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Authors' contributions
MH, MK, and YT carried out experiments. MH, TN, ST, and OS participated in the design of the study. MK and YT performed the statistical analysis. MH and SH wrote the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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ORIGINAL CONTRIBUTIONS

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Role of Complement 3a in the Synthetic Phenotype and Angiotensin II-Production in Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats

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BACKGROUND

Spontaneously hypertensive rats (SHR)-derived vascular smooth muscle cells (VSMCs) show exaggerated growth with a synthetic phenotype and angiotensin II (Ang II)-production. To evaluate the contribution of complement 3 (C3) or C3a toward these abnormalities in SHR, we examined effects of a C3a receptor inhibitor on proliferation, phenotype, and Ang II-production in VSMCs from SHR and Wistar–Kyoto (WKY) rats.

METHODS

Expression of pre-pro-C3 messenger RNA (mRNA) and C3 protein was evaluated by reverse transcription-PCR and western blot analyses, and C3a receptor mRNA was evaluated by reverse transcription-PCR analysis in quiescent VSMCs from SHR and WKY rats. We examined the effects of the C3a inhibitor, SB290157, on proliferation and the expression of phenotype-marker and Krueppel-like factor 5 (KLF-5) mRNAs in VSMCs from SHR and WKY rats. We examined effects of C3a receptor inhibitor, SB290157, on Ang II-production in conditioned medium of VSMCs from SHR and WKY rats by a radioimmunoassay.

RESULTS

Expression of pre-pro-C3 mRNA and C3 protein was significantly higher in SHR VSMCs than WKY VSMCs. SB290157 significantly inhibited proliferation of VSMCs from SHR, but not in cells from WKY rats. Relative to WKY VSMCs, SB290157 significantly increased the low expression of SM22α mRNA and decreased the high expression of osteopontin mRNA in SHR VSMCs. SB290157 significantly decreased the high expression of KLF-5 and Ang II-production in VSMCs from SHR, but not in cells from WKY rats.

CONCLUSIONS

C3a induces exaggerated growth, a synthetic phenotype and Ang II-production in SHR-derived VSMCs. C3a may be primarily involved in cardiovascular remodeling in hypertension.

Keywords: *angiotensin II; blood pressure; complement 3; hypertension; Kruppel-like factor 5; phenotype; proliferation; spontaneously hypertensive rat; vascular smooth muscle cell*

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Patients with essential hypertension, a hereditary polygenic disease, are eventually complicated with stroke, cardiovascular remodeling, and nephrosclerosis. These complications are practical targets for therapy of essential hypertension with antihypertensive medicines. Spontaneously hypertensive rats (SHR), a genetic animal model for essential hypertension, show exaggerated growth of cardiovascular organs in comparison with normotensive Wistar–Kyoto (WKY) rats.[1,2] Enhanced DNA synthesis and organ hypertrophy have been described in SHR even as early as the day of birth.[3–5] SHR-derived vascular smooth muscle cells (VSMCs) in culture show a higher specific growth rate, abnormal contact inhibition, accelerated entry into S phase of the cell cycle, and nonspecific hyperproliferation in response to various growth factors in comparison to cells from WKY rats.[6,7] These behaviors may reflect intrinsic abnormalities in SHR that are not caused by pressure overload because there is no blood pressure in culture. Therefore, these characteristics of VSMCs from SHR appear to be associated with genetic abnormalities. We found that SHR-derived VSMCs generate angiotensin II (Ang II) in homogenous cultures.[8] We have reported that the mechanism underlying this enhanced generation of Ang II in SHR-derived VSMCs appears to be a change from the contractile to the synthetic phenotype in comparison to cells from WKY rats.[9,10]

It is possible that genetic abnormalities are involved in the exaggerated growth and synthetic phenotype of VSMCs from SHR. We investigated the genes that are responsible and found by microarray analysis that the messenger RNA (mRNA) encoding complement 3 (C3) is expressed only in VSMCs from SHR and is associated with both the synthetic phenotype and exaggerated growth.[11] At the same time, we demonstrated

