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ORIGINAL ARTICLE

# The angiotensin II receptor antagonist, losartan, enhances regulator of G protein signaling 2 mRNA expression in vascular smooth muscle cells of Wistar rats

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**Angiotensin II (Ang II) reportedly enhances regulator of G-protein signaling 2 (RGS2), thus making a negative feedback loop for Ang II signal transduction. However, few studies have reported whether Ang II receptor (ATR) antagonists influence RGS2 mRNA expression. We investigated RGS2 mRNA expression when Ang II binding to ATR was blocked with Ang II subtype-1 receptor ($AT_1R$) blockers using vascular smooth muscle cells from the thoracic aorta of male Wistar rats. RGS2 mRNA expression significantly increased with Ang II stimulation, and this increase was almost completely abolished by olmesartan, a potent $AT_1R$-specific blocker. Ang II subtype-2 receptor ($AT_2R$) was not involved in Ang II-mediated RGS expression. In contrast, the $AT_1R$ blocker, losartan, partially decreased Ang II-mediated RGS2 mRNA expression because this antagonist directly stimulated RGS2 mRNA expression in Ang II-free medium. EXP3174, which is an active metabolite of losartan, almost completely blunted Ang II-mediated RGS2 mRNA expression without direct stimulation of RGS2 mRNA expression. Moreover, pretreatment with olmesartan abolished Ang II-mediated RGS2 mRNA expression. Treatment with a protein kinase C inhibitor partially decreased losartan-mediated RGS2 mRNA expression. These results suggest that $AT_1R$ blockers inhibit RGS2 mRNA expression in response to Ang II via an $AT_1R$-mediated mechanism. However, the $AT_1R$ blocker, losartan, behaves as a direct agonist for RGS2 mRNA expression via $AT_1R$ through protein kinase C-dependent and -independent pathways. In conclusion, losartan exhibits dual effects on RGS2 mRNA expression, and the direct upregulation of RGS2 mRNA expression may provide a new strategy for the treatment of hypertension.**

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**Keywords:** angiotensin II; $AT_1R$ antagonist; $AT_2R$; RGS2; vascular smooth muscle cell

## INTRODUCTION

Angiotensin II (Ang II) activates an impressive array of signaling pathways in vascular smooth muscle cells (VSMCs) predominantly via the seven-transmembrane, heterotrimeric G-protein-coupled receptor, Ang II subtype-1 receptor ($AT_1R$). The binding of Ang II to $AT_1R$ causes a biphasic response with a rapid and transient activation of phosphatidylinositol-specific phospholipase C, thus producing inositol trisphosphate and diacylglycerol, followed by a prolonged activation of phospholipase D.[1,2] The production of inositol trisphosphate occurs within seconds and reaches maximal activation at 15 s, after which it returns to baseline levels. Ang II-stimulated $AT_1R$ has a critical role in the physiological and pathophysiological regulation of the cardiovascular system, for example, blood pressure control, by affecting arterial tone, electrolytes and fluid balance. It is also involved in cardiac dysfunction, vascular sclerosis and intracellular crosstalk in hormone transduction, such as insulin sensitivity or sympathetic nerve activity.[3,4]

$AT_1R$ activity is regulated, at least in part, by regulators of G-protein signaling (RGS) proteins, which mostly decrease G-protein function by promoting the guanosine triphosphatase activity of their Gα subunits.[5,6] Among the RGS protein family, RGS2 displays regulatory selectivity for the Gαq subclass of G-proteins and has an important role in cardiovascular pathophysiology.[7–12] RGS2 is expressed in many cardiovascular or renal tissues, including the heart, kidneys and blood vessels.[13] Silencing the RGS2 gene enhances Ang II signaling transduction.[14] In fact, RGS2-knockout mice exhibit a strong hypertensive phenotype, renovascular abnormalities, persistent constriction of the resistance vasculature and prolonged response of the vasculature to vasoconstrictors *in vivo* and *in vitro*.[15]

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In addition, recent studies have reported that Ang II upregulates RGS2 mRNA expression in human VSMCs, suggesting the existence of a negative feedback loop.[16] We have demonstrated that arterial RGS2 mRNA is upregulated by Ang II infusion in Dahl salt-resistant rats, and this feedback mechanism is almost completely abolished in Dahl salt-sensitive rats.[17] The suppressed RGS2 mRNA response to Ang II may be responsible, in part, for the susceptibility of Dahl salt-sensitive rats to Ang II-mediated kidney damage[17,18] Considering these data, it seems quite interesting to investigate the relationship between $AT_1R$ blockers and RGS2 expression because the blockers exhibit antihypertensive action with cardiovascular protection in various clinical settings. In the present study, we investigated the role of $AT_1R$ in RGS2 mRNA expression in response to Ang II and examined how the $AT_1R$ blocker, losartan, affected RGS2 mRNA expression using Wistar rat VSMCs in culture.

