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Effect of angiotensin II on matrix metalloproteinase-2 secretion in human umbilical vein endothelial cells

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Abstract:	Matrix metalloproteinase (MMP), which is secreted from vascular cells, is an enzyme degrading extracellular matrix protein. MMP molecules, including MMP-2, are involved in the destabilization of atherosclerotic plaque and plaque rupture during the development of cardiovascular disease (CVD). Angiotensin II (Ang-II), a vascular stimulant associated with CVD progression, has been demonstrated to be mainly involved in cardiovascular remodeling of atherosclerosis and cardiac hypertrophy. This study was performed to investigate the regulation of MMP-2 by Ang-II in human umbilical vein endothelial cells (HUVECs). Ang-II significantly increased MMP-2 secretion and MMP-2 messenger RNA expression in HUVECs. The effects of Ang-II were suppressed by the coexistence of telmisartan, a blocker of the Ang-II receptor type 1 (AT1 receptor), or PD123319, a blocker of Ang-II receptor type 2 (AT2 receptor). Especially, PD123319 showed marked suppression of the effect of Ang-II on MMP-2. Therefore, Ang-II-induced upregulation of MMP-2 in HUVECs was considered to be mainly achieved through AT2 receptors, although AT1 and AT2 receptors were expressed in HUVECs, but the detailed mechanisms remain undefined. These findings suggests that Ang-II can enhance MMP-2 mainly through AT2 receptors in endothelial cells, but the significance of circulating MMP-2 as a cardiovascular biomarker requires confirmation in further clinical studies.
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Professor Michael R. Rosen, MD
Editor-in-chief, Journal of Cardiovascular Pharmacology

Dear Editor,
RE: JCVP-17-415R2

Thank you for your favorable evaluation and helpful comments
I wish to submit an original article as a revised version (JCVP-17-415R2) for publication in *Journal of Cardiovascular Pharmacology*, titled "Effect of angiotensin II on matrix metalloproteinase-2 secretion in human umbilical vein endothelial cells." The paper was coauthored by Ryo Sato, MSci, Chika Hiraishi, MD.

The resubmitted manuscript has been re-revised in response to comments of Editor and Reviewer 2. This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. All authors have read and approved the manuscript. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. Details about competing interests are provided separately. I would like to recommend potential reviewers as shown in the on-line submission system.

Thank you for your consideration. I look forward to hearing from you.

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Thank you for your comments and suggestions to our paper. We would response to your comments item by item as follows.

To reviewer #1:
No further comments

Thank you for your favorable comments.

To reviewer 2:

1. The reviewer understands that 20pg/mL of Ang II was added with telmisartan and with PD 123319. However the question was the concentration of Ang II similar in the telmisartan and PD 123310 experiments.

Thank you for your helpful comments.
The concentration of Ang II was similar in the telmisartan and PD 123310 experiments.

Page 9, lines 21-23 in the revised manuscript:
Besides, it was of interest that the increased MMP-2 secretion by the Ang-II incubation at 20 pg/mL for 24 hours was markedly suppressed by the treatment of 0.1-μM PD123319, a selective AT2 receptor blocker, in HUVECs (Figure 5B).

2. Besides the addition of Ang II did telmisartan induce release of Ang II by HUVECs ? Otherwise the reviewer appreciates that the study limitations are clearly underlined in the revised manuscript

Thank you for your comments. We would describe the discussion for the study limitations in this regard as follows (Page 13, lines 4-10).

Furthermore, beside the addition of Ang II, whether telmisartan induced the release of Ang II by HUVEC remains to be elucidated because the concentration of Ang-II was not measured in the experimental medium. The inhibitory effect of telmisartan on Ang-II induced MMP-2 production was not significantly different between the concentrations of telmisartan (0.5, 1.0 and 2.0 μM). It might suggest that telmisartan could not induce the release of Ang-II by HUVECs.

Abstract

Matrix metalloproteinase (MMP), which is secreted from vascular cells, is an enzyme degrading extracellular matrix protein. MMP molecules, including MMP-2, are involved in the destabilization of atherosclerotic plaque and plaque rupture during the development of cardiovascular disease (CVD). Angiotensin II (Ang-II), a vascular stimulant associated with CVD progression, has been demonstrated to be mainly involved in cardiovascular remodeling of atherosclerosis and cardiac hypertrophy. This study was performed to investigate the regulation of MMP-2 by Ang-II in human umbilical vein endothelial cells (HUVECs). Ang-II significantly increased MMP-2 secretion and MMP-2 messenger RNA expression in HUVECs. The effects of Ang-II were suppressed by the coexistence of telmisartan, a blocker of the Ang-II receptor type 1 (AT1 receptor), or PD123319, a blocker of Ang-II receptor type 2 (AT2 receptor). Especially, PD123319 showed marked suppression of the effect of Ang-II on MMP-2. Therefore, Ang-II-induced upregulation of MMP-2 in HUVECs was considered to be mainly achieved through AT2 receptors, although AT1 and AT2 receptors were expressed in HUVECs, but the detailed mechanisms remain undefined. These findings suggests that Ang-II can enhance MMP-2 mainly through AT2 receptors in endothelial cells, but the significance of circulating MMP-2 as a cardiovascular biomarker requires confirmation in further clinical studies.

Effect of angiotensin II on matrix metalloproteinase-2 secretion in human umbilical vein endothelial cells

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Conflict of interest disclosures:

Professor Hiroshi Yoshida received honoraria for speaking activities from Astellas, Amgen, Bayer, Kowa, Mochida, MSD, Sanofi, Shionogi, and Takeda , but these were not related to this study. Ryo Sato and Chika Hiraishi have no potential conflict of interest to disclose.