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that C3 also changes renal mesangial cells to the synthetic phenotype.[12] We investigated mechanisms underlying the C3-induced phenotypic changes and found that C3 stimulates Kruppel-like factor 5 (KLF-5) promoter activity through extracellular signal-regulated kinase (ERK) signaling.[13]

C3 is a 190 kDa glycoprotein that consists of two polypeptide chains, which are produced from liver, monocytes, and macrophages, and it is essential for eliciting the complement response.[14] C3 from pre-pro-C3 mRNA is secreted after cleavage of the heterodimer, and then C3 is proteolytically cleaved into C3a (molecular weight 9,000) and C3b (molecular weight 185,000). C3a is an anaphylotoxin, and C3b serves as an opsonizing agent.[15] We have demonstrated that the increases in Ang II-production in VSMCs in homogeneous culture are associated with changes to the synthetic phenotype in VSMCs.[8,16]

In the current study, in order to evaluate whether C3 or C3a is associated with the exaggerated growth of the synthetic phenotype and increased Ang II-production in VSMCs from SHR, we examined effects of the C3a receptor inhibitor on proliferation, phenotype, and Ang II-production in VSMCs from SHR and WKY rats.

METHODS

Ethics and animals. Our investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, 1996). The ethics committee of the Nihon University School of Medicine examined every research protocol involving the use of living animals. SHR/Izm and WKY/Izm rats were obtained from Japan SLC (Hamamatsu, Japan).

Cell culture and establishment of quiescence. VSMCs were obtained from aortic explants from 3-week-old prehypertensive male SHR/Izm and WKY/Izm rats. VSMCs were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (Gibco Life Technologies, Gaithersburg, MD), 100 U/ml penicillin, and 100 mg/ml streptomycin. Experiments were performed on cells between the 5th and 10th passages. Trypsinized cells were plated into 24-well culture dishes at a density of 10^5 cells/cm^2. They were allowed to grow in DMEM containing 10% calf serum for 24 h, and the culture medium was then changed to DMEM with 0.2% calf serum. The cells were incubated in this medium for 48–72 h to establish quiescence.

Proliferation of VSMCs. VSMCs from SHR and WKY rats were inoculated and grown into DMEM containing 5% calf serum in the absence or presence of 0.1 μmol/l SB290157 in 24-well culture dishes at a density of 10^5 cells/cm^2. Cells were trypsinized with 0.05% trypsin at 24, 48, and 72 h after inoculation, and cell numbers were counted in a Coulter counter (Coulter Electronics, Luton, UK).

Semiquantitative reverse transcription-PCR analysis. Total RNAs from samples were extracted from quiescent VSMCs from SHR and WKY rats with ISOGEN (Nippon Gene, Tokyo, Japan). Primer sequences are listed in **Table 1**. 18S ribosomal RNA was used as an internal control. To confirm that no genomic DNA was co-amplified by PCR, control reverse transcription-PCR experiments were performed with each set of primers but without reverse transcriptase; no product was amplified. For semiquantative analysis of mRNA, the kinetics of the PCR reaction were monitored; the number of cycles at which the PCR products were detectable on the gel was compared between samples.[17] Serial tenfold dilutions of complementary DNA (100, 10, and 1 ng) were amplified; the PCR products were detectable at earlier cycles with increasing amounts of complementary DNA. PCR was performed for 30 cycles in a DNA thermal cycler (Perkin-Elmer Cetus, Waltham, MA), and products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized by ultraviolet illumination.

Western blot analysis for C3 protein in VSMCs. VSMCs (5×10^4 cells/cm^2) were disrupted with lysis buffer (50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Triton X-100). Total proteins were extracted and purified with 100 μl of chloroform and 400 μl of methanol. Protein samples were boiled for 3 min and subjected to electrophoresis on 8% polyacrylamide gels and then transblotted to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Blots were incubated with rabbit polyclonal antibodies specific for C3 (Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse monoclonal antibody specific for α-tubulin (Sigma, St Louis, MO) as an internal control, and were then incubated with goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulin G (Bio-Rad Laboratories). Immunocomplexes were detected by enhanced chemiluminescence (ECL, Amersham, UK).