## METHODS

### Cell culture

VSMCs were isolated from the thoracic aortas of 6-week-old male Wistar rats using the explant method. Briefly, aortic walls were cut into $2 \times 2$ mm strips. The tissues were placed in 100 mm diameter tissue culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM; low glucose) with L-glutamine, phenol red (Wako Pure Chemical Industries, Tokyo, Japan). DMEM was supplemented with 10% fetal bovine serum (FBS), $10^5$ U $l^{-1}$ penicillin and 100 mg $l^{-1}$ streptomycin (Wako Pure Chemical Industries). The medium was changed every 3 days. The strips were maintained at 37 °C in a humidified, 5% $CO_2$ atmosphere, and after 10 days, the VSMCs growing out from the strips were collected using 0.05% trypsin/0.02% ethylenediaminetetraacetate. The collected cells were cultured in the same medium, and semiconfluent cells between passages 4 and 10 were used for the experiments.

A total of $10^6$ cells were placed in a 35-mm-diameter, 6-well plate and cultured in DMEM containing 10% FBS. When the cells reached a semiconfluent growth state, the medium was changed to FBS-free medium with or without a given concentration of test compounds. The cells were collected with trypsin/ethylenediaminetetraacetate, and the collected cells were stored at − 80 °C until assay.

### Angiotensin II receptors and RGS2 mRNA expression

First, we investigated RGS2 mRNA expression in VSMCs in response to 100 nmol $l^{-1}$ Ang II (Sigma-Aldrich, St Louis, MO, USA). VSMCs in a semiconfluent growth state were incubated in 10% FBS (Wako Pure Chemical Industries)-DMEM (Sigma-Aldrich) with 100 nmol $l^{-1}$ Ang II at 37 °C for 0–6 h. The cells were collected with trypsin and ethylenediaminetetraacetate (Wako Pure Chemical Industries) at each time point. The cells were centrifuged at 3000 r.p.m. for 5 min at room temperature. The pellets were quickly plunged into liquid nitrogen, and the samples were stored under − 80 °C until RGS2 mRNA determination.

Ang II exerts its actions via two Ang II receptors (ATR), that is, $AT_1R$ and Ang II subtype-2 receptor ($AT_2R$). We attempted to investigate the role of $AT_1R$ in RGS2 mRNA expression following Ang II stimulation. VSMCs were pre-incubated for 2 h at 37 °C in FBS–DMEM with the $AT_1R$-specific antagonist, olmesartan (Daiichi Sankyo, Tokyo, Japan). Then, the cells were stimulated with 100 nmol $l^{-1}$ Ang II for 2 h. The cells were collected and processed to determine RGS2 mRNA expression levels. In this experiment, we used 200 nmol $l^{-1}$ olmesartan because the dose is reportedly enough to block $AT_1R$.[19,20]

To evaluate the role of $AT_2R$ in Ang II-mediated RGS2 mRNA expression, we investigated the effects of PD123319, an $AT_2R$ antagonist (Sigma-Aldrich), and of CGP42112A, an $AT_2R$ agonist (Sigma-Aldrich), on RGS2 mRNA expression in response to Ang II. The cells were pre-incubated with 200 nmol $l^{-1}$ PD123319 or 200 nmol $l^{-1}$ CGP42112A for 2 h and were then stimulated with 100 nmol $l^{-1}$ Ang II for 2 h. RGS2 mRNA was determined as described above in the $AT_1R$ blocker section.

### Losartan and RGS2 mRNA expression

Because it is well reported that the $AT_1R$ antagonist, losartan, exhibits both $AT_1R$-dependent and $AT_1R$-independent functions, we attempted to examine whether RGS2 is involved in the benefits seen with losartan.[21] We examined the effects of losartan on Ang II-mediated RGS2 mRNA expression. VSMCs were pre-incubated in medium with 500 nmol $l^{-1}$ losartan (Merck, Whitehouse Station, NJ, USA) for 2 h and then stimulated with 10 or 100 nmol $l^{-1}$ Ang II for 2 h. The cells were collected and processed for RGS2 mRNA determination.

Next, we examined the time course of RGS2 mRNA expression after stimulation with losartan. In this experiment, VSMCs were cultured in medium with 500 nmol $l^{-1}$ losartan for 0–6 h. The cells were collected at each incubation period for RGS2 mRNA determination.

In addition, it has been reported that ~17% of losartan is converted to its 10-fold active metabolite, EXP3174 (5-carboxylic acid metabolite of losartan).[21] EXP3274 behaves as a potent antagonist against $AT_1R$. This may partially explain the actions of losartan on ATR antagonism. Therefore, we examined the effects of EXP3174 (Merck) on Ang II-mediated RGS2 mRNA expression. The cells were pre-incubated with 500 nmol $l^{-1}$ EXP3174 for 2 h and then stimulated with Ang II for 2 h. The cells were collected to determine the RGS2 mRNA expression levels.