Abstract

Matrix metalloproteinase (MMP), which is secreted from vascular cells, is an enzyme degrading extracellular matrix protein. MMP molecules, including MMP-2, are involved in the destabilization of atherosclerotic plaque and plaque rupture during the development of cardiovascular disease (CVD). Angiotensin II (Ang-II), a vascular stimulant associated with CVD progression, has been demonstrated to be mainly involved in cardiovascular remodeling of atherosclerosis and cardiac hypertrophy. This study was performed to investigate the regulation of MMP-2 by Ang-II in human umbilical vein endothelial cells (HUVECs). Ang-II significantly increased MMP-2 secretion and MMP-2 messenger RNA expression in HUVECs. The effects of Ang-II were suppressed by the coexistence of telmisartan, a blocker of the Ang-II receptor type 1 (AT1 receptor), or PD123319, a blocker of Ang-II receptor type 2 (AT2 receptor). Especially, PD123319 showed marked suppression of the effect of Ang-II on MMP-2. Therefore, Ang-II-induced upregulation of MMP-2 in HUVECs was considered to be mainly achieved through AT2 receptors, although AT1 and AT2 receptors were expressed in HUVECs, but the detailed mechanisms remain undefined. These findings suggests that Ang-II can enhance MMP-2 mainly through AT2 receptors in endothelial cells, but the significance of circulating MMP-2 as a cardiovascular biomarker requires confirmation in further clinical studies.

Key words: angiotensin II, Ang-II receptor type 2, human umbilical vein endothelial cells, matrix metalloproteinase-2

Introduction

Cardiovascular disease (CVD) is one of the leading causes of death worldwide.[1,2] Among CVDs, acute coronary syndrome (ACS) is considered a disease spectrum ranging from unstable angina to acute myocardial infarction and sudden death.[3,4] Biomarkers reflecting the pathological stages of the disease process of CVD have been studied to establish analysis of the pathological condition, risk stratification, selection of therapeutic drugs, and evaluation of therapeutic efficacy.[4-7] Matrix metalloproteinase (MMP), a kind of enzyme that degrades the extracellular matrix, is secreted from the vessel wall cells. It has been revealed that MMP contributes to atherosclerotic plaque vulnerability and ventricular remodeling relevant to heart failure.[5-6] Various inflammatory cytokines are expected to be biomarkers for diagnosis, therapeutic decision-making, and prognostic prediction of CVD because the highly sensitive detection of plaque formation and rupture with the relevant cytokines may reflect pathological features of CVD.[4,5] In MMP subtypes, gelatinases (MMP-2 and MMP-9) that selectively degrade the basement membrane of vascular cells are primarily involved in the remodeling of cardiovascular lesions, and the stromelysin group (MMP-3) that activates these gelatinases may be also involved.[8-11]

Angiotensin II (Ang-II), a vasopressor substance, has been demonstrated to be mainly involved in cardiovascular remodeling of cardiac hypertrophy and atherosclerosis through the regulation of cell proliferation and extracellular matrix production.[12,13] The renin-angiotensin-aldosterone system (RAS), including Ang-II was found as a vasopressor system, and recently, the role of RAS in the vascular tissue is considered important in the formation of atherosclerotic lesions in addition to the regulation of

blood pressure.[12,13] The effects of RAS function exert the induction of inflammatory reactions, endothelial dysfunction, and cell proliferation in the cardiovascular system.[12-14] RAS is involved in the exacerbation of atherosclerotic lesions through many mechanisms, and it plays an important role in the onset and subsequent progression of CVD.[15] Monocytes and macrophages produce angiotensin converting enzyme (ACE) in the atherosclerotic lesions, and Ang-II enhances ACE production through the increased inflammation and subsequently increases Ang-II locally leading to the activation of Ang-II receptors. Furthermore, these ACE and Ang-II, expressed in the marginal region of atherosclerotic plaques, are suggested to possibly facilitate the plaque destabilization (13).

Stimulating effects of Ang-II on cardiovascular cells are mediated mainly by the Ang-II receptor type 1 (AT1 receptor). However, physiological functions of Ang-II receptor type 2 (AT2 receptor) remains to be clarified, but this receptor has been reported to exert vasodilation and anti-proliferation of cells in contrast to the AT1 receptor, which induces vasoconstriction, cellular proliferation, aldosterone secretion, sodium retention, sympathetic stimulation, cardiac hypertrophy, and the production of reactive oxidation species.[16-18]

Effects of Ang-II on pathological molecules related to CVD have been extensively investigated in smooth muscle cells, but they are not well defined in vascular endothelial cells. In particular, the regulation of gelatinases, including MMP-2, in terms of the effects of Ang-II in vascular endothelial cells is not well defined. Consequently, the expression and secretion of MMP-2 induced by Ang-II in vascular endothelial cells were investigated in the present study.

Methods

The effects of Ang-II on MMP-2 and MMP-9 expressions and secretion were examined in human umbilical vein endothelial cells (HUVECs). HUVECs were cultured at 37°C under the conditions of 5% carbon dioxide in endothelial cell basal medium-2 with a medium reagent set (Cambrex/Lonza, Walkersville, MD, USA) and fetal calf serum (FCS) inactivated for 30 minutes at 56°C. For the experiments, HUVECs (5×10^5 cells/mL) were plated in RPMI-16 medium with inactivated 10% FCS in 12 wells for 24 hours and then stimulated by adding Ang-II at the indicated concentrations and times in the medium. Thereafter, the culture medium was collected, and concentrations of MMP-2 and MMP-9 proteins in the medium were measured by using the Quantikine enzyme-linked immunoabsorbent assay (R&D Systems, Minneapolis, MN, USA).