Measurement of Ang II in conditioned medium. VSMCs (10^6) from SHR and WKY rats were inoculated in 10 cm^2 wells with

Table 1 | PCR primers and product sizes

Target mRNA		Primer Sequence	PCR product (bp)
Pre-pro-C3	5′	5′-CAGCAGACCTCAGTGACCAA-3′	351
	3′	5′-ATAGCTGTCAGCCAGGTGCT-3′	
C3a receptor	5′	5′-GACCTACACTCAGGGC-3′	376
	3′	5′-ATGACGGACGGGATAAG-3′	
SM22α	5′	5′-TTGAAGGCCAATCACGTGCTT-3′	312
	3′	5′-AAGCCAGTGAAGGTGCCTGAG -3′	
Osteopontin	5′	5′-TGGCTTACGGACTGAGGTCA-3′	486
	3′	5′-GACCTCAGAAGATGAACTCT-3′	
KLF-5	5′	5′-ACCTACTTTCCCCCATCACC-3′	205
	3′	5′-CCGGGTTACTCCTTCTGTTG-3′	
18S rRNA	5′	5′-CGACGACCCATTCGAACGTCT-3′	312
	3′	5′-GCTATTGGAGCTGGAATTACCG-3′	

KLF-5, Kruppel-like factor 5; mRNA, messenger RNA; pre-pro-C3, pre-pro-complement 3; rRNA, ribosomal RNA.

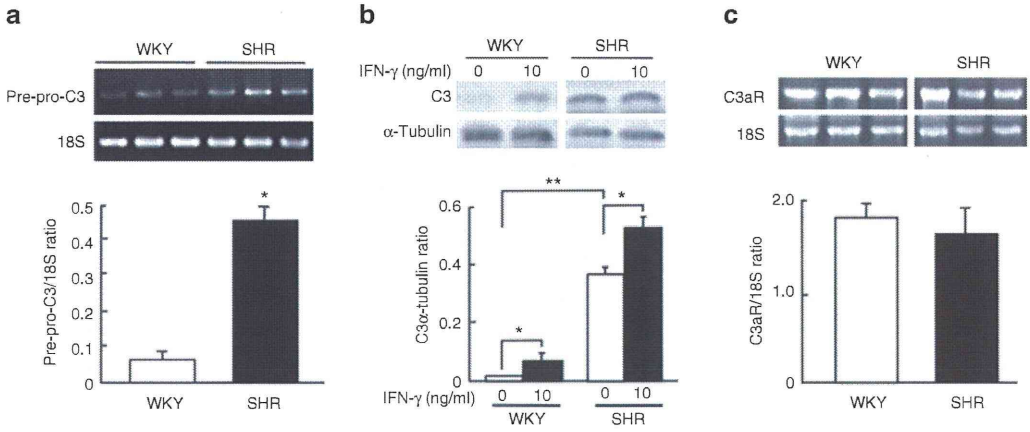

Figure 1 | Expression of complement 3 (C3) and the C3a receptor in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. Total RNA was extracted from quiescent VSMCs of 3-week-old SHR and WKY rats. Semiquantitative determination of the abundance of (**a**) C3 and (**c**) C3a receptor messenger RNA (mRNA) was performed by reverse transcription and PCR analysis. The ratio of each mRNA to 18S ribosomal RNA (rRNA) was evaluated by analysis of amplified PCR products. Data were expressed as mean ± s.e.m. ($n = 6$ from two analyses). (**b**) Western blot analysis of expression of C3 protein in cultured VSMCs from SHR and WKY rats without or with interferon-γ (IFN-γ). Quiescent VSMCs from 3-week-old SHR and WKY rats were incubated without (control) or with 10 ng/ml IFN-γ for 20 h. The ratio of abundance of IFN-γ protein to α-tubulin was evaluated by densitometric analysis. Data are means ± s.e.m. ($n = 3$). *$P < 0.05$, **$P < 0.01$ between indicated column.

