptapeptide. In agreement, Langeveld et al. (18) demonstrated a significant reduction in neointimal thickness and stenosis in the abdominal aorta of rats infused with ANG-(1-7) following stent implantation compared with saline-treated controls. These studies effectively demonstrate that ANG-(1-7) is an endogenous regulator of vascular cell growth.

Our previous study indicates that ACE2 plays a critical role in the regulation of vascular growth by altering the tissue concentrations of the angiotensin peptides (14). Chronic blockade of AT1 receptors with olmesartan resulted in a fivefold increase in ACE2 expression in the thoracic aorta of spontaneously hypertensive rats when compared with the aortas of vehicle-treated animals. This observation correlates with the results obtained in VSMCs in the current study, demonstrating losartan inhibition of the ANG II-mediated decrease in ACE2. The resultant elevation in plasma ANG-(1-7) in the olmesartan-treated rats was associated with a significant reduction in medial hypertrophy but not lumen size. Conversely, no change in media-to-lumen ratio was observed following treatment with atenolol or hydralazine at concentrations that reduced blood pressure to similar levels as olmesartan but did not increase ACE2 or plasma ANG-(1-7) concentrations. These results demonstrate that the reduction in vascular remodeling was independent of blood pressure but required upregulation of ACE2 and a concomitant elevation of circulating ANG-(1-7). Thus AT1 receptor blockers may not only decrease blood pressure by preventing the action of ANG II but also by ANG-(1-7)-mediated reduction in vascular hypertrophy.
**Perspective**

ACE2 is a key regulator maintaining the systemic and tissue levels of ANG II and ANG-(1-7) to balance the pressor/mitogenic and depressor/growth inhibitory arms of the renin-angiotensin system. The results of the present study identify distinct receptor systems that differentiate the actions of ANG II and ANG-(1-7) in the regulation of ACE2. ACE2 is reduced by ANG II, through activation of AT1 receptors, whereas ANG-(1-7) blocks the downregulation of ACE2 by ANG II, through the AT1 receptor. This indicates that ACE2 and the ACE2-mediated conversion of ANG II to ANG-(1-7) will be increased following treatment with AT1 receptor antagonists such as losartan, suggesting that both the reduction in responses to ANG II and an increase in ANG-(1-7) may participate in the effects of angiotensin receptor blockers. Furthermore, the studies described demonstrate that angiotensin peptides regulate ACE2 through activation of MAP kinase-phosphatase signaling pathways. Inhibitors of protein kinases are currently under investigation as novel molecular targets for the treatment of cardiovascular disease (17). The results of the current study suggest that agonists of protein phosphatases or agents that regulate these enzymes should also be considered as potential therapeutic interventions.

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