We assessed the effects of losartan on RGS2 mRNA expression in VSMC $AT_1R$, which were blocked with the $AT_1R$-specific antagonist, olmesartan. The cells were pre-incubated with 200 nmol $l^{-1}$ olmesartan (Daiichi Sankyo) for 30 min to block $AT_1R$. We examined the dose-dependency of RGS2 mRNA expression in response to 0–500 nmol $l^{-1}$ losartan. In these experiments, 200 nmol $l^{-1}$ olmesartan was utilized because the $IC_{50}$ of $AT_1R$ antagonism is one-tenth less with olmesartan than it is with losartan.[19,20]

### PKC inhibition and RGS2 mRNA expression

As it has been reported that the increase in RGS2 mRNA expression in response to Ang II stimulation is partially mediated by PKC activation,[16] we examined the role of PKC activity on RGS2 mRNA in response to losartan in Ang II-free medium. VSMCs were pre-incubated for 30 min in FBS-free DMEM containing 10 μmol $l^{-1}$ of the PKC inhibitor, GF109203X (Sigma-Aldrich), for 30 min, and then, 500 nmol $l^{-1}$ losartan or 100 nmol $l^{-1}$ Ang II was added to the medium. After 2 h incubation, the cells were collected to determine the RGS2 mRNA expression levels.

### RGS2 mRNA Determination

*RNA extraction.* Total RNA was extracted from the cells using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. In brief, the cells collected were re-suspended in phosphate-buffered saline. Then, the cells were lysed in the lysis/binding buffer (4.5 mol $l^{-1}$ guanidine-HCl, 50 mmol $l^{-1}$ Tris-HCl, 30% Triton X-100 (w/v), pH 6.6) and vortexed for 15 s. The homogenates were subsequently transferred to a High Pure filter tube (High Pure RNA Isolation Kit) and centrifuged to ensure that the RNA/DNA adhered to the filter. The filters were then incubated with a DNase I buffer for 15 min to digest DNA, after which the filters were washed repeatedly with Wash Buffer I/II according to the manufacturer's instructions. Finally, RNA was eluted with an elution buffer and stored at − 80 °C until mRNA determination. Total RNA concentration and purity were determined using a spectrophotometer at wavelengths of 260 and 280 nm. The ratio of $OD_{260}/OD_{280}$ was greater than 1.90.

*cDNA synthesis.* The highly purified RNA was used for cDNA synthesis using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's protocol. In brief, 2.5 μmol $l^{-1}$ of anchored-oligo $(dT)_{18}$ primer, 60 μmol $l^{-1}$ of random hexamer primer, and an RNA template were mixed and denatured by heating at 65 °C for 10 min using a thermal block cycler with a heating lid. The tube was quickly cooled in an ice-chilled container. The reaction mixture containing 1 × Transcriptor reverse transcriptase reaction buffer, 20 U of Protector RNase inhibitor, 1 mmol $l^{-1}$ of each deoxynucleotide mixture, and 10 U Transcriptor reverse transcriptase was placed in each tube. The tubes were mixed carefully and heated at 55 °C for 30 min and at 85 °C for 5 min. The reaction was terminated by placing the

tubes in ice-chilled water, and the tubes were stored at −80 °C until the determination of their mRNA concentrations.

*Real-time PCR.* Real-time PCR was performed using a LightCycler TaqMan Master Kit and a LightCycler ST300 system (Roche Diagnostics).[17] The primers were designed by Nihon Gene Research Laboratories, Tokyo, Japan (Table 1). The probes were selected from probes 1–165 of the Universal ProbeLibrary for the LightCycler (Roche Diagnostics). The PCR reaction mixture comprised 4 µl of 5× concentrations of LightCycler TaqMan Master mix, 200 nmol $l^{-1}$ of forward and reverse primers, 100 nmol $l^{-1}$ of the Universal ProbeLibrary probe and 5 µl cDNA template. Finally, the total volume was adjusted to 20 µl with PCR-grade distilled water. The conditions for multiplication are presented in Table 2.

### Statistical analysis

The results are expressed as the mean ± s.e. Statistical significances were analyzed by breakdown and one-way analysis of variance using the STATISTICA program (StatSoft, Tulsa, OK, USA). A *P*-value $<0.05$ was considered statistically significant.

### Guidelines for handling rats

The institutional committee for animal research of the University of Tokyo approved this study. Our experiments were performed in accordance with the National Institutes of Health guidelines.

## RESULTS

### Angiotensin II receptors and RGS2 mRNA expression

Ang II increased RGS2 mRNA expression in VSMCs at 1 h after the stimulation and by 229% at 2 h compared with the basal level ($P<0.05$, Figure 1). Thereafter, the increase gradually declined over 4 h. Therefore, we utilized a 2-h incubation for the experiments, except in the specified experimental conditions.

To examine the role of $AT_1R$ on Ang II-mediated RGS2 mRNA expression, we investigated the effect of the specific $AT_1R$ blocker, olmesartan, on RGS2 mRNA expression in VMSCs[19,20] (Figure 2). In Ang II-free conditions, olmesartan did not influence RGS2 mRNA expression (left two bars). Ang II-stimulated RGS2 mRNA expression in olmesartan-free medium, and this increase was completely abolished by pretreatment with olmesartan (right two bars).