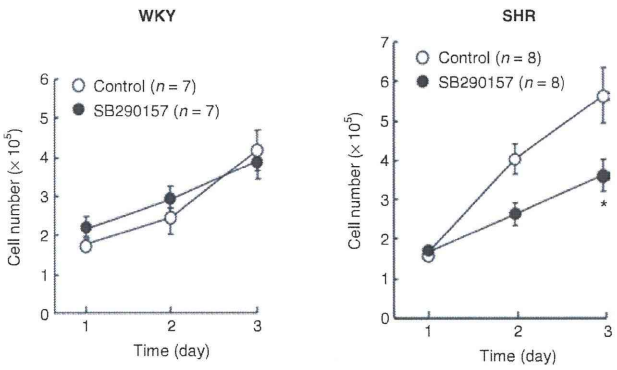

Figure 2 | Effects of the complement 3a receptor inhibitor, SB290157, on proliferation of vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. VSMCs were inoculated at 5×10^4 cells/cm^2 in DMEM containing 5% calf serum without (control) or with 0.1 μmol/l SB290157. Cells were replenished with DMEM containing 5% calf serum and 0.1 μmol/l SB290157 every 24 h. They were trypsinized at 24, 48, and 72 h after inoculation, and cell number was determined in a Coulter counter. Data are means ± s.e.m. ($n = 8$). *$P < 0.01$ vs. control. DMEM, Dulbecco's modified Eagle's medium.

DMEM containing 10% calf serum and incubated for 24 h. The cells were then washed twice with phosphate-buffered saline, and incubated first for 24 h with serum-free DMEM and then for 48 h with 2 ml of fresh serum-free DMEM in the absence or presence of 0.1 μmol/l SB290157. The culture medium was collected and centrifuged at 600*g* for 10 min, and the resulting supernatant (conditioned medium) was collected and treated with 1 mmol/l each of aprotinin, leupeptin, and pepstatin A as well as 0.1 mmol/l phenylmethylsulfonyl fluoride. Samples were applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, MA), and peptides were eluted with 3 ml of methanol-water-trifluoroacetic acid (80:19.9:0.1, vol/vol). The eluate was dried in a vacuum centrifuge and subjected to a radioimmunoassay for Ang II. The antiserum to Ang II (GE Healthcare, Buckinghamshire, UK) showed <1% cross-reactivity with Ang I, and 100% cross-reactivity with Ang III (heptapeptide), Ang II (3–8) (hexapeptide), and Ang II (4–8) (pentapeptide).

Statistical analysis. Values are reported as the mean ± s.e.m. Statistical analysis was done with Student's *t*-test for unpaired data, two-way analysis of variance, or Duncan's multiple range test.

RESULTS

Expression of C3 and the C3a receptor in VSMCs

To assess C3 and receptor systems, we evaluated expression of pre-pro-C3 mRNA, C3 protein and C3a receptor mRNA in VSMCs from SHR and WKY rats. The abundance of pre-pro-C3 mRNA was significantly ($P < 0.01$) higher in the VSMCs from SHR than in cells from WKY rats (**Figure 1a**). C3 protein was not expressed in cells from WKY rats, although it was increased with 10 ng/ml interferon-γ, whereas the abundance of C3 protein was signicantly ($P < 0.05$) higher in VSMCs from SHR rats than WKY rats, and C3 was also increased significantly ($P < 0.05$) with interferon-γ (**Figure 1b**). VSMCs from SHR and WKY rats expressed C3a receptor mRNA. There were no differences in the abundances of C3a receptors between either strain (**Figure 1c**).

Effects of the C3a receptor inhibitor on proliferation of VSMCs

Figure 2 shows the effects of C3a receptor inhibitor, SB290157, on proliferation of VSMCs from SHR and WKY rats. The increase in cell numbers in cultures grown in DMEM containing 5% calf serum was higher in VSMCs from SHR than in cells from WKY rats. SB290157 significantly ($P < 0.05$) inhibited the increase in the number of VSMCs in SHR rats, whereas SB290157 did not affect the increase in the number of cells in WKY rats.

