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ORIGINAL ARTICLE

The counterregulating role of ACE2 and ACE2-mediated angiotensin 1–7 signaling against angiotensin II stimulation in vascular cells

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To clarify the role of endogenous angiotensin (Ang)-converting enzyme 2 (ACE2) and its cleavage product, Ang 1–7, in the atherogenic stimulation of vascular cells, we investigated the effect of pharmacological inhibition of ACE2 and Mas, an Ang 1–7 receptor, on cellular responses against Ang II stimulation. We measured extracellular signal-regulated kinase (ERK) 1/2 phosphorylation by western blot, smooth muscle cell (SMC) proliferation by WST assay and the adhesion of monocytes labeled with PKH67 to endothelial cells (ECs) by fluorescence microplate reader. Cells were pretreated with Ang 1–7, olmesartan (Ang II type 1 receptor (AT1) blocker), DX600 (ACE2 inhibitor), D-Ala7-Ang1–7 (D-Ala; Mas antagonist), or combinations of treatments before the application of Ang II. Treatment with Ang II increased phosphorylated ERK 1/2 of SMC and EC, proliferation of SMC and adhesion of monocyte to EC, which were blocked by olmesartan. Pretreatment with DX600 either did not accelerate or only slightly accelerated these cellular responses. However, when Ang II signaling through AT1 was reduced by olmesartan, the additional treatment with DX600 significantly blunted some of the effect of olmesartan. Similarly, pretreatment with D-Ala reduced the inhibitory effect of olmesartan in response to Ang II stimulation. Endogenous ACE2 in vascular cells may contribute to counteracting the Ang II-mediated cellular response partly by upregulating the Ang 1–7 signaling through Mas.
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Keywords: ACE2; adhesion; angiotensin 1–7; cell proliferation; Mas

INTRODUCTION

The proliferation of vascular smooth muscle cells (VSMCs) and the dysfunction and apoptosis of endothelial cells (ECs) characterize the initial stages of the atherosclerotic disease process. [1] These pathophysiological changes in ECs and VSMCs are enhanced by an interaction with local autocrine, paracrine and intracrine factors, which promote local inflammatory reactions. Angiotensin II (Ang II), which is a major regulator of blood pressure by regulation of resistant vessels and sodium retention, is one of the most important factors involved in the promotion of the atherogenic response in vessel walls. Ang II binds to Ang II type 1 receptor (AT1) and exhibits atherogenic activities, such as cell proliferation or differentiation, through the activation of the extracellular-regulated kinase (ERK) 1/2.[2]

Recently, the Ang-converting enzyme 2 (ACE2)-Ang 1–7-Mas axis has received researchers' attention as a counterregulator of the ACE-Ang II-AT1 axis. ACE2 is expressed in vascular cells, including VSMCs and ECs, and was reported to be upregulated in the aorta of the spontaneous hypertensive rat in response to Ang receptor blocker (ARB) treatment.[3] In the renin–Ang system, ACE2 functions as a mono-coxypeptidase by mainly contributing to the generation of Ang 1–7 from Ang II, which shows a much higher catalytic efficiency than the conversion of Ang I to Ang 1–9.[4] Many studies have shown the pivotal role of Ang 1–7 in the cardiovascular system by the exogenous administration of the peptide into mammalian cells or animals,[5–8] including exerting an antiproliferative effect on SMCs[8] and counter-regulating of Ang II signaling in ECs.[7] Recently, a G-protein-coupled receptor, Mas, was identified as a functional receptor of Ang 1–7.[9] It has been reported that Mas-deficient mice with a FVB/N genetic background exhibit both an elevation in blood pressure and endothelial dysfunction, suggesting that the physiological role of Ang 1–7 signaling in maintaining vascular homeostasis is mediated through Mas.[10] This accumulating evidence suggests that ACE2 in vascular walls may protect from atherosclerosis not only by reducing Ang II signaling but also by promoting Ang 1–7 signaling through Mas. However, it remains unclear whether and how the endogenously expressed ACE2 in vascular cells has a protective role against atherogenic stimulation. Thus, in this study, we used pharmacological inhibition of ACE2 or Mas expressed in vascular cells to investigate the physiological role of endogenous ACE2 and ACE2-mediated Ang 1–7 signaling in atherogenic stimulation by Ang II.