**Table 1 Design of primers for amplification of RGS2 mRNA**

| *Primers designed* | *Sequence (5′→3′)* |
|---|---|
| RGS2 FP[1] | AACTTTTATCAAGCCTTCTCCTGA |
| RGS2 RP[2] | ACGCTCTGAATGCAGCAAG |
| GAPDH FP[1] | AATGTATCCGTTGTGGATCTGA |
| GAPDH RP[2] | GCTTCACCACCTTCTTGATGT |

Abbreviations: FP[1], forward primer; RGS2, regulator of G-protein signaling 2; RP[2], reverse primer.

**Table 2 Times and temperatures applied for the real-time PCR configuration using the TaqMan probe and the LightCycler**

| *Parameters* | *Target temp* | *Incubation time* | *Transition rate* | *Acquisition mode* |
|---|---|---|---|---|
| Denaturation | 95 °C | 10 min | 20 °C $s^{-1}$ | None |
| Amplification[a] | 95 °C | 10 s | 20 °C $s^{-1}$ | None |
| | 60 °C | 25 s | 20 °C $s^{-1}$ | Single |
| Cooling | 40 °C | 30 s | 20 °C $s^{-1}$ | None |

[a]45 cycles amplification to determine RGS2 (regulator of G-protein signaling 2) mRNA.

Next, to determine the role of $AT_2R$ in the upregulation of RGS2 mRNA in response to Ang II stimulation, we examined the effects of the $AT_2R$ antagonist, PD123319, or the agonist, CGP42112A, on Ang II-mediated RGS2 mRNA upregulation (Figure 3). In Ang II-free medium, neither PD123319 nor CGP42112A influenced RGS2 mRNA expression (left three bars). In Ang II-plus medium, RGS2 mRNA significantly increased, and this increase was influenced by neither PD123319 nor CGP42112A (right three bars). These data indicated that $AT_2R$ was not involved in the upregulation of RGS2 mRNA in response to Ang II stimulation.

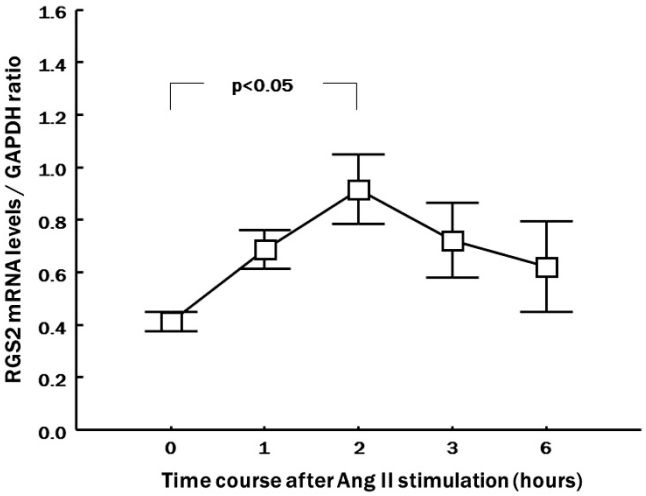


**Figure 1** Time course of Ang II-mediated RGS2 mRNA expression. VSMCs cultured in Ang II-free medium (at 0 h) were stimulated with Ang II during 6 h. After each incubation period, the cells were collected, and the content of RGS2 mRNA was determined as described in the text. Values are expressed as the mean ± s.e. ($n=5$). Differences were assessed by one-way analysis of variance followed by *post hoc* LSD test. Ang II, angiotensin II; LSD, least significant difference; RGS2, regulator of G-protein signaling 2.

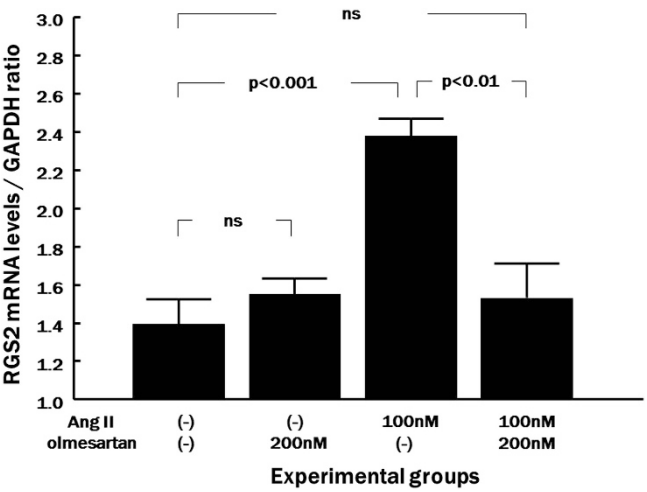


**Figure 2** $AT_1R$ inhibition by olmesartan and RGS2 mRNA expression. Ang II (−), medium without Ang II; olmesartan (−), medium without olmesartan. The cells were pre-incubated in medium with olmesartan, and then, stimulated with Ang II. The cells were collected for RGS2 mRNA determination. Values are expressed as the mean ± s.e. ($n=6$). Differences were assessed by one-way analysis of variance. Ang II, angiotensin II; NS, not significant; RGS2, regulator of G-protein signaling 2.

