Smooth muscle cell-specific Hif-1α deficiency suppresses angiotensin II-induced vascular remodelling in mice

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Aim
Vascular remodelling is mediated by vascular smooth muscle cell (VSMC) proliferation and hypertrophy, both processes of which are linked to medial thickening and fibrosis. Here, we show that hypoxia-inducible factor-1α (Hif-1α) expressed in smooth muscle cells (SMCs) is involved in angiotensin II (Ang II)-induced vascular remodelling in an in vivo model.

Methods and results
To clarify the role of Hif-1α in vascular remodelling, we created mice lacking the Hif-1α gene in SMCs (SMKO mice). Ang II infusion induced medial thickening and vascular fibrosis, accompanied by Hif-1α up-regulation, in the aortae of control mice, but not in those of SMKO mice. In accordance with those results, our in vitro studies showed that the deletion of SMC-derived Hif-1α suppressed the Ang II-induced hypertrophy of VSMCs, and our in vivo studies showed that the Ang II-induced expression of fibrosis-related genes in aortae was suppressed by SMC-specific Hif-1α deficiency. In addition, the SMC-specific Hif-1α deficiency suppressed Ang II-induced macrophage infiltration and Ang II-induced expression of inflammation-related genes in aortae. The superoxide production observed in the aortae of control mice with Ang II was suppressed in those of SMKO mice with Ang II, and this finding was consistent with the results of little Ang II-induced c-Src phosphorylation in SMKO mouse aortae. Loss- and gain-of-function analysis in in vitro experiments confirmed that VSMC-derived Hif-1α functions as an intrinsic modulator of vascular remodelling-related gene expression.

Conclusion
Our results revealed that SMC-derived Hif-1α is a crucial mediator of Ang II-induced vascular remodelling.

Keywords
Hypoxia-inducible factor-1 (HIF-1) • Vascular smooth muscle • Vascular remodelling • Angiotensin II

1. Introduction
Angiotensin II (Ang II), the primary active component of the renin–angiotensin system, is a multifunctional hormone responsible for cellular processes that result in cardiovascular remodelling, such as reactive oxygen species (ROS) production, migration, proliferation, hypertrophy, fibrosis, and inflammation. The effects of Ang II are mediated by two Ang II receptors, Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R). AT1R is expressed much more than AT2R in blood vessels and is the main contributor to the progression of vascular diseases. In vascular smooth muscle cells (VSMCs), AT1R activation promotes the proliferation, migration, and hypertrophy of VSMCs, all of which are linked to medial thickening. In addition, the Ang II-induced phosphorylation of the extracellular signal-regulated kinase (Erk) pathway and the PI3 K/AKT pathway contributes to the expression of inflammatory cytokines, such as monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6). Moreover, superoxide, a ROS, is generated by activating the Ang II-AT1R-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase signalling axis and acts to activate the aforementioned phosphorylation pathways. ROS production in VSMCs also contributes to vascular remodelling.

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Indeed, chronic Ang II infusion into mice by using osmotic pumps enhanced ROS production and inflammation through the induction of those cytokines in blood vessels, and mice presenting with medial thickening and vascular fibrosis in their aortae as well as blood pressure elevation are generally used as an animal model of vascular remodelling diseases. However, in VSMCs, it remains arguable how Ang II signalling is linked to medial thickening, vascular fibrosis, oxidative stress, and vascular inflammation in an in vivo model.

Hypoxia-inducible factor-1 (Hif-1) is a transcription factor consisting of hypoxia-induced Hif-1α and constitutively expressed Hif-1β, and is distributed ubiquitously in the human body. Hif-1 regulates various cellular responses, such as proliferation, survival, energy metabolism, chemotaxis, inflammation, fibrosis, and angiogenesis. It has been shown that Ang II and several cytokines induce Hif-1α in the absence of a hypoxic condition, and that Ang II induces Hif-1α via Erk1/2 phosphorylation or the inhibition of prolyl hydroxylase (PHD), an enzyme contributing to Hif-1α degradation. In VSMCs. There are also reports that Hif-1α phosphorylation by Erks increases its nuclear accumulation and transcriptional activity, and Hif-1α is induced via c-Src phosphorylation (pTyr481). Although it has been demonstrated that chronic Ang II infusion into mice induces Hif-1α in the aortic wall, it remains unclear whether and how Hif-1α activation in VSMCs contributes to vascular remodelling in living systems.