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METHODS

Cell culture

A10 cells (rat thoracic VSMCs), human umbilical vein ECs (HUVECs) and THP-1 cells (human acute monocytic leukemia cell line) were obtained from Dainippon Sumitomo Pharma (Osaka, Japan). The growth medium included Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) and antibiotics (Gibco BRL, Gaithersburg, MD, USA) for A10 cells, endothelial basal medium 2 Bulletkit (Nissui Pharmaceutical, Tokyo, Japan) for HUVECs and RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics for THP-1 cells. Cell passages 8–14 for A10 cells and 4–8 for HUVECs were used for all experiments.

Quantitative real-time reverse transcriptase-PCR

RNA from VSMCs and HUVECs was purified using an RNA isolation protocol (SV Total RNA Isolation System, Promega, Madison, WI, USA). A High-Capacity cDNA Reverse Transcription kit was used according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA) to synthesize complementary DNA. Quantitative PCR was carried out using real-time detection technology and analyzed on a model 7900 Sequence Detector (Applied Biosystems) with specific primers and fluorescent probes for ACE2 and Mas (TaqMan Gene Expression Assays, Applied Biosystems). Levels of mRNA were compared at various time points after normalization to concurrent 18S rRNA amplification.

Protein analysis

After 24 h of serum depletion, subconfluent VSMCs and HUVECs were incubated with Ang II (10^{-7} M; Bachem Bioscience, King of Prussia, PA, USA) for 10 min followed by immunoblot assays for phosphorylated ERK 1/2. In certain experiments, cells were pretreated with either Ang 1–7 (10^{-7} M; Bachem Bioscience), an ARB; olmesartan (10^{-6} M; Sankyo Laboratories, Rahway, Japan), an ACE2 inhibitor; DX600 (10^{-6} M; Phoenix Pharmaceuticals, Belmont, CA, USA), a Mas receptor antagonist; D-Ala7-Ang 1–7 (D-Ala; 10^{-5} M; Bachem Bioscience) or a combination for 10–20 min before the application of Ang II. The phosphorylation state of ERK 1/2 was measured by western blotting. Cells were lysed in a buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% w/v sodium dodecyl sulfate, 10% glycerol, 50 mM DTT and 0.1% w/v bromophenol blue. The lysate was then sonicated for 5 s and boiled for 5 min, followed by centrifugation at 12 000 *g* for 5 min to remove insoluble debris. Proteins were electrophoretically transferred to nitrocellulose filters. The filters were blocked in Tris-buffered saline solution containing 1% Tween 20 and 5% non-fat dried milk. The filters were washed and incubated with primary antibodies. The primary antibodies used were rabbit polyclonal antiphosphorylated extracellular signal-regulated protein kinase (ERK) antibody and rabbit polyclonal antiphospho ERKs antibody (Cell Signaling Technology, Danvers, MA, USA). Filters were thoroughly washed in Tris-buffered saline solution containing 1% Tween 20 and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare Life Sciences, Waukesha, WI, USA). Bands were visualized using an enhanced chemiluminescence system (GE Healthcare Life Sciences).

Cell proliferation assay

The proliferation of VSMCs stimulated by Ang II was determined by estimating viable cells with the Cell Counting Kit-8 (Dojindo, Japan). VSMCs in 100 µl medium were seeded onto a 96-well microplate at 10 000 cells per well and were incubated overnight at 37 °C. Then, the cells were treated with 10^{-7} M Ang 1–7, 10^{-6} M olmesartan, 10^{-6} M DX600, or 10^{-5} M D-Ala or a combination of treatments for 60 min before the application of Ang II. After incubation for 24 h, 10 µl of Cell Counting Kit-8 solution was added to each well, and the plates were further incubated for 4 h at 37 °C. The absorbance at 450 nm was measured with an ImmunoReader NJ-2000.