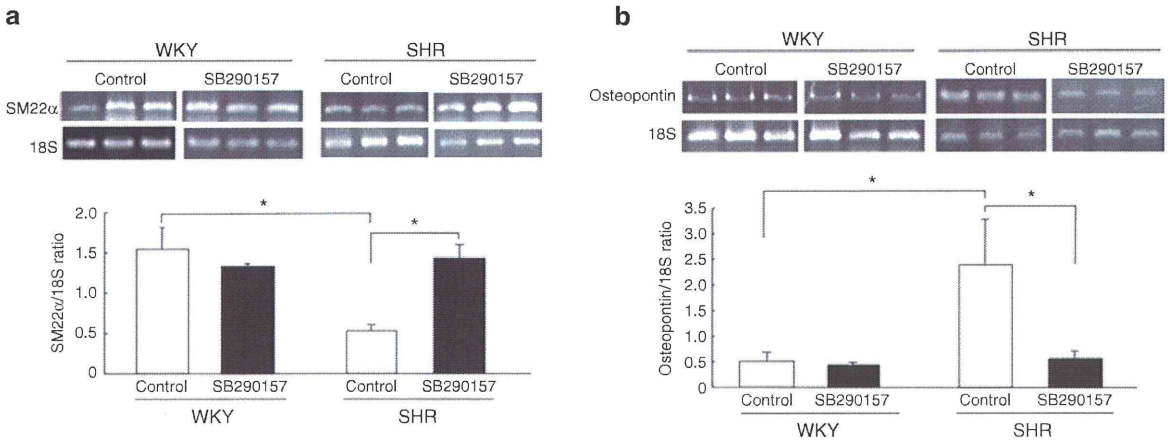

Figure 3 | Effects of the complement 3a receptor inhibitor, SB290157, on expression of the mRNAs of the phenotypic markers, (**a**) SM22α and (**b**) osteopontin, in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. Quiescent VSMCs from 3-week-old SHR and WKY rats were incubated without (control) or without 0.1 μmol/l SB290157 for 24 h. Total RNA was extracted and the abundance of SM22α and osteopontin mRNAs was evaluated by reverse transcription and PCR analysis. The ratio of abundance of each mRNA to 18S rRNA was evaluated by densitometric analysis. Data are means ± s.e.m. ($n = 8$ from two analyses). *$P < 0.05$ between indicated columns. mRNA, messenger RNA; rRNA, ribosomal RNA.

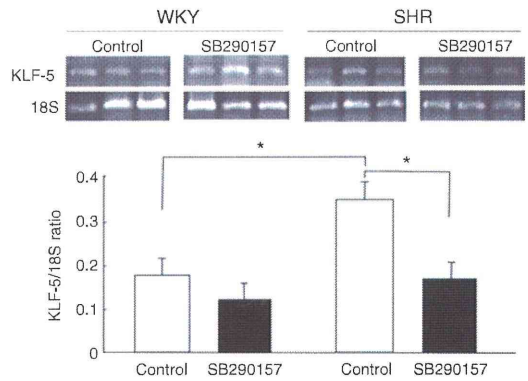

Figure 4 | Effects of the complement 3a receptor inhibitor, SB290157, on expression of Kruppel-like factor 5 (KLF-5) mRNAs in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. Quiescent VSMCs from 3-week-old SHR and WKY rats were incubated without (control) or without 0.1 μmol/l SB290157 for 24 h. Total RNA was extracted and the abundance of KLF-5 mRNAs was evaluated by reverse transcription and PCR analysis. The ratio of abundance of each mRNA to 18S rRNA was evaluated by densitometric analysis. Data are means ± s.e.m. ($n = 8$ from two analyses). *$P < 0.05$ between the indicated columns. mRNA, messenger RNA.

Effects of the C3a receptor inhibitor on the phenotype of VSMCs

Basal abundances of SM22α mRNA as a marker for the contractile phenotype of VSMCs were significantly ($P < 0.05$) lower in VSMCs from SHR rats than in cells from WKY rats (**Figure 3a**). Basal abundances of osteopontin mRNA as a marker for the synthetic phenotype of VSMCs were significantly ($P < 0.05$) higher in VSMCs from SHR rats than in cells from WKY rats (**Figure 3b**). These findings indicate that the phenotype of VSMCs from SHR is more synthetic than in cells from WKY rats in serum deprivation. The C3a receptor inhibitor SB290157 significantly ($P < 0.05$) increased the abundance of SM22α mRNA (**Figure 3a**) and decreased the abundance of osteopontin mRNA in VSMCs from SHR rats (**Figure 3b**), but these did not occur in cells from WKY rats, indicating that endogenous C3a induces the synthetic phenotype of SHR-derived VSMCs.