To test the effects of Ang-II receptor blockers on Ang-II-induced changes in MMP-2 secretion from HUVECs, cultured HUVECs were plated in 12 wells. After 24 hours, they were incubated in the RPMI-1640 culture medium supplemented with the inactivated 10% FCS and the addition of 0.5 μM of telmisartan (Wako Chemical Company, Tokyo, Japan), an AT1 receptor blocker, or 0.1 μM of PD123319 (Abcam, Cambridge, UK), a selective AT2 receptor blocker, to the culture medium. After 1 hour of incubation, the cells were stimulated by Ang-II (20 pg/mL). Then, MMP-2 protein concentrations in the collected culture medium were measured 24 hours later. As a side note, the selectivity of PD 123319 to AT2 receptor was reported previously (24).

To test mRNA expression of MMP-2 and MMP-9, total RNA was extracted from HUVECs cultured in the absence and presence of Ang-II by using TRIzol reagent

(Thermo Fisher Scientific, Waltham, MA, USA), and complement (c)-DNA was synthesized from the RNA by the reverse transcription (RT) reaction. Then RT-polymerase chain reaction (PCR) tests were performed as follows. Real-time PCR was conducted in a 20-µL reaction volume with 0.2 µM of each primer and 2.5 mM of magnesium chloride, using KAPA SYBR® FAST qPCR Kits (Kapa Biosystems, Woburn, MA, USA) and the real-time PCR system 7500Fast (Applied Biosystems, Foster City, CA, USA). The real-time PCR program was composed of thermal denaturation for 10 seconds at 95°C, annealing for 40 seconds at 95°C, elongation reaction for 30 seconds at 60°C, and amplification for 40 cycles. We used the 18s ribosomal RNA as a control.

Alternatively, using the cDNA and specific primers (Table 1) for MMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, the cells were subjected to PCR reaction with Taq DNA polymerase (Takara, Tokyo, Japan) and dNTP mix (Takara) in the Gene Amp PCR system 9700 (Applied Biosystems) for semi-quantitative RT-PCR. The amplification program was followed by heating for 1 minute at 94°C, and then 30 cycles consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C, and extension for 1 minute at 72°C. The amplified products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide, and detected under ultraviolet irradiation. The signal intensity was measured by ODYSSEY FC (LI-COR Biosciences, Lincoln, NE, USA). In addition, the expression of subtypes of Ang-II receptor in HUVECs was also investigated, as aforementioned, except for 3% agarose gel electrophoresis.

Real-time PCR for MMPs was performed using TaqMan mix (Applied Biosystems) and TaqMan probes of MMP-2 (Hs01548727_m1) and MMP-9 (Hs00234579_m1) (Applied

Biosystems). Relative quantification of the gene expression was performed using the comparative cycle threshold (CT) method. The comparative CT [CT(MMP-2)/CT(18sRNA)] was calculated for each sample, and then relative quantification was estimated. The sequences of used primers for semi-quantitative RT-PCR and the sizes of the predicted PCR products are presented in Table 1.

Statistical analysis

The Mann-Whitney U test was used to compare data between the two groups, and a comparison among the three groups was performed by analysis of variance (post-hoc analysis: Fisher-PLSD). Statistical significance was determined at $p<0.05$.

Results

HUVECs were incubated for 24 hours with 5 pg/mL of Ang-II, and consequently, MMP-2 secretion from HUVECs was modestly increased but not significantly. When HUVECs were incubated for 24 hours with 20 pg/mL of Ang-II, MMP-2 secretion was significantly increased (Figure 1A). The time-course study showed that Ang-II incubation at 20 pg/mL significantly increased MMP-2 secretion by approximately 1.5-fold after 8 hours, and it increased even more by 2-fold after 24 hours (Figure 1B). However, the apparent MMP-9 secretion was not observed by incubating Ang-II at 5 or 20 pg/mL for 24 hours (data not shown). To determine why the difference between MMP-2 and MMP-9 was observed in HUVECs despite the same MMP group of gelatinase, mRNA expressions of MMP-2 and MMP-9 were evaluated by real-time PCR. The exponential amplification of MMP-2 mRNA was found, but that of MMP-9 mRNA

was not found (Figure 2).

The real-time PCR study indicated that MMP-2 mRNA expression in HUVECs was significantly increased by the incubation of Ang-II (20 pg/mL), as shown in Figure 3. The average comparative CT value by Ang-II stimulation was significantly decreased from 3.00 ± 0.01 to 2.57 ± 0.33 (Figure 3A). To verify this result, the semi-quantitative RT-PCR study was performed by using GAPDH as an internal control. Signal strength ratios of MMP2/GAPDH of electrophoresis images were measured. The significantly increased expression of MMP-2 mRNA by the stimulation of Ang-II (20 pg/mL) was similarly observed in the real time PCR study (Figure 3B). These results suggest that Ang-II may stimulate MMP-2 expression at the transcriptional level.