THP-1 preparation and adhesion assay of THP-1 cells to HUVECs

Staining of THP-1 cells with PKH67 was carried out according to the manufacturer's instructions (Sigma). Before the application of Ang II, HUVECs were treated with 10^{-7} M Ang 1–7, 10^{-6} M olmesartan, 10^{-6} M DX600, or 10^{-5} M D-Ala or a combination of treatments for 60 min. The stained THP-1 cells and HUVECs were activated by exposure to Ang II (10^{-7} M) for 4 h. Then, the THP-1 cells were seeded onto confluent HUVECs monolayers. After 10 min, non-adherent cells were washed off three times with phosphate-buffered saline. Finally, we counted adherent THP-1 cells with fluorescence microscopy using two different filters: excitation at 490 nm and emission at 502 nm.

Statistical analysis

Data were analyzed with Stat View version 4.51. and are presented as mean ± s.e.m. Statistical analysis was carried out using analysis of variance with Fisher's *post-hoc* comparison. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effects of ACE2 and Ang 1–7 on phosphorylation of ERK 1/2 stimulated by Ang II

We confirmed ACE2 and Mas expression from RNA isolated from VSMCs and HUVECs by real-time PCR, and the expression levels of these genes were not altered by any pharmacological treatment used in this study (data not shown). To clarify the role of endogenous ACE2 and its cleavage product Ang 1–7 in Ang II-induced mitogen-activated protein kinase activation, VSMCs and HUVECs were stimulated by Ang II and were pharmacologically inhibited by AT1 (olmesartan), ACE2 (DX600) and Mas (D-Ala).

In both VSMCs and HUVECs, the stimulation by Ang II caused significant increase in ERK 1/2 phosphorylation, which was blocked by pretreatment with olmesartan (Figures 1a and b, lanes B and D). DX600 did not significantly alter the phosphorylation of ERK 1/2 stimulated by Ang II (Figures 1a and b, lane C). However, when AT1 was blocked by olmesartan, the additional treatment with DX600 significantly increased ERK 1/2 activation (Figures 1a and b, lane E). This effect was blunted in response to Ang 1–7 treatment, and this blunting was diminished by the Mas blockade of D-Ala (Figures 1a and b, lanes F and H). D-Ala partly reversed the inhibitory effect of olmesartan on Ang II-induced ERK 1/2 phosphorylation, suggesting that Ang 1–7-mediated Mas activation might be a negative regulator of Ang II-mediated mitogen-activated protein kinase activation (Figures 1a and b, lane G).

Effects of ACE2 and Ang 1–7 on proliferation of SMCs

The WST assay was carried out using the Cell Counting Kit-8 to investigate the involvement of ACE2-Ang 1–7-Mas axis in Ang II-mediated VSMCs proliferation. Incubation of VSMCs with Ang II for 24 h caused a significant increase in the cell numbers compared with the control VSMCs, which was completely inhibited by pretreatment with olmesartan (Figure 2, lanes B and D). Pretreatment with DX600 slightly but significantly accelerated the Ang II-mediated proliferation (Figure 2, lane C).

The inhibition of ACE2 by DX600 partly blunted the inhibitory effect of olmesartan on Ang II-mediated proliferation of SMCs (Figure 2, lane E). This effect of DX600 was reduced by the additional treatment with Ang 1–7, which was blocked by D-Ala (Figure 2, lanes F and H). Inhibition of Ang 1–7 signaling by D-Ala partly reversed the inhibitory effect of olmesartan against Ang II-mediated cell proliferation (Figure 2, lane G).

Effects of ACE2 and Ang 1–7 on adhesion of monocyte adhesion to ECs

We investigated the co-culture of THP-1 cells and HUVECs to evaluate the effects of inflammatory cell adhesion to ECs. The co-culture of THP-1 cells and HUVECs after Ang II stimulation caused a significant increase in adherent THP-1 cells compared with control, which was blocked by olmesartan (Figure 3, lanes B and D).

