Angiotensin II regulates ACE and ACE2 in neurons through p38 mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 signaling

Liang Xiao, Karla K. V. Haack, and Irving H. Zucker
Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, Nebraska

Submitted 14 November 2012; accepted in final form 20 March 2013

Xiao L, Haack KK, Zucker IH. Angiotensin II regulates ACE and ACE2 in neurons through p38 mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 signaling. Am J Physiol Cell Physiol 304: C1073–C1079, 2013. First published March 27, 2013; doi:10.1152/ajpcell.00364.2012.—Brain ANG II plays an important role in modulating sympathetic function and homeostasis. The generation and degradation of ANG II are carried out, to a large extent, through the angiotensin-converting enzyme (ACE) and ACE2, respectively. In disease states, such as hypertension and chronic heart failure, central expression of ACE is upregulated and ACE2 is decreased in central sympathoregulatory neurons. In this study, we determined the expression of ACE and ACE2 in response to ANG II in a neuronal cell culture and the subsequent signaling mechanism(s) involved. A mouse catecholaminergic neuronal cell line (CATH.a) was treated with ANG II (30, 100, and 300 nM) for 24 h, and protein expression was determined by Western blot analysis. ANG II induced a significant dose-dependent increase in ACE and decrease in ACE2 mRNA and protein expression in CATH.a neurons. This effect was abolished by pretreatment of the cells with the p38 MAPK inhibitor SB-203580 (10 μM) 30 min before administration of ANG II or the ERK1/2 inhibitor U-0126 (10 μM). These data suggest that ANG II increases ACE and attenuates ACE2 expression in neurons via the ANG II type 1 receptor, p38 MAPK, and ERK1/2 signaling pathways.

MATERIALS AND METHODS

Cell culture. CATH.a neurons were purchased from American Type Culture Collection (Manassas, VA). The neuronal cells were grown in RPMI 1640 medium containing 8% horse serum, 4% fetal bovine serum, and 100 IU/l penicillin at 37°C in 5% CO2 in a humidified atmosphere. Before treatment, the neuronal cells were grown in RPMI 1640 medium containing 8% horse serum, 4% fetal bovine serum, and 100 IU/l penicillin at 37°C in 5% CO2 in a humidified atmosphere. Before treatment, the neuronal cells were allowed to differentiate in serum-free medium for 48 h. For immunofluorescence, neurons were grown on 12-mm poly-l-lysine-coated coverslips in 24-well plates at a density of 1 x 10⁶ cells/well. For real-time RT-PCR and Western blotting, the neurons were plated at a density of 1 x 10⁶ cells/well in six-well culture plates. The neurons were treated with ANG II (30, 100, and 300 nM) for 4 h for real-time RT-PCR or 24 h for immunofluorescence and Western blot analysis. An AT1R antagonist (losartan, 10 μmol/l), an AT2R antagonist (PD-123319, 10 μmol/l), a p38 MAPK inhibitor (SB-203580, 10 μmol/l), or an ERK1/2 inhibitor (U-0126, 10 μmol/l) was given 30 min before ANG II treatment.

Immunofluorescence confocal microscopy. After appropriate treatment and incubation with ANG II, the media were aspirated and the cells were washed with ice-cold PBS. The neurons were fixed with 4% paraformaldehyde for 10 min. The cells were washed five times with PBS at room temperature and blocked using 10% normal donkey serum (NDS), 1% BSA, and 1% Triton in PBS for ≥1 h at room temperature. Primary antibody-directed anti-ACE or anti-ACE2 (catalog nos. sc-20791 and sc-20998, Santa Cruz Biotechnology) was diluted (1:200) in 1% NDS, 1% BSA, and 1% Triton in PBS and

Address for reprint requests and other correspondence: I. H. Zucker, Dept. of Cellular and Integrative Physiology, 985850 Nebraska Medical Center, Omaha, NE 68198-5850 (e-mail: izucker@unmc.edu).

http://www.ajpcell.org 0363-6143/13 Copyright © 2013 the American Physiological Society
ACE AND ACE2 BALANCE IN NEURONS

ANG II modulates ACE and ACE2 expression in CATH.a neurons. ACE and ACE2 were detected in the membrane and cytoplasm of CATH.a neurons by laser confocal immunofluorescence. ACE and ACE2 were found in the cell membrane and cytoplasm. After 24 h of treatment with 30, 100, and 300 nM ANG II, ACE expression was increased in a dose-dependent manner (Fig. 1, top). In contrast, ACE2 expression was decreased after the same ANG II treatments (Fig. 1, bottom). The changes in protein expression were quantified by Western blot analysis (Fig. 2). ACE protein was significantly increased after 100 nM (0.45 ± 0.06) and 300 nM (0.62 ± 0.05) ANG II compared with the control group (0.27 ± 0.02). ACE2 protein level was significantly reduced with 300 nM ANG II (0.22 ± 0.01) compared with the control group (0.34 ± 0.01). ACE and ACE2 gene transcription was also determined by real-time RT-PCR, as shown in Fig. 3. ACE mRNA was increased twofold at 100 nM ANG II. ACE2 mRNA levels were decreased by ~60% by 100 nM ANG II. ACE mRNA was augmented by 3.7-fold and ACE2 was reduced by ~75% by 300 nM ANG II.