Then, which Ang-II receptor subtype was implicated in the effects of Ang-II on MMP-2 in HUVECs was tested by treatment with Ang-II receptor blockers. Additionally, the expression of Ang-II receptor subtypes in HUVECs was investigated, and AT1 and AT2 receptors were found in HUVECs (Figure 4). Since 0.1 to 1 μM was regarded to be optimal concentrations of telmisartan, an AT1 receptor blocker, in cell experiments,[19] 0.5 μM telmisartan was used at first in the present study. The increased MMP-2 secretion by Ang-II incubation at 20 pg/mL for 24 hours was significantly suppressed by the treatment of 0.5-μM telmisartan in HUVECs (Figure 5A). In addition, Inhibitory effects of telmisartan on Ang-II induced MMP-2 production were not significantly different among the concentrations (0.5, 1.0, and 2.0 μM) of telmisartan (data not shown). Besides, it was of interest that the increased MMP-2 secretion by the Ang-II incubation at 20 pg/mL for 24 hours was markedly suppressed by the treatment of 0.1-μM PD123319, a selective AT2 receptor blocker, in HUVECs (Figure 5B). Namely, the

addition of telmisartan suppressed Ang-II-induced upregulation of MMP-2 secretion by 13.7%; however, the addition of PD123319 suppressed that by 65.1%, and the inhibition rate was greater in PD123319 treatment than in telmisartan treatment (Table 2). Hence, Ang-II-induced upregulation of MMP-2 in HUVECs was considered to arise mainly through Ang-II AT2 receptors, although Ang-II AT1 and AT2 receptors were expressed in HUVECs.

Discussion

MMP plays a central role in the degradation of tissue extracellular matrix, leading to arterial plaque rupture and left ventricular remodeling after myocardial infarction, which is attributed to increased MMP-2 and MMP-9 expressions.[20] The expression and function of MMP-2 in vascular endothelial cells are not well defined. MMP-2 belongs to the extracellular matrix degradation enzyme family of end protease, which functions in the degradation of the extracellular matrix in various disease processes.

The role of Ang-II has been considered to maintain homeostasis of the water-electrolyte balance and blood pressure, but recent studies have shown that Ang-II plays an important role in the progression of atherosclerotic lesions.[12] Plaque destabilization develops due to rupture of the fibrous cap and vascular endothelial injury, which is accelerated by the increased MMP expression.[10,11] The relevance of Ang-II to atherosclerotic pathogenesis has been demonstrated in several animal models. In a study with apolipoprotein E knockout mice, the administration of Ang-II was reported to cause the formation of atherosclerosis and induce the formation of abdominal aortic aneurysms.[21] Whether cytokines and adhesion molecules involved in the inflammatory

response of vessel walls are controlled by Ang-II has not been well studied.[22]

The secretion of MMP-2 from HUVECs in response to Ang-II was increased in a dose-response and time-dependent manner. By contrast, there was no significant change in the secretion of MMP-9. This difference between MMP-2 and MMP-9 was supported by real-time PCR data because MMP-2 mRNA showed an exponential amplification pattern but MMP-9 mRNA did not, which is similar to that reported in a previous report.[23] Ang-II may enhance MMP-2 expression at the transcription level because Ang-II increased the MMP-2 mRNA expression in HUVECs.

The present study showed that AT1 and AT2 receptors were expressed as Ang-II receptor subtypes in HUVECs. A previous paper also reported the presence of AT1 and AT2 receptors in endothelial cells.[17,24] Telmisartan can block the AT1 receptor more selectively than the AT2 receptor, and thereby, telmisartan might act mainly on AT1 receptors in HUVECs, resulting in the suppression of the increased MMP-2 expression by Ang-II. The addition of telmisartan suppressed Ang-II-induced upregulation of MMP-2 secretion by 13.7%; however, the addition of PD123319, a selective AT2 receptor blocker, suppressed it even further by 65.1%. Therefore, increased MMP-2 expression and secretion by Ang-II may be driven by Ang-II receptors, especially the AT2 receptor. Ang-II is considered to destabilize atherosclerotic plaque resulting from promoting the degradation of extracellular matrix by MMP increased by oxidative stress (13). Ang-II-induced production of MMP-2 was suppressed by both AT1-blocker and AT2-blocker, and it indicated that Ang-II-induced production of MMP-2 was operated through Ang-II receptors. In addition, inhibition rates of Ang-II-induced production of MMP-2 was marked by the treatment of AT2-blocker in comparison with

AT1-blocker. It suggested that AT2 receptor might be more significant for the regulation of MMP-2 production induced by Ang-II in HUVECs. Otherwise, AT2 receptor might be upregulated by the Ang-II stimulation. Possibly in these manners, AT2-blocker markedly suppressed Ang-II-induced production of MMP-2, but it remains to be cleared.

A previous study showed that Ang-II induces an increase in MMP-2 expression in idiopathic ascending aortic aneurysms via the AT1 receptor and Jun amino-terminal kinase pathway.[25,26] The accumulation of hypoxia-inducible factor (HIF)-1 alpha can induce the expression of vascular endothelial growth factor, which, in turn, may promote increased MMP-2 expression and activity in neighboring endothelial cells.[27] MMP-2 is a key molecule in the destabilization of atherosclerotic plaques through reducing the extracellular matrix and promoting the apoptosis of smooth muscle cell.[28,29] In this pathological process, Ang-II-induced MMP-2 upregulation might evoke vascular inflammation in conjunction with the accelerative release of inflammatory cytokines in HUVECs. Neovascularization mediated by the HIF-1 alpha/MMP-2 pathway might compensate cellular function under the condition of ischemia and hypoxia. In this regard, the AT2 receptor blocking-mediated suppression of MMP-2 upregulation induced by Ang-II might be similar with the improvement of ischemic conditions, but this remains to be clarified. In the JAPAN-ACS report, serum MMP-2 levels were significantly increased in association with changes in the coronary plaque volume.[30] Therefore, serum MMP-2 levels might be a biomarker for coronary atherosclerotic diseases.