To clarify the contribution of Hif-1α in medial VSMCs to the Ang II-induced vascular remodelling, we created mice lacking the Hif-1α gene, specifically in SMCs. The present study demonstrated that the Ang II-induced medial thickening with VSMC hypertrophy and vascular fibrosis in the aortae was suppressed in Hif-1α-deficient mice. Furthermore, Ang II-induced macrophage infiltration and superoxide production were also ameliorated in the aortic wall of Hif-1α-deficient mice. Consistent with those results, the expression of genes related to vascular remodelling, including fibrosis, inflammation, and the Ang II-AT1R-endothelin implemented by the University of Tokushima, Tokushima, Japan, and this investigation conformed to US National Institutes of Health (NIH Publication, 8th Edition, 2011). Hif-1α-deficient mice were mated with SM22α-Cre transgenic mice to produce SM22α-Cre/+; Hif-1α-deficient mice (SMKO mice) and SM22α-Cre+/−; Hif-1α-deficient mice (control mice). The mice were divided into four groups (n = 10–12): control mice without Ang II (Wako, Osaka, Japan) infusion, control mice with Ang II infusion, SMKO mice without Ang II infusion, and SMKO mice with Ang II infusion. The mice were anaesthetized with an i.p. injection of pentobarbital sodium and the aorta or the heart was isolated for further analysis at the end of the experiments (qRT–PCR, western blotting, histomorphology, and immunofluorescence).

Role of SMC-derived Hif-1α in vascular remodelling

2. Methods

2.1 Animals and animal experiments

All experimental procedures conformed to the guidelines for animal experimentation implemented by the University of Tokushima, Tokushima, Japan, and this investigation conformed to US National Institutes of Health (NIH Publication, 8th Edition, 2011). Hif-1α-deficient mice were mated with SM22α-Cre transgenic mice to produce SM22α-Cre/+; Hif-1α-deficient mice (SMKO mice) and SM22α-Cre+/−; Hif-1α-deficient mice (control mice). The mice were divided into four groups (n = 10–12): control mice without Ang II (Wako, Osaka, Japan) infusion, control mice with Ang II infusion, SMKO mice without Ang II infusion, and SMKO mice with Ang II infusion. The mice were anaesthetized with an i.p. injection of both pentobarbital sodium and 1% lidocaine hydrochloride (Xylocaine; Astra-Zeneca, London, UK) was injected subcutaneously for local analgesia. The absence of the pedal withdrawal reflex was checked frequently to ensure adequacy of anaesthesia with plans to use more if the mice exhibited any signs of pain during the operation. Heart rate and respiration rate were also monitored during the procedures. Ang II dissolved in normal saline was continuously infused at 1.7 μg/kg/min for 4 weeks by means of a subcutaneously implanted osmotic minipump (Alzet model 2004; Alza Corp., Mountain View, CA, USA). Systolic blood pressure (SBP) was measured by a computerized tail-cuff system (BP-98A; Softron, Tokyo, Japan). Urinary albumin excretion was assessed as the ratio of urinary albumin (μg) measured by a Mouse Albumin ELISA Kit (AKRAL-021; Shibayagi, Shibukawa, Japan) to urinary creatinine (mg) (277–10501; Wako) (n = 8–10). After 4 weeks of Ang II infusion, all the mice were euthanized with an i.p. injection of pentobarbital sodium (150 mg/kg) and the aorta or the heart was isolated for further analysis at the end of the experiments (qRT–PCR, western blotting, histomorphology, and immunofluorescence).