Effect of p38 MAPK and ERK1/2 inhibition on ACE and ACE2 expression. Because ANG II increases phosphorylation of p38 MAPK and ERK1/2, it was of interest to determine the influence of these proteins on ACE and ACE2. ACE and ACE2 gene transcription was measured following p38 MAPK or ERK1/2 inhibition. Significant interaction (P < 0.0001) was observed between MAPK inhibitor pretreatments and ANG II treatments (Fig. 3). Although baseline ACE and ACE2 mRNA levels were not affected by the p38 MAPK inhibitor SB-203580 or the ERK1/2 inhibitor U-0126, both abolished the ANG II modulation of ACE and ACE2 gene transcription. Post hoc analysis showed a significant difference in relative ACE mRNA levels for 300 nM ANG II (3.68 ± 0.37 with vehicle vs. 0.95 ± 0.11 and 0.89 ± 0.16 with SB-203580 and U-0126, respectively, both P < 0.05). Significant differences in ACE2 mRNA were also found between the vehicle-treated group (0.27 ± 0.13) and neurons treated with the p38 MAPK inhibitor (1.08 ± 0.12) or the ERK1/2 inhibitor (1.07 ± 0.11) at 300 nM ANG II.

Protein expression following MAPK inhibition was measured by Western blot analysis. As shown in Fig. 4, similar to the data for real-time RT-PCR, the p38 MAPK inhibitor or the ERK1/2 inhibitor normalized the dose-dependent upregulation of ACE and downregulation of ACE2 without affecting the baseline expression of the two enzymes.

The effects of SB-203580 and U-0126 pretreatments were confirmed by immunoblotting the phosphorylated and total proteins for p38 and ERK. As shown in Fig. 5, dose-dependent phosphorylation of p38 MAPK was prevented by pretreatment with SB-203580 (Fig. 5A), while U-0126 inhibited ERK1 and ERK2 activation (Fig. 5B).

Effect of angiotensin receptor blockade on ACE and ACE2 expression. Since both major subtypes of the ANG II receptors have been associated with MAPK signaling activation in different cell types or tissues (12, 28), AT1R and AT2R antagonists were used to determine which subtype is involved in the modulation of ACE and ACE2. As shown in Fig. 6, significant interactions were observed between ANG II and its receptor antagonist treatments for ACE expression. The AT1R antagonist losartan normalized the dose-dependent upregulation of ACE (0.66 ± 0.01 and 0.40 ± 0.04 with vehicle and losartan, respectively, at 300 nM ANG II, P < 0.05), while AT2R antagonism with PD-123319 had no effect. ACE2 expression was significantly decreased in neurons treated with vehicle + 300 nM ANG II (0.37 ± 0.01 and 0.23 ± 0.03 at 0 and 300 nM ANG II, respectively, P < 0.05) and neurons treated with PD-123319 + 300 nM ANG II (0.34 ± 0.02 and 0.22 ± 0.03...
at 0 and 300 nM ANG II, respectively, \( P < 0.05 \). Losartan completely blocked the effects of ANG II on ACE2. These data suggest a dominant role of the AT1R in the regulation of ACE and ACE2 expression in CATH.a neurons.

**DISCUSSION**

In the current study we hypothesized that ANG II upregulates ACE and downregulates ACE2 expression in CATH.a neurons. These cellular data support the putative effects of ANG II on ACE and ACE2 expression in CATH.a neurons.
ANG II on ACE and ACE2 gene transcription through an AT1R-p38 MAPK and ERK1/2 signaling pathway.

The localization of ACE and ACE2 has been mapped in various sites in the brain in humans and experimental animals (40, 42). ACE in the brain is associated with the endothelium of cerebral blood vessels (4, 13), epithelial cells of the choroid plexus, and plasma membranes of astrocytes in the circumventricular organs (42). ACE is also expressed at moderate levels in neurons in the PVN, and rostral ventrolateral medulla has been reported previously and has been associated with increased oxidative stress and hyperactivated ANG II-AT1R signaling in hypertension and heart failure (5, 33, 38). Activation of p38 MAPK and ERK1/2 by ANG II has also been observed in other tissues, including heart (24, 31), vascular smooth muscle (25, 35), and kidney (14, 26). Several different upstream signaling pathways may link AT1R activation to phosphorylation of p38 MAPK or ERK1/2. The Src family of protein tyrosine kinases has been shown to play a key role in the coupling of cell surface receptors with MAPK activation (14, 27) and NADPH oxidase-derived O2·−, which also serves as a second messenger for MAPK activation (14, 27).