The study limitations include unknown mechanisms of AT2 receptor pathway for the regulation of MMP2 in endothelial cells. In the present study, HUVECs were used,

but human aortic endothelial cells (HAoECs) also should be tested to investigate effects of Ang-II on MMP-2 production. In the preliminary experiments, HAoECs provided less clear effects of Ang-II in comparison with HUVECs. Therefore, the experiment protocol should be rearranged when HAoECs are used. Furthermore, beside the addition of Ang II, whether telmisartan induced the release of Ang II by HUVEC remains to be elucidated because the concentration of Ang-II was not measured in the experimental medium. The inhibitory effect of telmisartan on Ang-II induced MMP-2 production was not significantly different between the concentrations of telmisartan (0.5, 1.0 and 2.0 μM). It might suggest that telmisartan could not induce the release of Ang-II by HUVECs. Additionally, the clinical significance of serum MMP2 measurements should be investigated by clinical studies in the future.

Conclusions

The present study shows that Ang-II, which is involved in CVD progression, may enhance MMP-2 mainly through AT2 receptors in vascular endothelial cells. These results also suggest that the vascular endothelial MMP-2 enhanced by Ang-II can be a biomarker for CVDs, but this requires confirmation by further animal and clinical studies.

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Figure legends

Figure 1 Ang-II-induced MMP-2 secretion in cultured HUVECs

(A) Cultured HUVECs (n=4) were incubated in the presence of different concentrations of Ang-II for 24 hours. The data were compared by analysis of variance (post-hoc analysis: Fisher-PLSD) (n=4). Statistical significance was determined at $p<0.05$.

(B) HUVECs were incubated with Ang-II (20 pg/mL) for 8 hours and 24 hours. Then, MMP-2 secretion was measured in collected incubation medium. The MMP-2 secretion was compared between the absence and presence of Ang-II (n=4). The data were compared by the Mann-Whitney U test (n=4). Statistical significance was determined at $p<0.05$.

Ang-II, angiotensin II; HUVECs, human umbilical vein endothelial cells; MMP, metalloproteinase

Figure 2 MMP-2 and MMP-9 mRNA expression in HUVECs

Expressions of MMP-2 and MMP-9 were evaluated by real-time polymeras chain reaction with 18s ribosomal RNA used as an internal control. MMP-2 mRNA was expressed in HUVECs but MMP-9 was not.

MMP, metalloproteinase; mRNA, messenger RNA; HUVECs, human umbilical vein endothelial cells

Figure 3 Ang-II can enhance MMP-2 expression at the transcriptional level

(A) Real-time PCR results of MMP-2 mRNA expression

Cultured HUVECs were incubated with the absence and presence of Ang-II (20

pg/mL) for 24 hours. The data were compared by the Mann-Whitney U test (n=3). Statistical significance was determined at $p<0.05$.

(B) Semi-quantitative RT-PCR results of MMP-2 mRNA expression

RNA was extracted from HUVECs incubated with the absence and presence of 20 pg/mL of Ang-II. After the RT reaction and amplification by PCR, the products were subjected to 1.5% agarose. GAPDH was used as an internal control. The signal intensity MMP-2/GAPDH was determined from electrophoresis images. MMP-2 mRNA was significantly increased by the incubation of 20 pg/mL of Ang-II, which is in line with the results of real-time PCR. The data were compared by the Mann-Whitney U test (n=3). Statistical significance was determined at $p<0.05$.

Ang-II, angiotensin II; MMP, metalloproteinase; PCR, polymerase chain reaction; mRNA, messenger RNA; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Figure 4 RT-PCR results of mRNA expression of AT1 and AT2 receptors

The RT-PCR products for mRNA expression of AT1 and AT2 receptors were subjected to 1.5% agarose gel electrophoresis. The reference represents 100-bp DNA ladder.

RT, reverse transcription; PCR, polymerase chain reaction; mRNA, messenger RNA

Figure 5 Effects of Ang-II on MMP-2 secretion from HUVECs in the pretreatment of Ang-II receptor blockers

HUVECs were plated in 12 wells, and after 24 hours, they were incubated in the

culture medium supplemented with the absence or presence of (A) telmisartan (0.5 μM) or (B) PD123319 (0.1 μM). After 1 hour of incubation, they were stimulated by Ang-II (20 pg/mL). Then, MMP-2 secretion in the collected culture medium was measured 24 hours later. Data between the absence and presence of Ang-II receptor blockers when Ang-II was administered were compared by the Mann-Whitney U test (n=4). Statistical significance was determined at $p<0.05$.