In some experiments, hydralazine (12.5 mg/L) (hydralazine hydrochloride; Hydril 753; Sigma Aldrich) was added to drinking water to adjust SBP of control mice with Ang II to that of SMKO mice with Ang II.

2.2 Superoxide detection and measurement of NADPH oxidase activity in aortae

We directly evaluated superoxide production in the aortic wall by in situ dihydroethidium (DHE) staining as previously described (n = 6–8) and measured tissue NADPH oxidase activity as previously described (n = 6–8).

2.3 Measurement of cell volume by cell analyser

VSMC volume was determined by measuring the mean of forward scatter (FS) of propidium iodide negative cells with a cell analyser (EPICS XL-MCL; BECKMAN COULTER).

2.4 Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Statistical significance was assessed by Student’s t-test for two comparisons, by a two-way analysis of variance (ANOVA), or by a one-way ANOVA followed by the Bonferroni post hoc test or Dunnett test for multiple comparisons as described in each Figure, where values of P < 0.05 or P < 0.01 were considered significant; n is the number of individual experiments.

An extended version of the materials and methods is available in Supplementary material online.

3. Results

3.1 Induction of Hif-1α expression in vivo and haemodynamic changes by chronic Ang II infusion into mice

The deficiency of the Hif-1α gene in SMCs significantly reduced Hif-1α mRNA and protein expression in mouse whole aortae. Although chronic Ang II infusion induced the expression of both Hif-1α mRNA and protein in the aortae of control mice, it had no such effect on SMKO mice (Figure 1A–C). Hif-1α immunofluorescence confirmed that SMKO mice hardly had any Hif-1α protein in the aortic media, and that Ang II induced Hif-1α nuclear accumulation in the adventitia of SMKO mouse aortae and in both the media and adventitia of control mouse aortae (see Supplementary material online, Figure S1).

Chronic Ang II infusion causes not only vascular remodelling, but also heart hypertrophy and hypertension. We found that SMC-specific Hif-1α deficiency did not affect the ratio of heart weight to body weight in the Ang II-infused mice, but suppressed the Ang II-induced SBP elevation 4 weeks after the start of Ang II infusion (Figure 1D and E). In the absence of any treatment, no differences in SBP were observed between control mice and SMKO mice; neither were there any differences in urinary albumin excretion (Figure 1F). The results indicate that SMC-derived Hif-1α contributes to haemodynamic changes with Ang II infusion, but not under normal conditions.
3.2 Hif-1α deficiency in SMCs suppressed Ang II-induced vascular remodelling in mice

Masson's trichrome staining of the aortic sections revealed that medial thickening and perivascular fibrosis were induced by Ang II infusion in control mice, but not in SMKO mice (Figure 2A–C and see Supplementary material online, Figure S2). In female mice, SMC-specific Hif-1α deficiency suppressed the Ang II-induced vascular remodelling (see Supplementary material online, Figure S3). Ang II-induced perivascular fibrosis in the coronary arteries was also suppressed by SMC-specific Hif-1α deficiency (see Supplementary material online, Figure S4). The results indicate that Hif-1α in VSMCs contributes to Ang II-induced medial thickening and vascular fibrosis in aortae, and those effects have no gender difference. In accordance with those results, the mRNA expression in the aortae of plasminogen activator inhibitor-1 (PAI-1) and collagen I, two molecules implicated in fibrosis, was enhanced by Ang II infusion in control mice, but those effects were suppressed in SMKO mice (Figure 2D).