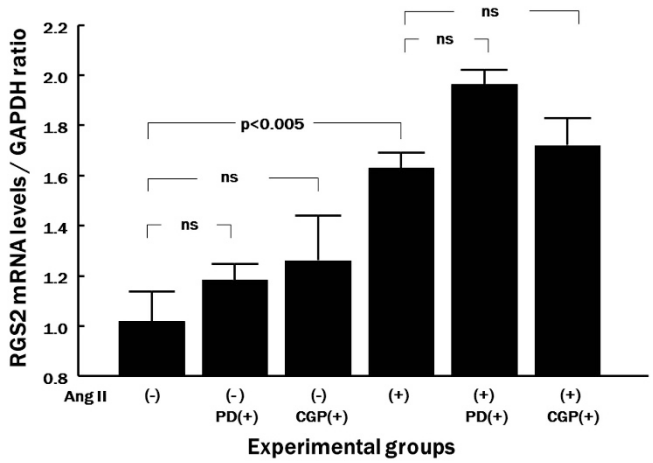


**Figure 3** Effects of $AT_2R$ antagonist or agonist on Ang II-mediated RGS2 mRNA expression. A (−), culture medium without Ang II; A (+), medium with 100 nmol $l^{-1}$ Ang II; PD (+), culture medium with 200 nmol $l^{-1}$ PD123319; and CGP (+), culture medium with 200 nmol $l^{-1}$ CGP42112A. The cells were cultured in medium with the reagent for 2 h and then stimulated with Ang II for 2 h. The cells were collected for RGS2 mRNA determination. Values are expressed as the mean ± s.e. ($n=6$). Differences were assessed by one-way analysis of variance. Ang II, angiotensin II; NS, not significant; RGS2, regulator of G-protein signaling 2.

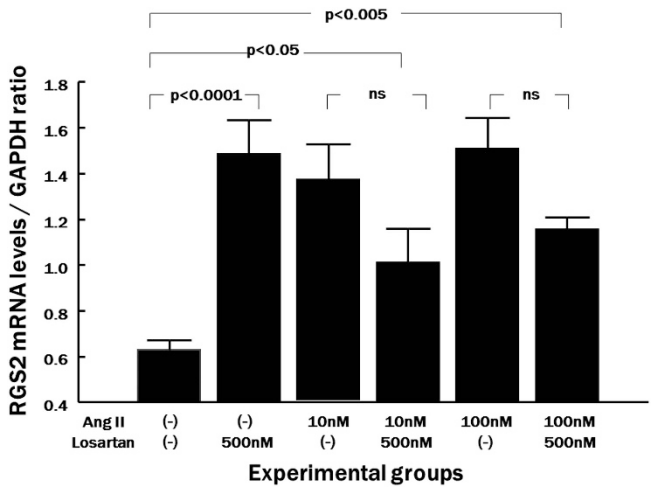


**Figure 4** The $AT_1R$ inhibitor losartan and RGS2 mRNA expression. A (−), medium without Ang II; L (−), medium without losartan. The cells were pre-incubated with losartan for 2 h and then stimulated with Ang II. The cells were collected for RGS2 mRNA determination. Values are expressed as the mean ± s.e. ($n=6$). Differences were assessed by one-way analysis of variance. Ang II, angiotensin II; NS, not significant; RGS2, regulator of G-protein signaling 2.

### Losartan and RGS2 mRNA expression in VSMCs

We investigated the influence of the $AT_1R$ inhibitor, losartan, on RGS2 mRNA expression in response to Ang II (Figure 4). In losartan-free medium, Ang II significantly stimulated RGS2 mRNA expression in a dose-dependent manner. Moreover, losartan directly stimulated RGS2 mRNA expression in Ang II-free medium and tended to blunt the Ang II-mediated RGS2 mRNA expression, although the difference was not significant. These data indicated that losartan exhibited the direct stimulation of RGS2 mRNA expression in Ang II-free medium and partially blocked the upregulation of RGS2 mRNA in response to Ang II.

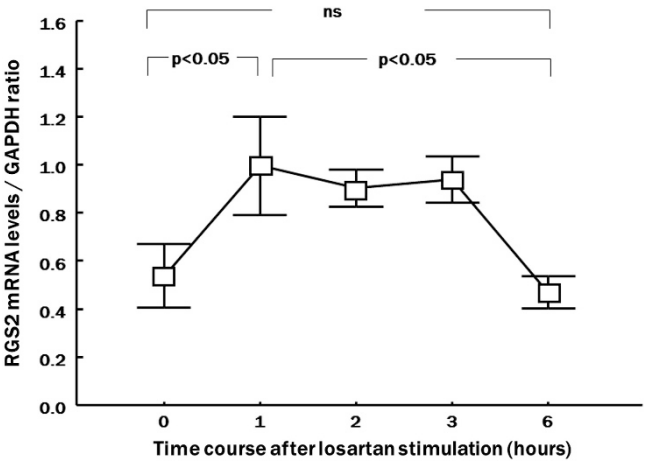


**Figure 5** Time course of RGS2 mRNA expression induced by losartan. Time course of RGS2 mRNA expression induced by losartan was shown. The cells were stimulated in 500 nmol $l^{-1}$ losartan for 0 to 6 h. Values are expressed as the mean ± s.e. ($n=6$). Differences were assessed by one-way analysis of variance. NS, not significant; RGS2, regulator of G-protein signaling 2.