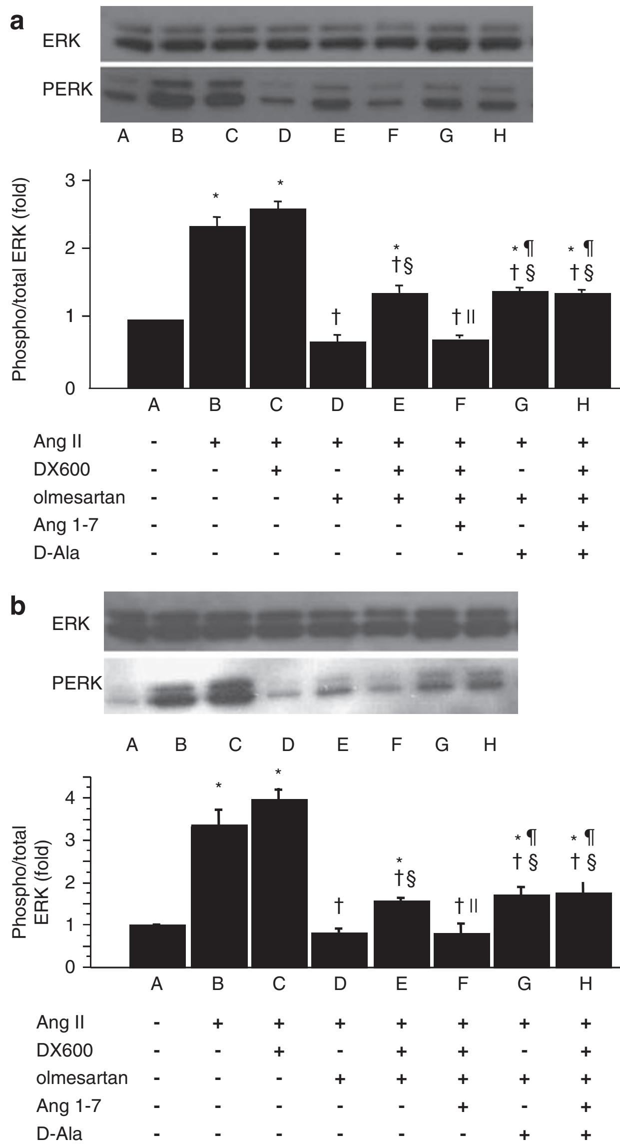

Figure 1 (**a**) Phosphorylation of ERK 1/2 in VSMCs stimulated by Ang II. A representative western blot is shown above the graph. (**b**) Phosphorylation of ERK 1/2 in HUVECs stimulated by Ang II. A representative western blot is shown above the graph (n=3, each). *$P<0.01$ *vs.* lane A, †$P<0.01$ *vs.* lane B, lane C, §$P<0.01$ *vs.* lane D, ‖$P<0.01$ *vs.* lane E, ¶$P<0.01$ *vs.* lane F.

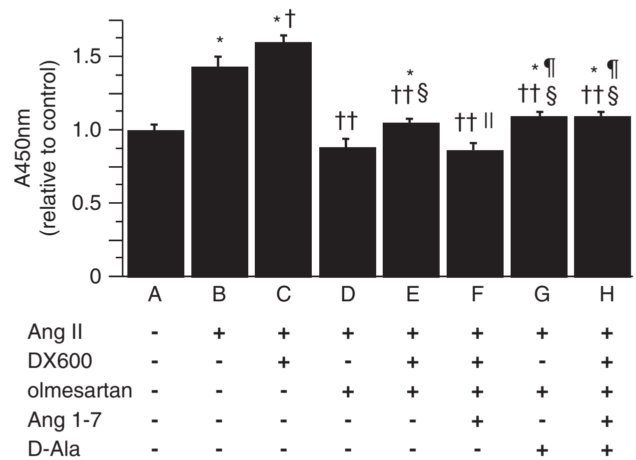

Figure 2 Cell proliferation in VSMCs stimulated by Ang II. The WST assay was carried out using the Cell Counting Kit-8, and absorbance at 450 nm was measured as an indicator of cell proliferation (n=5, each). *$P<0.01$ *vs.* lane A, †$P<0.05$ *vs.* lane B, ††$P<0.01$ *vs.* lane B, lane C, §$P<0.05$ *vs.* lane D, ‖$P<0.05$ *vs.* lane E, ¶$P<0.05$ *vs.* lane F.