Because VSMC hypertrophy contributes to medial thickening, we measured cell volume with a cell analyser in an in vitro system. We first confirmed the extent of Hif-1α deletion in VSMCs isolated from the aortae of SMKO mice (SMKO VSMCs) compared with VSMCs from control mice (control VSMCs) by measuring the levels of genomic DNAs and transcripts (see Supplementary material online, Figure S5A and B). Hypertrophy was induced by incubation with Ang II for 72 h in VSMCs isolated from the aortae of control mice (control VSMCs), but not in VSMCs from SMKO mice (SMKO VSMCs). Even without any treatments, SMKO VSMCs were smaller than control VSMCs (Figure 2E). We also examined the effect of Hif-1α knockdown on the hypertrophy of human aortic smooth muscle cells (HASMCs) after confirming the extent of Hif-1α deletion by Hif-1α siRNA treatment (see Supplementary material online, Figure S5C). Ang II-induced HASMC hypertrophy was also suppressed by Hif-1α knockdown (Figure 2F). Together, the results indicate that Hif-1α in VSMCs contributes to Ang II-induced vascular remodelling via VSMC hypertrophy.

3.3 SMC-specific Hif-1α deficiency suppressed Ang II-induced vascular remodelling independently of blood pressure change

As shown in Figure 3, although the administration of hydralazine, a vasodilator, suppressed the Ang II-induced SBP elevation in control mice to levels comparable with that in SMKO mice, hydralazine did not affect the Ang II-induced vascular remodelling. The results suggest that SMC-specific Hif-1α deficiency suppresses Ang II-induced vascular remodelling independently of the change of SBP by Ang II infusion in SMKO mice.

Figure 1 Hif-1α expression in mouse whole aortae and haemodynamic changes by chronic Ang II infusion into the mice. (A) Hif-1α mRNA expression in aortae was measured by real-time quantitative RT–PCR and normalized to β-actin expression (n = 10–12). Hif-1α protein expression was assessed by western blotting. (B) Representative immunoblot. The rightmost two lanes show the band patterns for the cell lysates of untreated and DMOG- (DMOG 1 mmol/L, 4 h) treated control mouse VSMCs, respectively, as a positive control to confirm Hif-1α-specific bands. (C) Densitometric analysis (n = 10–12). Statistical significance was assessed by a two-way ANOVA and multiple comparisons were corrected by Bonferroni’s t-test. Means ± SEM, **P < 0.01. (D) Ratio of heart weight (mg) to body weight (g) (n = 10–12). (E) SBP was measured by the tail-cuff method before and after Ang II infusion for 2 and 4 weeks (n = 10–12). (F) Urinary albumin excretion was measured by ELISA and normalized to urinary creatinine excretion (n = 8–10). Statistical significance was assessed by a two-way ANOVA and multiple comparisons were corrected by Bonferroni’s t-test. Means ± SEM, **P < 0.01 vs. sham, ##P < 0.01 vs. control mice with Ang II.
3.4 SMC-specific Hif-1α deficiency suppressed Ang II-induced vascular inflammation

SMC-specific Hif-1α deficiency also suppressed Ang II-induced macrophage infiltration in aortic adventitia (Figure 4A) and this was accompanied by the suppression of Ang II-induced mRNA expression of macrophage markers, CD68 and F4/80, and inflammatory cytokines, MCP-1, interleukin-1β (IL-1β), IL-6, and TNF-α in the aorta (Figure 4B). The results indicate that Hif-1α deficiency in SMCs of mice suppresses Ang II-induced vascular inflammation.

3.5 SMC-specific Hif-1α deficiency suppressed Ang II-induced superoxide production in aortic wall

Superoxide production in Ang II-induced vascular remodelling was reported to be enhanced by the Ang II-AT1R-NADPH oxidase signalling axis.5,16 DHE staining of the aortic sections showed that chronic Ang II infusion enhanced superoxide production in the aortic media of control mice, but not in those of SMKO mice (Figure 5A and B). The lucigenin chemiluminescence assay also denoted that SMC-specific Hif-1α deficiency suppressed Ang II-induced NADPH oxidase activity in the...
aortae (Figure 5C), and this was accompanied by the suppression of Ang II-induced mRNA expression of Nox1 and p22phox, two components of NADPH oxidase (Figure 5D). The results suggest that Hif-1α deficiency in SMCs of mice suppresses Ang II-induced superoxide production in the aortic media via suppression of the Ang II-AT1R-NADPH oxidase signalling axis.