To clarify these points, we examined RGS2 mRNA expression in response to Ang II during 0–6 h (Figure 5). RGS2 mRNA expression was immediately increased with losartan stimulation, and the expression peaked at 1 h. This increase was maintained over 3 h after stimulation. The time course apparently differed from that of Ang II stimulation.

Losartan is converted into a metabolite, EXP3174, in humans, and this compound works as a potent $AT_1R$ blocker.[21] We examined the influence of this metabolite on RGS2 mRNA expression (Figure 6). In Ang II-free conditions, losartan stimulated expression, but the active metabolite did not stimulate RGS2 mRNA expression (left graph). Intriguingly, EXP3174 almost completely abolished the Ang II-mediated RGS2 mRNA expression, whereas losartan partially blunted the expression (right graph).

To examine whether $AT_1R$ is involved in RGS2 mRNA expression by losartan, we blocked $AT_1R$ with olmesartan and then assessed the alterations of losartan-mediated RGS2 mRNA expression (Figure 7). In olmesartan-free medium, losartan stimulated RGS2 mRNA expression in a dose-dependent manner (left graph). In contrast, this increase was completely abolished when the cells were pre-incubated with olmesartan (right graph).

### PKC inhibition and RGS2 mRNA expression in VSMCs

To determine whether PKC is involved in the Ang II- or losartan-mediated RGS2 mRNA expression, the cells were pre-incubated with the PKC inhibitor, GF109203X, and then stimulated with Ang II or losartan for 2 h. Pre-treatment with GF109203X decreased the baseline RGS2 mRNA expression by 50 ± 1% (Figure 8). GF109203X significantly decreased the Ang II-mediated RGS2 mRNA expression by 79 ± 1%. Similarly, this inhibitor attenuated the losartan-mediated RGS2 mRNA expression (55 ± 1%). It was also noted that the generation was significantly greater than RGS2 mRNA synthesis using a PKC inhibitor alone. These data suggested that the PKC inhibitor, GF109203X, exhibits a greater reduction on RGS2 mRNA biosynthesis induced by Ang II compared with that induced by losartan (79% inhibition vs 55%). Thus, it was suggested that RGS2 mRNA expression might comprise two components: a PKC-dependent and a PKC-independent component. With respect to losartan,

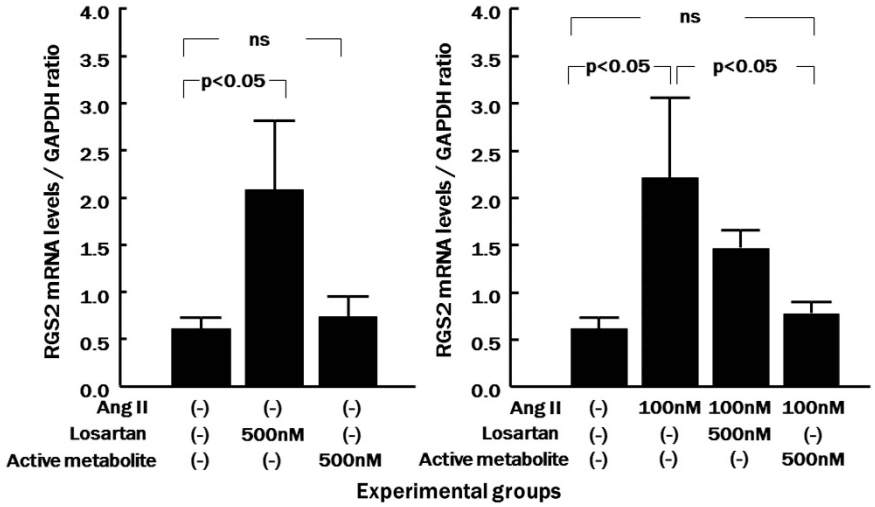


**Figure 6** Effects of the metabolite of losartan, EXP1374, on RGS2 mRNA expression. Ang II (−), medium without Ang II; Losartan (−), medium without losartan; Active metabolite (−), medium without active metabolite of losartan EXP1374. The cells were incubated in medium with the reagent for 2 h and then stimulated with Ang II. The cells were collected for RGS2 mRNA determination. Values are expressed as the mean ± s.e. ($n=6$). Differences were assessed by one-way analysis of variance. Ang II, angiotensin II; NS, not significant; RGS2, regulator of G-protein signaling 2.