In the current study we elucidated an AT1R-p38 MAPK and ERK1/2 signaling pathway in the modulation of ACE and ACE2 expression in neurons. Central AT1R-dependent phosphorylation of p38 MAPK or ERK1/2 in the subfornical organ, PVN, and rostral ventrolateral medulla has been reported previously and has been associated with increased oxidative stress and hyperactivated ANG II-AT1R signaling in hypertension and heart failure (5, 33, 38). Activation of p38 MAPK and ERK1/2 by ANG II has also been observed in other tissues, including heart (24, 31), vascular smooth muscle (25, 35), and kidney (14, 26). Several different upstream signaling pathways may link AT1R activation to phosphorylation of p38 MAPK or ERK1/2. The Src family of protein tyrosine kinases has been shown to play a key role in the coupling of cell surface receptors with MAPK activation (14, 27).

For this reason, ACE and ACE2 expression in neurons and glial cells inside the blood-brain barrier is important. On the other hand, ACE2 is also present in brain centers that control cardiovascular function and is associated with AT1R expression (8, 16). In the present study, we demonstrated that ACE and ACE2 are expressed in the cell membrane and cytoplasm of CATH.a neurons and that ANG II modulates these two enzymes in opposite directions. The subcellular localization of ACE2 agrees with previous in vitro studies (8, 13); however, it is not clear if ACE is localized in the cytoplasm as well as in the membrane. The increase in the ratio of ACE to ACE2 correlates with results in animal models of heart failure or hypertension (1, 13). This mechanism may serve a positive-feedback function in hyperactivated central ANG II signaling and exacerbation of the diseases through augmented sympathoexcitation. It has previously been shown that knocking down the expression of the AT1aR causes downregulation of ACE2 in the brain stem of normal mice without affecting ACE expression (16). We speculate that the effects of the AT1R on ACE and ACE2 expression may be different in the basal state and following ANG II stimulation, since some of our findings are statistically different only at high doses of ANG II, which may trigger a series of signaling pathways.

In the current study we elucidated an AT1R-p38 MAPK and ERK1/2 signaling pathway in the modulation of ACE and ACE2 expression in neurons. Central AT1R-dependent phosphorylation of p38 MAPK or ERK1/2 in the subfornical organ, PVN, and rostral ventrolateral medulla has been reported previously and has been associated with increased oxidative stress and hyperactivated ANG II-AT1R signaling in hypertension and heart failure (5, 33, 38). Activation of p38 MAPK and ERK1/2 by ANG II has also been observed in other tissues, including heart (24, 31), vascular smooth muscle (25, 35), and kidney (14, 26). Several different upstream signaling pathways may link AT1R activation to phosphorylation of p38 MAPK or ERK1/2. The Src family of protein tyrosine kinases has been shown to play a key role in the coupling of cell surface receptors with MAPK activation (14, 27) and NADPH oxidase-derived O2·−, which also serves as a second messenger for MAPK activation (14, 27).

Our laboratory has shown that ANG II induces O2·− production through AT1R and NADPH oxidase activation in CATH.a neurons. It has previously been shown that knocking down the expression of the AT1aR causes downregulation of ACE2 in the brain stem of normal mice without affecting ACE expression (16). We speculate that the effects of the AT1R on ACE and ACE2 expression may be different in the basal state and following ANG II stimulation, since some of our findings are statistically different only at high doses of ANG II, which may trigger a series of signaling pathways.

In the current study we elucidated an AT1R-p38 MAPK and ERK1/2 signaling pathway in the modulation of ACE and ACE2 expression in neurons. Central AT1R-dependent phosphorylation of p38 MAPK or ERK1/2 in the subfornical organ, PVN, and rostral ventrolateral medulla has been reported previously and has been associated with increased oxidative stress and hyperactivated ANG II-AT1R signaling in hypertension and heart failure (5, 33, 38). Activation of p38 MAPK and ERK1/2 by ANG II has also been observed in other tissues, including heart (24, 31), vascular smooth muscle (25, 35), and kidney (14, 26). Several different upstream signaling pathways may link AT1R activation to phosphorylation of p38 MAPK or ERK1/2. The Src family of protein tyrosine kinases has been shown to play a key role in the coupling of cell surface receptors with MAPK activation (14, 27) and NADPH oxidase-derived O2·−, which also serves as a second messenger for MAPK activation (14, 27).
neurons (21). Therefore, $O_2^-$ may also participate in the regulation of ACE and ACE2 expression. In the current study we used a relatively high concentration of ANG II (300 nM), and the changes in ACE and ACE2 proteins occurred at 24 h after administration of ANG II. This suggests that this effect may not occur under physiological conditions but may reflect disease states such as heart failure or hypertension, where ANG II levels in the brain may be high. It is highly possible that ANG II concentration in the medium was not sustained at the given dose during the treatment. We speculate that ANG II may trigger a series of downstream effects, including oxidative stress, that modulate cell function for a longer period of time. We observed changes in ACE and ACE2 mRNA levels within 4 h. However, changes in protein expression were not detected until 8 h after ANG II was added (data not shown) and were still observed after 24 h.