Effects of the C3a receptor inhibitor on expression of KLF-5 mRNA in VSMCs

Figure 4 shows the effects of the SB290157 C3a receptor inhibitor on expression of KLF-5 mRNA in VSMCs from SHR and WKY rats. Basal abundances of KLF-5 mRNA were significantly higher ($P < 0.05$) in VSMCs from SHR than in cells from WKY rats. SB290157 significantly ($P < 0.05$) decreased the abundance of KLF-5 mRNA in VSMCs from SHR rats, but SB290157 did not affect the abundance of KLF-5 mRNA in cells from WKY rats.

Effects of the C3a receptor inhibitor on Ang II-production in VSMCs

Figure 5 shows the effects of the SB290157 C3a receptor inhibitor on Ang II-production in VSMCs from SHR and WKY rats. Basal production of Ang II was significantly higher ($P < 0.05$) in VSMCs from SHR than in cells from WKY rats. Incubation with SB290157 significantly ($P < 0.05$) decreased production of Ang II in VSMCs from SHR, but it did not affect production of Ang II in cells from WKY rats.

DISCUSSION

In the present study, we confirmed that SHR-derived VSMCs abundantly express pre-pro-C3 mRNA and produce C3 protein when compared to cells from WKY rats. We previously evaluated the expression of 1,300 transcripts by microarray analysis with total mRNA extracted from the mid-layer of smooth aortic muscle from 3-week-old prehypertensive SHR and WKY rats *in vivo*. mRNAs encoding six genes including pre-pro-C3 were expressed only in aortic smooth muscle from SHR, and not in smooth muscle from WKY rats. Moreover,

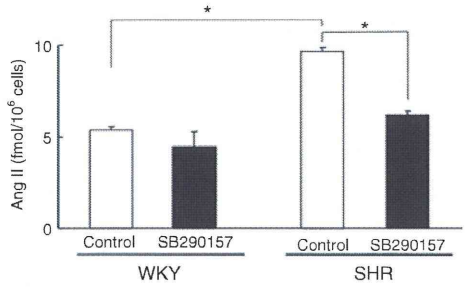

Figure 5 | Effects of the complement 3a receptor inhibitor, SB290157, on production of angiotensin II (Ang II) in conditioned medium of vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. VSMCs (10^6) were incubated first for 24 h with serum-free DMEM and then for 48 h with 2 ml of fresh serum-free DMEM in the absence or presence of 0.1 μmol/l SB290157. The culture medium was collected and subjected to radioimmunoassay for Ang II. Data are means ± s.e.m. ($n = 6$). *$P < 0.05$ between indicated columns. DMEM, Dulbecco's modified Eagle's medium.

pre-pro-C3 mRNA was detected only in cultured VSMCs from SHR, not in cells from WKY rats *in vitro*.[11] In the microarray analysis, there were no differences in the levels of mRNA for C4, C8, and C9 in aortic smooth muscle from SHR and WKY rats. These results indicated that the abundant expression of C3 in SHR-derived VSMCs is independent of the complement generating pathways.

Ueda *et al.*[18] showed that C3 is produced by human aortic VSMCs but not by a human smooth muscle cell line or VSMCs from human umbilical cord vein. Thus C3-production may differ between smooth muscle cell types. Thus since the enhanced expression of pre-pro-C3 in VSMCs from SHR might be associated with genetic alterations, we therefore performed the direct sequencing of PCR products for promoter lesions of pre-pro-C3 in genomic DNA extracted from liver of SHR and WKY rats. However, there were no sequence differences in pre-pro-C3 promoter lesions between the two strains (data not shown). It is possible that the enhanced expression of pre-pro-C3 in VSMCs from SHR is associated with epigenetic alterations. Further investigation is required to clarify the different expression of pre-pro-C3 in VSMCs from SHR and WYK rats.