Ang-II, angiotensin II; MMP, metalloproteinase; HUVECs, human umbilical vein endothelial cells

Figure 1

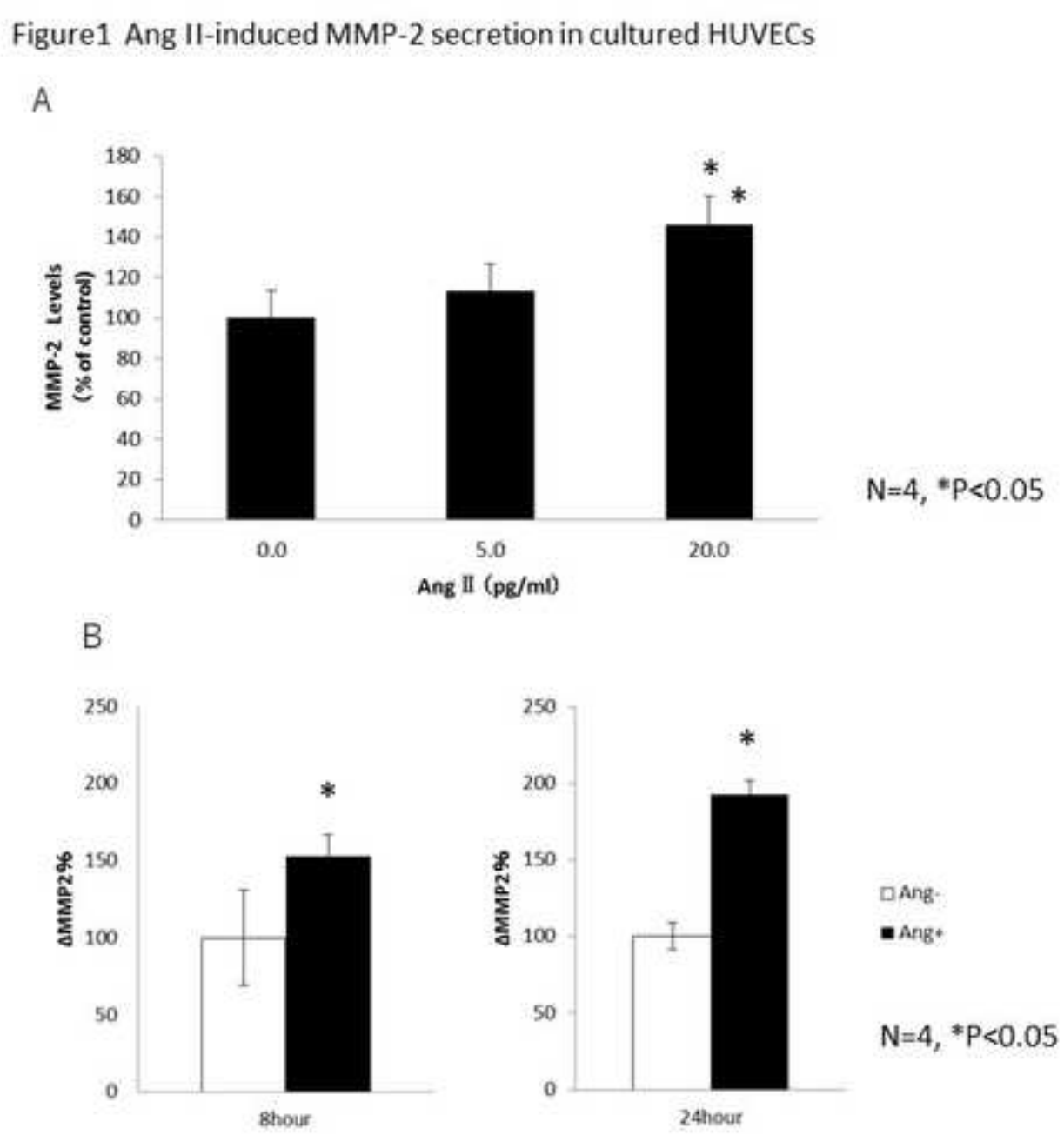


Figure 2 MMP-2 and MMP-9 mRNA expression in HUVECs

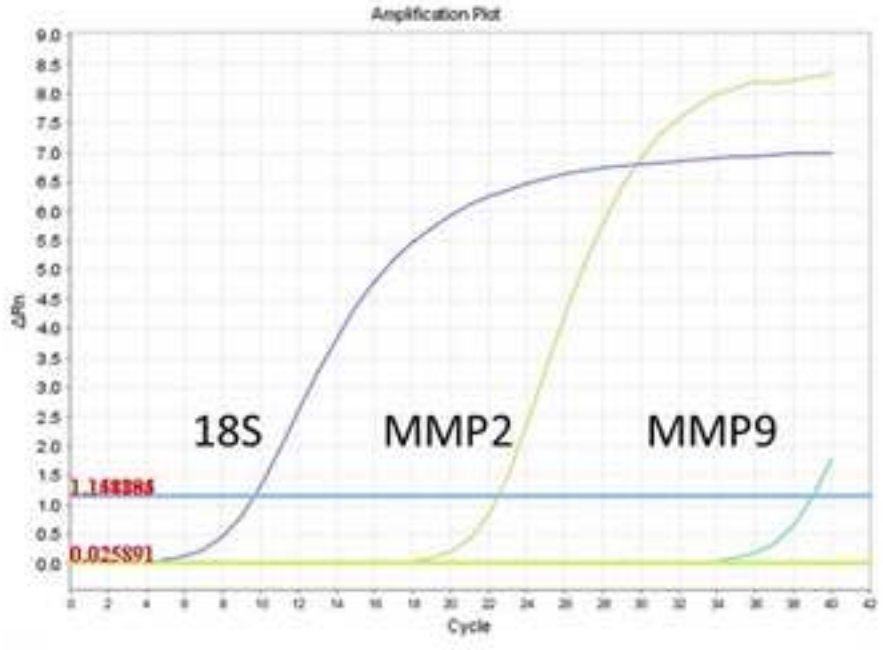


Figure 3 Ang-II can enhance MMP-2 expression at the transcriptional level.