The influences of the AT$_2$R on MAPK activation are different from those for the AT$_1$R. AT$_1$R activation induces sustained activation of ERK1/2 in the NG-108 neuronal cell line (28). However, it has been shown to play an inhibitory role in ERK1/2 activation in cardiomyocytes during ischemia-reperfusion injury (37). We did not measure AT$_1$R and AT$_2$R expression in the current study. Our previous work clearly showed that ANG II upregulates AT$_1$R transcription and protein expression at 100 nM within 4 h in this neuronal cell line (21, 22). Recent work by Herrera et al. (11) has called into question the validity of AT$_1$R measurements, at least in mice, because of nonspecificity of commercial antibodies. While it is certainly true that many AT$_1$R antibodies may not be completely specific, data from Gao et al. (9) clearly show that the use of a blocking peptide abolished the AT$_1$R band in the Western blot in the brain. However, the effect of ANG II on AT$_2$R expression is not as clear in CATH.a neurons. Functional AT$_2$R effects have been observed, in that AT$_2$R-specific agonists increased potassium current in these neurons (10). In the current study the AT$_2$R antagonist PD-123319 did not significantly alter the effects of ANG II on ACE and ACE2 expression, whereas losartan reversed the effects of ANG II, strongly suggesting a dominant AT$_1$R mechanism.

One of the limitations of the current study is that it is still unclear which transcriptional mechanisms downstream of p38 MAPK and ERK1/2 participate in the modulation of ACE and ACE2. Our laboratory has shown that several transcription factors, including activator protein-1, NF-$\kappa$B, and cAMP responsive element-binding protein, are involved in the regulation of AT$_1$R expression in response to ANG II (20, 22; unpublished observations). Similar factors may also be involved in the regulation of both converting enzymes. Hypoxia-
inducible factor 1α has been shown to inhibit ACE2 and promote ACE expression in pulmonary artery smooth muscle cells during hypoxic pulmonary hypertension (41). A study by Cardinale et al. (2) showed that chronic subcutaneous infusion of ANG II resulted in an increase in ACE and a decrease in ACE2 expression in the PVN, which were normalized by inhibition of NF-kB. Another recent study found that an upregulation of ACE in local tissues (heart, kidney, and aorta) of spontaneously hypertensive rats via histone code modifications, which was normalized by the AT1R antagonist valsartan (15). In addition, although mRNA and protein levels were measured in these experiments, the enzyme activities need to be determined. Finally, whether this mechanism is operative in vivo is not known. Because of the complex interaction among RAS components, it is technically challenging to dissect and test one of the RAS components in vivo without affecting the others.

In conclusion, the current findings suggest that ACE and ACE2 expression is regulated by ANG II via AT1R and p38 MAPK and ERK1/2 signaling pathways in CATH.a neurons. This may help explain the bidirectional ACE and ACE2 expression in the central nervous system in heart failure and hypertension.

REFERENCES

The final version of the manuscript; I.H.Z. is responsible for conception and K.K.V.H. prepared the figures; L.X. drafted the manuscript; L.X. and K.K.V.H. prepared the figures; L.X. drafted the manuscript; L.X. and I.H.Z. edited and revised the manuscript; L.X., K.K.V.H., and I.H.Z. approved the final version of the manuscript; I.H.Z. is responsible for conception and design of the research.

GRANTS

This work was funded by National Heart, Lung, and Blood Institute Grants P01 HL-62222 and F32 HL-116172-01 to K. K. V. Haack.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.X. and K.K.V.H. performed the experiments; L.X. and K.K.V.H. analyzed the data; L.X. and I.H.Z. interpreted the results of the experiments; L.X. and K.K.V.H. prepared the figures; L.X. drafted the manuscript; L.X. and I.H.Z. edited and revised the manuscript; L.X., K.K.V.H., and I.H.Z. approved the final version of the manuscript; I.H.Z. is responsible for conception and design of the research.

REFERENCES