In the present study, we examined effects of a C3a receptor inhibitor on proliferation, phenotype, and Ang II-production of VSMCs from SHR and WKY rats, to evaluate whether C3 or C3a is associated with the exaggerated growth in the synthetic phenotype and Ang II-production in SHR-derived VSMCs. Expression of SM22α mRNA was relatively low, whereas expression of osteopontin mRNA was relatively high in quiescent VSMCs from SHR compared to cells from WKY rats, indicating that SHR-derived VSMCs are rather synthetic. SB290157 increased the low expression of SM22α and decreased the high expression of osteopontin mRNAs in VSMCs from SHR. These findings indicate that endogenous C3a acts to induce the synthetic phenotype of SHR-derived VSMCs as a final effector.

C3 has roles in immune defenses but has also been reported to exert several other biological functions. C3 produced by endothelial cells, monocytes/macrophages, and fibroblasts induces atherosclerosis via activation of several cytokines.[19] C3 is synthesized by embryonic cells and participates in cell differentiation and proliferation.[20] C3 enhances growth of cancer cells,[21] contributes to contraction of VSMCs directly or indirectly through the activation of basophils,[22] and activates MAP kinase in endothelial cells.[23] Schraufstatter *et al.*[24] recently demonstrated that C3a maintains dedifferentiation of mesenchymal stem cells by the phosphorylation of ERK1/2. Thus the C3 and C3a system may induce the synthetic phenotype of VSMCs by its dedifferentiating effects on the mesenchymal cells.

Concerning mechanisms underlying the synthetic phenotype of VSMCs from SHR compared to cells from WKY rats, the expression of KLF-5 mRNA was higher in SHR-derived VSMCs, and was inhibited by the C3a receptor antagonist in the present experiments. These results indicate that the increased expression of the C3–C3a system induces KLF-5, which promotes the synthetic phenotype of SHR-derived VSMCs. In the cardiovascular system, KLF-5 is expressed abundantly in embryonic VSMCs but is decreased in the adult vasculature.[25] KLF-5 expression is markedly upregulated in activated VSMCs and myofibroblasts during atherosclerosis.[25,26] KLF-5 is implicated in alterations in VSMCs during the change to the synthetic phenotype, which includes alterations in gene expression, at the site of vascular injury.[27] We previously demonstrated that exogenous C3a increased the expression of KLF-5 mRNA and promoter activity via ERK signaling in VSMCs.[13] These findings and the present study suggest that endogenous C3a induces the synthetic phenotype of SHR VSMCs from the stimulation of KLF-5.

Production of Ang II was increased in VSMCs from SHR, which was suppressed with the C3a receptor antagonist, indicating that increases in the endogenous C3–C3a system are associated with increased Ang II-production. We have previously demonstrated that the increased Ang II-production in SHR-derived VSMCs is associated with the Ang II-generating proteinases such as cathepsin D and angiotensin-converting enzyme.[7] VSMCs that display the synthetic phenotype have a reduced fractional volume of myofilaments and many synthetic organelles such as Golgi, mitochondria, and endoplasmic reticulum, which generate proteolytic enzymes including cathepsin D and angiotensin-converting enzyme, which produce Ang II, as well as growth factors and cytokines.[28,29] It is possible that endogenous C3a contributes to the increased Ang II-production through the induction of the synthetic phenotype of SHR-derived VSMCs.

Taken together, SHR-derived VSMCs primarily produce C3–C3a, but this does not occur in cells from WKY rats. Endogenous C3a promotes transcriptional activation of KLF-5 to induce the exaggerated growth with a synthetic phenotype and the production of Ang II in VSMCs from SHR.

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ORIGINAL ARTICLE

Protective effects of statin on cardiac fibrosis and apoptosis in adrenomedullin-knockout mice treated with angiotensin II and high salt loading