A Real time PCR results of MMP-2 mRNA expression

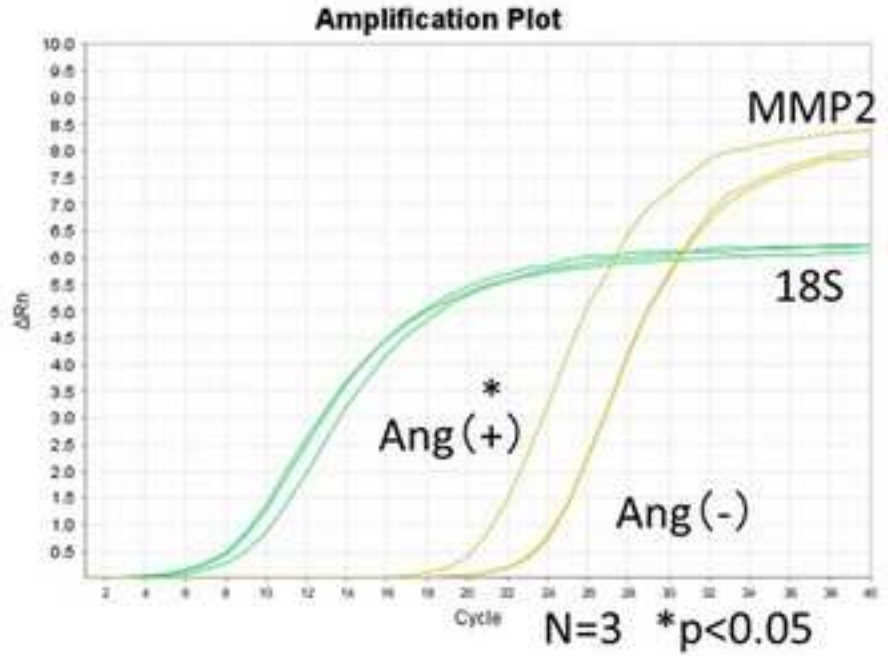


Figure 3B

Figure 3　Ang-II can enhance MMP-2 expression at the transcriptional level.

B　Semi-quantitative RT-PCR results of MMP-2 mRNA expression

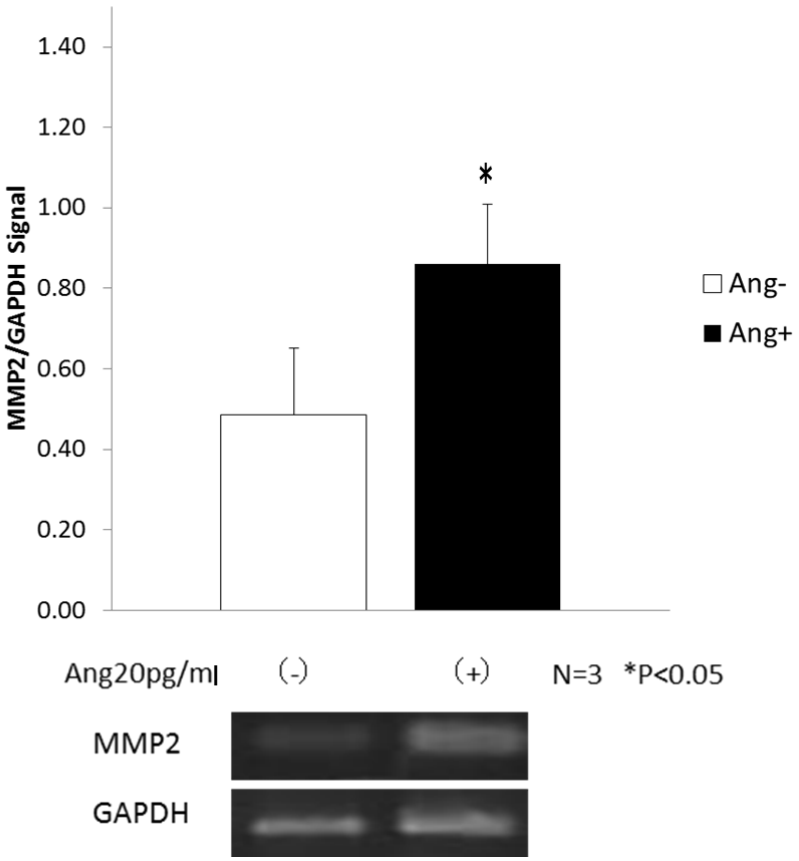
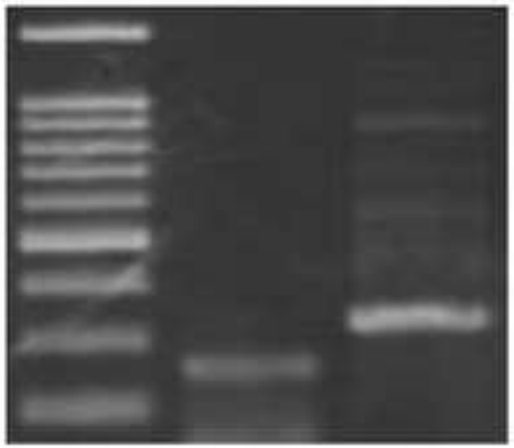


Figure 4 RT-PCR results of mRNA expression of AT1 and AT2 receptors



Reference AT1 receptor AT2-receptor

Figure 5 Effects of Ang-II on MMP-2 secretion from HUVECs in the pretreatment of Ang-II receptor blockers