Pretreatment with DX600 did not alter the Ang II mediated cell adhesion (Figure 3, lane C). However, under the inhibition of Ang II signaling by olmesartan, DX-600 obviously increased the adherent THP-1 cells (Figure 3, lane E). Ang 1–7 blocked the reversing effect of DX600, and the effect of Ang 1–7 was reduced by the Mas blockade of D-Ala (Figure 3, lanes F and H). D-Ala partly reduced the antiadherent effect of olmesartan in response to Ang II stimulation (Figure 3, lane G).

DISCUSSION

This study was designed to investigate the role of endogenous ACE2 and its cleavage product Ang 1–7 in cell signaling and subsequent atherogenic responses in Ang II-stimulated vascular cells. The single treatment of an ACE2 inhibitor had little effect on Ang II-mediated ERK phosphorylation, proliferation of VSMCs and monocyte adhesion to HUVECs. However, when AT1 signaling was reduced by the administration of ARB, the inhibition of ACE2 significantly reversed some of the suppressing effects of these Ang II-induced cellular responses. The activation of Ang 1–7-mediated Mas signaling might be involved in the observed effect of ACE2, as indicated by the results of Mas inhibition or Ang 1–7 administration (Figure 4).

The inhibitory effect of Ang 1–7 on Ang II stimulation has been supported by several previous studies using the exogenous administration of the peptide into vascular cells. Freeman *et al.*[8] reported that Ang 1–7 inhibited the proliferation of VSMCs induced by Ang II. In human ECs, Ang 1–7 counterregulated the Ang II–induced phosphorylation of c-Src, ERK 1/2 and SHP-2, and the activation of reduced form of nicotinamide adenine dinucleotide phosphate oxidase by Mas receptor.[7] Thus, it was postulated that endogenous ACE2 in vascular cells might have some protective effects in Ang II-induced vascular injury by producing Ang 1–7 from Ang II. Our results showed that the effect of ACE2 inhibition in the Ang II-induced cellular response was revealed only when the Ang II signaling was reduced by ARB, possibly because of the relatively small amount of Ang 1–7 produced by ACE2 compared with the concentration of the peptide used in previous reports. It has been reported that treatment with ARB increased ACE2 expression in vascular walls;[3] however, ACE2 mRNA expression was not altered by administration of olmesartan in our study probably because of the short duration of treatment. A long ARB-treatment period might enhance the role of ACE2 by increasing Ang 1–7 production.

When we considered the function of ACE2 *in vivo*, it should be noted that the catalytic property of ACE2 is not limited to the renin–Ang system.[4] In particular, apelin 13 and apelin 36, which are hydrolyzed and rendered inactive by ACE2, have recently received researchers' attention because these peptides contribute to protection from the development of cardiovascular dysfunction through their receptor apelin.[11] Thus, the reduction of apelin by ACE2 might counteract its beneficial role in the renin–Ang system in vascular walls. Studies using genetic disruption or pharmacological inhibition of ACE2 in atherosclerotic animals might be useful to determine its physiological role in atherosclerosis.