3.6 Hif-1α in VSMCs is an intrinsic modulator of genes related to vascular remodelling

To investigate whether Hif-1α intrinsically mediates the expression of fibrosis- or inflammation-related genes in VSMCs, dimethyloxalylglycine (DMOG), a competitive inhibitor of PHD, was used to specifically induce the accumulation of Hif-1α proteins. We first confirmed that Hif-1α protein expression was induced by DMOG, reaching its peak
after incubation for 4 h, and found that this effect was abolished by Hif-1α siRNA treatment (Figure 6A and B). PAI-1 and collagen I mRNA expression, which was induced by chronic Ang II infusion in mouse aortae (Figure 2D), was also enhanced by DMOG after incubation for 24 h. Those effects, however, were suppressed by Hif-1α siRNA treatment (Figure 6C) in HASMCs. Hif-1α siRNA treatment did not affect the DMOG-induced expression of IL-1β and IL-6 in HASMCs. The results confirm that SMC-derived Hif-1α is an intrinsic modulator of the expression of extracellular matrix-related genes involved in vascular remodelling.

3.7 SMC-specific Hif-1α deficiency affects Ang II-induced phosphorylation pathway for Hif-1α induction in aortae

We showed that Ang II increased Hif-1α protein expression following the induction of c-Src phosphorylation (pTyr418) and Erk1/2 phosphorylation in HASMCs (Figure 7A). The increase in Hif-1α protein expression was suppressed by preincubation with the MEK inhibitor, PD98059 (Figure 7B), and by preincubation with Src kinase inhibitors, PP1 and PP2 (Figure 7C). The results suggest that Ang II induces Hif-1α protein expression via c-Src phosphorylation (pTyr418) and Erk1/2 phosphorylation in the in vitro system (Figure 7D). Our in vivo investigation also demonstrated that Ang II infusion induced c-Src phosphorylation (pTyr418) in the aortae of control mice, but not in those of SMKO mice (Figure 7E). These lines of evidence suggest that SMC-specific Hif-1α deficiency affects at least the Ang II-induced c-Src phosphorylation (pTyr418) for Hif-1α induction in mouse aortae, and agree well with the decreased superoxide production caused by Hif-1α deficiency in aortic VSMCs of Ang II-infused mice.

4. Discussion

In the present study, we investigated the role of Hif-1α expressed in VSMCs in Ang II-induced vascular remodelling in an in vivo experimental
mechanisms. (i) PAI-1 and collagen I mRNA expression was enhanced in the whole aortae of control mice with Ang II infusion, but those effects were suppressed in SMKO mice with Ang II infusion. In our in vitro study, PAI-1 and collagen I mRNA expression was enhanced following Hif-1α induction by DMOG in HASMCs, but was suppressed by Hif-1α knockdown. Those results suggest that Hif-1α in VSMCs contributes to the Ang II-induced vascular fibrosis with regulation of fibrosis-related genes. (ii) The fact that Ang II induces hypertrophy in control VSMCs but not in SMKO VSMCs suggests that the Ang II-induced medial thickening in the aortae is mediated by VSMC hypertrophy via Hif-1α induction in VSMCs. (iii) SMC-specific Hif-1α deficiency suppressed Ang II-induced macrophage infiltration in aortae and Ang II-induced mRNA expression of macrophage markers and cytokines, including IL-1β, TNF-α, IL-6, and MCP-1, in the aortae. Those findings also suggest that Hif-1α in VSMCs is involved in Ang II-induced inflammatory responses in vascular remodelling.

Furthermore, our in vivo investigation showed that Ang II infusion induced c-Src phosphorylation (pTyr418), which was involved in the Ang II-AT1R signalling pathway and contributed to Hif-1α induction in VSMCs of control mice, but not in those of SMKO mice. This result agreed well with the decreased superoxide production in the aortae of SMKO mice with Ang II infusion, compared with the control mice with Ang II infusion. Thus, SMC-specific Hif-1α deficiency might suppress vascular remodelling signalling downstream of Hif-1α activated by Ang II and Ang II-induced vascular remodelling also by suppressing Ang II-AT1R signalling in the chronic phase. This means that the feedback regulation by Hif-1α may be involved in Ang II-AT1R-induced vascular remodelling signalling in the chronic phase.