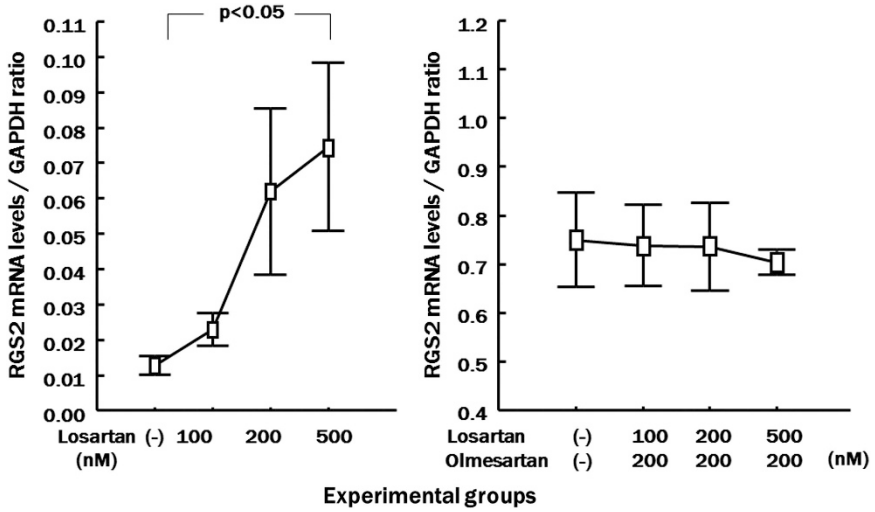


**Figure 7** Olmesartan pretreatment and losartan-mediated RGS2 mRNA expression. Losartan (−), medium without losartan; Olmesartan (−), medium without losartan. Dose-dependent effect of losartan on RGS2 mRNA expression in VSMCs was examined in an olmesartan-free condition (left graph) and in an olmesartan-plus condition (right graph). The cells were cultured in olmesartan for 30 min and then stimulated with Ang II. The cells were collected, and the RGS2 mRNA content was determined. Values are expressed as the mean ± s.e. ($n=6$). Differences were assessed by one-way analysis of variance followed by *post hoc* LSD test. Ang II, angiotensin II; LSD, least significant difference; RGS2, regulator of G-protein signaling 2.

the PKC-independent component is much greater than the PKC-dependent component.

## DISCUSSION

In the present study, we demonstrated that Ang II increased RGS2 mRNA expression. The expression reached a maximum at 2 h after incubation in medium containing 100 nmol $l^{-1}$ Ang II and gradually declined to baseline during the next 4 h. Because RGS2 lowers G-protein-mediated Ang II signal transduction, the increase in RGS2 mRNA expression in response to Ang II attenuates intracellular Ang II signaling. These data indicated that the inhibitory effect on RGS2 mRNA expression presumably constitutes a negative feedback mechanism for Ang II intracellular signaling, as suggested by investigators from another laboratory.[16]

The increase in RGS2 mRNA expression in response to Ang II stimulation was almost completely abolished by the $AT_1R$-specific antagonist, olmesartan. Moreover, EXP3174, a metabolite of losartan and an $AT_1R$-specific antagonist, abolished the increase in the Ang II-mediated RGS2 mRNA expression. Ang II activates $AT_2R$ and counteracts the events mediated by the $AT_1R$ agonist. In the present study, however, we demonstrated that Ang II-mediated RGS mRNA expression was influenced by neither the $AT_2R$ antagonist, PD123319, nor the $AT_2R$ agonist, CGP42112A. These compounds did not influence RGS2 mRNA expression in Ang II-free medium as well.[22–24] These data clearly suggested that the Ang II-mediated RGS2 mRNA expression was solely an $AT_1R$-mediated event and that $AT_2R$ was not involved in the mechanism.

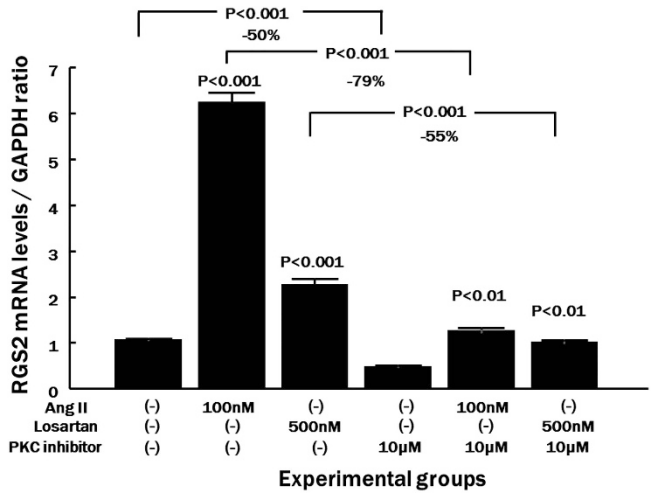


**Figure 8** Effect of PKC inhibition on RGS2 mRNA biosynthesis in VSMCs. Ang II (−), medium without Ang II; Losartan (−), medium without losartan; and PKC inhibitor (−), medium without GF109203X. The cells were pre-incubated in medium with GF109203X for 30 min to inhibit PKC and then incubated with Ang II or losartan for 2 h. The cells were collected, and the RGS2 mRNA content was determined. Values are expressed as the mean ± s.e. ($n=4$). Differences were assessed by one-way analysis of variance. Ang II, angiotensin II; RGS2, regulator of G-protein signaling 2; VSMC, vascular smooth muscle cell.

Moreover, we clearly demonstrated that losartan directly stimulated RGS2 mRNA expression in Ang II-free medium. The pretreatment by olmesartan completely abolished the increase in RGS2 mRNA induced by losartan. Such evidence strongly suggested that the direct action of losartan on RGS2 mRNA expression is an $AT_1R$-dependent event.