There are several limitations of this study. First, we used DX600 and D-Ala to inhibit ACE2 and Mas, respectively. Although the concentrations of these agents used in this study were not expected to induce

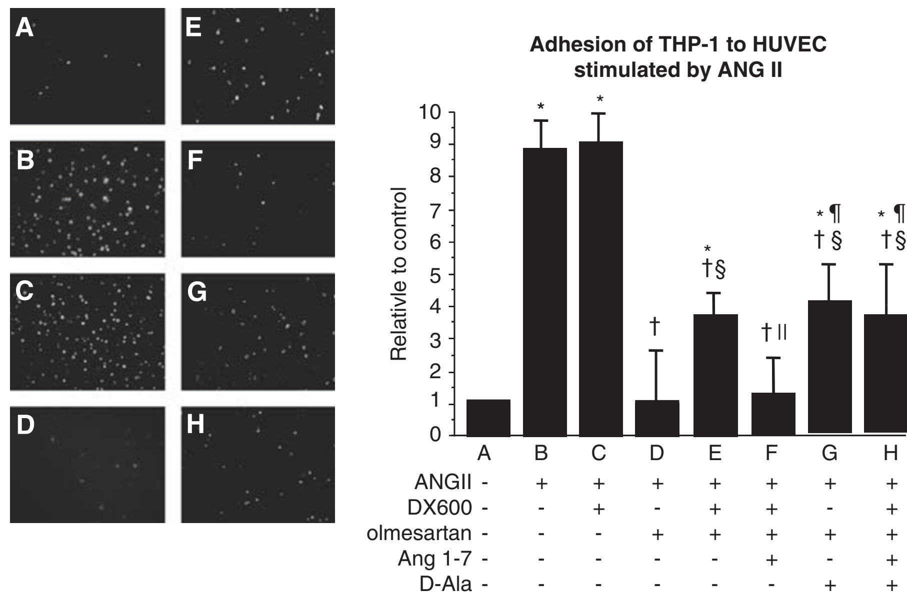

Figure 3 Adhesion of THP-1 cells to HUVECs after stimulation with Ang II. A representative microscopic photograph is shown to the left of the graph (original magnification ×400) (n=3, each). *P<0.01 vs. lane A, †P<0.01 vs. lane B, lane C, §P<0.05 vs. lane D, ‖P<0.05 vs. lane E, ¶P<0.05 vs. lane F.

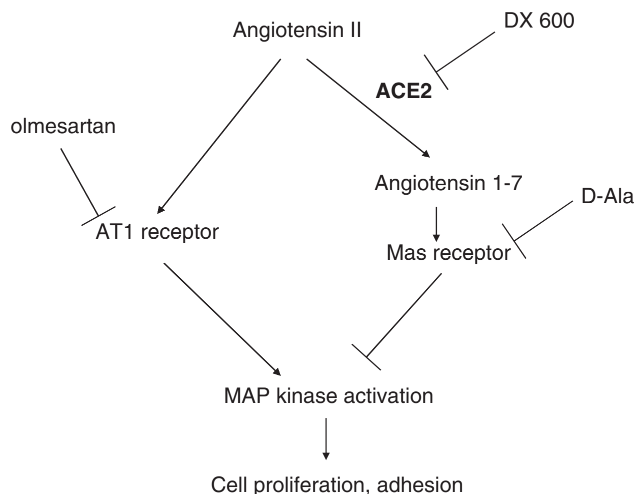

Figure 4 Summary of the overall mechanism observed in the present study. Endogenous ACE2 in vascular cells might contribute to the inhibition of AT1 receptor-induced mitogen-activated protein kinase activation and subsequent cellular proliferation and adhesion by upregulating the Ang 1–7-Mas pathway.

non-specific effects to cells, a molecular assessment such as one with small interfering RNA needs to be carried out to evaluate the specific effects of these genes. Second, we used A10, HUVECs and THP-1 in this study; the phenotypes of these cell lines differ somewhat from those of primary cells. Freshly isolated cells from animals are more desirable than cell lines for investigating physiological effects.

In conclusion, endogenous ACE2 in vascular cells might counteract Ang II-induced cellular signaling and atherogenic responses by increasing Ang 1–7 at least when ARB is administered. Further investigation would be required to determine whether ACE2 has protective effects against the development of atherosclerosis *in vivo*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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