In this study, we elucidated that SMC-specific Hif-1α deficiency suppressed Ang II-induced vascular remodelling. On the other hand, Matsuura et al. suggested that PHD inhibitors, which induce Hif-1α, suppress Ang II-induced vascular remodelling via down-regulation of vascular AT1R expression. However, they also suggested that Hif-1α does not contribute to AT1R expression in rat VSMCs because the hypoxia response element does not exist in the rat AT1R gene promoter region. PHD inhibitors administration may affect various vascular cells, not only VSMCs. We have demonstrated directly that SMC-derived Hif-1α contributes to Ang II-induced vascular remodelling in an in vivo genetic model.

We also found that SMC-specific Hif-1α deficiency suppressed Ang II-induced SBP elevation in the chronic phase. As it was reported that the suppression of Ang II-induced SBP elevation was correlates with the suppression of Ang II-induced vascular remodelling,21 we supposed that the suppression of Ang II-induced vascular remodelling in SMKO mice decreased vascular resistance, thereby resulting in the suppression of the SBP elevation. The most recent study, in which SMC-specific Hif-1α-deficient mice were also used, indicated the effects of i.v. Ang II administration on the dose-dependent increase in the mean blood pressure (MBP) in both their control mice and their mutant mice, and that MBP in the control mice never reached the MBP in the mutant mice.22 On the other hand, unlike their mouse model of acute Ang II infusion, we used a mouse model of chronic Ang II infusion to investigate the role of SMC-derived Hif-1α in vascular remodelling and examined SBP. We supposed that our result of SBP in the chronic phase depended on also the changes of vascular remodelling. The SMC-derived Hif-1α might have the different contribution to blood pressure elevation by Ang II depending on the acute phase or the chronic phase. Further investigation is required to verify this matter.
In addition, we found that Hif-1α mRNA expression in the heart was also decreased in our mutant mice (see Supplementary material online, Figure S6) as was indicated in their report. They showed that there were no changes in cardiac function between the control mice and the mutant mice, and we showed that the deficiency of SMC-derived Hif-1α did not affect heart weight but suppressed Ang II-induced vascular remodelling independently of the change of blood pressure. Thus, we supposed that the reduction of Hif-1α mRNA expression in the heart had no effect on vascular remodelling in SMKO mice.

Our results also showed that SMC-specific Hif-1α deficiency suppressed Ang II-induced urinary albumin excretion (Figure 1f). Because the change of urinary albumin excretion was correlated with that of SBP, the suppression of Ang II-induced SBP elevation in SMKO mice might contribute to the reduction of Ang II-induced kidney dysfunction in the mice. Zhu et al.23 showed that Hif-1α gene silencing attenuated Ang II-induced renal injury. Thus, SMC-derived Hif-1α in the kidney might also contribute to the Ang II-induced renal injury.

To summarize, we propose new mechanisms underlying Ang II-induced vascular remodelling via Hif-1α up-regulation in VSMCs. One clinical study suggested that intermittent hypoxia (IH) increases blood pressure in humans through a renin–angiotensin system-dependent mechanism.24 Because IH increases Hif-1α25 and we suggested that SMC-derived Hif-1α deficiency suppresses Ang II-induced hypertension in the chronic phase, SMC-derived Hif-1α might exert...
an influence on hypertension in patients presenting with obstructive sleep apnoea through a renin–angiotensin system-dependent mechanism. Moreover, because other clinical studies have shown that Hif-1α is induced in the human atherosclerotic region, including VSMCs, inhibition in VSMCs may be a novel therapeutic strategy to prevent vascular remodelling.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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