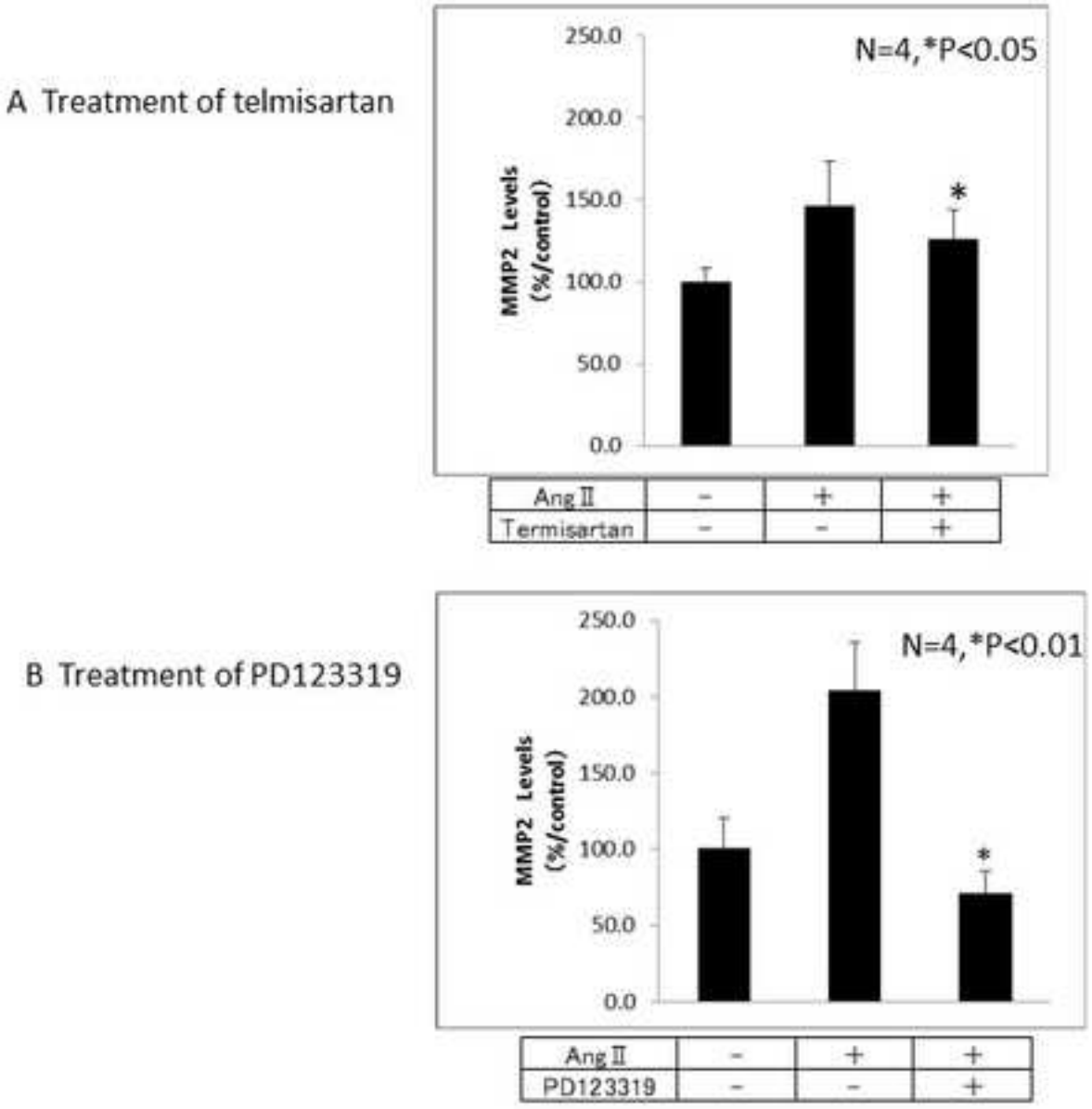


Table 1 Nucleotide sequences of used primers

target gene		sequence	amplicon size(bp)
MMP-2	Forward	5' - GGACTGCCCCCTGATGTCCA - 3'	191
	Reverse	5' - AGGTCCACGACGGCATCCAG - 3'	
GAPDH	Forward	5' - AAGGCTGAGAACGGGAAGCTTG - 3'	750
	Reverse	5' - GAGCTTGACAAAGTGGTCGTTGAG - 3'	
AT1-R	Forward	5' - GATGATTGTCCCAAAGCTGG - 3'	255
	Reverse	5' - TAGGTAATTGCCAAAGGGCC - 3'	
AT2-R	Forward	5' - TGGCTCTGTTTCTTAATGTT - 3'	293
	Reverse	5' - AGTAAAGAATAGGAATTGCAT - 3'	

Table 2 Inhibitory effects of Ang-II receptor blockers on MMP-2 secretion from HUVECs treated with Ang-II

Ang Ⅱ	+	+
Termisartan	+	−
PD123319	−	+
MMP−2 relative value(%)	86.3±12.0	34.9±6.2
Inhibition rate(%)	13.7±6.5	65.1±11.6*

N=4 *P<0.05

HUVECs were plated in 12-wells, and after 24 hours, were incubated in the culture medium supplemented with the absence or presence of telmisartan (0.5 μM) or PD123319 (0.1 μM), and after 1hour incubation, were stimulated by 20 pg/ml of Ang-II. Then, MMP-2 secretion in the collected culture medium was measured after 24 hours. The inhibition rates were calculated by relative values of MMP-2 in the medium with a control group (the absence of Ang-II receptor blockers) being 100 %. The results were compared by Mann-Whitney's U test (n= 4). Statistical significance was determined at $p < 0.05$.