Losartan is a prodrug, and ~17% of losartan is metabolized in the liver into the active metabolites, EXP3174 and EXP3179, with possible intracrine actions.[25–28] The partial reduction of Ang II-induced RGS2 mRNA expression with losartan might be owing to both $AT_1R$ inhibition and direct stimulation of RGS mRNA expression (Figure 5). To our knowledge, there have been no reports on such dual effects of losartan on RGS2 mRNA in VSMCs.

The mechanism of losartan-mediated RGS2 mRNA expression is not clear. However, we have shown that the increase in RGS2 mRNA expression following Ang II stimulation was composed of two components: a PKC-dependent and a PKC-independent component.[25] A total of 79% of RGS2 mRNA expression in response to Ang II was PKC-dependent, and 21% was still observed after PKC inhibition. In contrast, losartan directly stimulated RGS2 mRNA expression, and 55% of losartan-mediated RGS2 mRNA was PKC-dependent; 45% was independent of PKC activity. Almost 50% of RGS2 mRNA expression was PKC-dependent in the basal condition. The difference between Ang II and losartan stimulation in response to PKC inhibition clearly suggested that a significant part of losartan-mediated RGS2 mRNA expression was independent of the PKC mechanism. In this context, Grant *et al.*[16] recently demonstrated that among RGS proteins, only RGS2 was specifically regulated by Ang II and that the upregulation of RGS2 mRNA by Ang II was regulated via transcriptional levels through both PKC-dependent and PKC-independent pathways.

In the present study, we demonstrated that EXP3174 did not directly stimulate RGS2 mRNA expression. This indicated that EXP3174 may not be responsible for the direct effects of losartan if the increase in RGS2 mRNA biosynthesis by losartan is mediated by its metabolites in an *in vivo* state. Because there have been some reports of the effects of EXP3179 on intracellular signal transduction,[26–29] it is conceivable that the direct action of losartan may be due to EXP3179. In fact, an inverse agonistic action of losartan has been recognized. To express the inverse agonistic action, losartan binds to $AT_1R$, and the receptor is believed to undergo a conformational change that sends a negative signal for Ang II-dependent intracellular events. This inverse agonistic action may be explained by the PKC-independent RGS2 mRNA expression produced by losartan. More interestingly, the inverse agonistic action is reportedly attainable by the direct inhibition of PKC activity with EXP3179, a metabolite of losartan.[27] Thus, EXP3179 potentially exhibits the inverse agonistic action by upregulating the PKC-independent component of the losartan-mediated RGS2 mRNA expression. Unfortunately, however, in the present study, we were unable to obtain EXP3179 to test. It remains to be elucidated whether EXP3179 truly mediates RGS2 mRNA upregulation by losartan in an *in vivo* state.

In our preliminary studies, we found that some forms of calcium channel blockers directly increase RGS2 mRNA expression (unpublished data). This is not a class effect, and some structural moiety may be needed to stimulate RGS2 mRNA expression. In association with the knowledge on losartan, this property is very important in clinical settings because the RGS2 mRNA and inhibition of the signal transduction is potentially a new strategy for hypertension treatment. In fact, in clinical settings, there are differences in the effects on blood pressure reduction in association with insulin sensitivity among ATR antagonists.[30]

In the present study, we determined only the RGS2 mRNA levels and did not examine alterations in its activity. This was because RT-PCR is a sensitive and reproducible method for assessing RGS2 metabolism. However, it was technically difficult to quantitatively measure the very small amounts of RGS2 on western blot membranes.[17] We previously found that ERK1/2 phosphorylation after Ang II stimulation paralleled the alterations in RGS2 mRNA expression (unpublished data). This is in accordance with the data of Semplicini *et al.*[10] Considering these results, it is probable that the changes in RGS2 mRNA expression following losartan stimulation reflect the alteration of downstream intracellular Ang II signaling.

In conclusion, we demonstrated that there is a negative feedback loop between the Ang II-mediated signal transduction and regulation of Ang II signaling by RGS2. $AT_1R$-antagonism interrupts the feedback mechanism, thereby increasing the signaling through RGS2 downregulation. However, losartan directly stimulated RGS2 mRNA expression. The changes in RGS2 mRNA expression were influenced by PKC-dependent and PKC-independent mechanisms. The PKC-independent mechanism was owing to the direct action of losartan on RGS2 mRNA expression. This increase would buffer the decline in RGS2 mRNA expression following an $AT_1R$-specific inhibition. Such an effect has potentially critical implications in clinical settings.

## Perspectives

Ang II is the most critical factor in the pathogenesis of hypertension, and currently it is believed that RGS2 might have a role in cardiovascular regulation. The typical mechanism of action of $AT_1R$ blockers used to treat hypertension is blocking the activation of $AT_1R$. However, in the present study, RGS2 mRNA expression was upregulated by losartan via $AT_1R$ activation. Therefore, it is important to understand the correlation between Ang II and RGS2 and the mechanism by which blood pressure is regulated by RGS2. It is very likely that more information on RGS2 will provide exciting new opportunities for drug development and specificity.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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