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Statins exert pleiotropic effects, including antioxidative and cellular protective effects. Endogenous adrenomedullin (AM) induces anti-inflammatory, anti-fibrotic and proangiogenic effects. We examined the effects of simvastatin on cardiac fibrosis and apoptosis in AM heterozygous knockout ($AM^{+/-}$) mice treated with angiotensin (Ang) II and high salt loading. Seven-week-old $AM^{+/-}$ mice were infused with Ang II while on a high-salt diet with or without simvastatin for 2 weeks. Hearts were stained by hematoxylin-eosin or Masson's trichrome, and were immunostained with isolectin B_4 and α-smooth muscle actin antibodies. Expression of c-Kit and Sca-1 messenger RNA (mRNA) was evaluated by real-time PCR analysis. Apoptotic cells in hearts were identified by terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) staining. Hearts from Ang II/salt loading $AM^{+/-}$ mice showed marked perivascular fibrosis around coronary arteries. Treatment with simvastatin significantly inhibited the fibrosis around coronary arteries in Ang II/salt-loading $AM^{+/-}$ mice. Expression of c-Kit and Sca-1 mRNAs in hearts from Ang II/salt-loading $AM^{+/-}$ mice was significantly lower than in hearts from wild-type mice. Treatment with simvastatin significantly increased the suppressed expression of c-Kit and Sca-1 mRNAs. In addition, treatment with simvastatin significantly increased the number of isolectin B_4-positive capillary arteries in hearts from Ang II/salt-loading $AM^{+/-}$ mice. Ang II/high salt significantly increased apoptotic cells in hearts from $AM^{+/-}$ mice; this trend was reversed by treatment with simvastatin. Thus, statins have potent cardioprotective effects that may be associated with anti-fibrotic, proangiogenic and anti-apoptotic effects in Ang II/salt-loading $AM^{+/-}$ mice.
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Keywords: adrenomedullin; apoptosis; cardioprotection; knockout mouse; statin

INTRODUCTION

Adrenomedullin (AM) is a ubiquitously expressed multifunctional peptide that exerts vasodilatory, hypotensive[1] and neuromodulatory effects;[2] positive inotropic effects on cardiomyocytes;[3] natriuretic,[4] anti-apoptotic[5] and antioxidative stress effects;[6] and proangiogenic effects.[7] As AM is a pivotal intrinsic antioxidative substrate in tissues, AM insufficiency is associated with the pathogenesis of cardiovascular disease, hypertension, diabetes and renal disease. It has been reported that angiotensin II (Ang II) plus high salt (Ang II/salt) loading induce cardiac fibrosis in AM heterozygous knockout ($AM^{+/-}$) mice and that AM inhibits cardiac fibrosis in Ang II-induced hypertensive rats.[8,9] Thus, Ang II/salt loading in the $AM^{+/-}$ mouse is a useful model for cardiovascular damage, including cardiac fibrosis.

Statins have pleiotropic effects independent of their cholesterol-lowering effects.[10,11] Statins induce potent anti-oxidative effects, such as increases in the antioxidative molecule, hemoxidase-1 (HO-1).[12] Statins have also been reported to exert potent cardioprotective effects independent of their cholesterol-lowering effects.[10] Statins effectively improve cardiac function and increase the survival rate in patients with severe cardiac failure due to dilated cardiomyopathy without coronary arterial insufficiency.[13,14] In addition, statins upregulate and activate endothelial nitric oxide synthase (eNOS) and increase NO production through the PI3K-Akt-eNOS pathway.[15,16] Statins inhibit the formation of mevalonate and exhibit powerful anti-inflammatory effects, mediated by the inhibition of critical proteins such as Ras, Rho and NF-κB.[17] Thus, the anti-inflammatory effects of statins induce potent cardioprotective effects through the inhibition of Rho kinase.[18]

To further investigate the mechanisms underlying the cardioprotective effects of statin, we examined the effects of simvastatin on cardiac fibrosis and apoptosis in $AM^{+/-}$ mice treated with Ang II and high-salt diet.

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METHODS

Our investigation conformed to the standards of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The ethics committee of Nihon University School of Medicine examined every research protocol involving the use of living animals.

Animals

Adrenomedullin heterozygous knockout C57BL/6 male (AM$^{+/-}$) mice and C57BL/6 male (wild-type) mice were purchased from a laboratory at Tokyo University and used in this study.

Treatments for AM$^{+/-}$ mice

Seven-week-old AM$^{+/-}$ mice were infused with Ang II (640 ng kg^{-1} min^{-1}, Peptide Institute, Osaka, Japan) using osmotic mini-pumps (Alza, Cupertino, CA USA) while on a high-salt diet (8% NaCl) with or without oral simvastatin treatment (Banyu Pharmaceuticals, Tokyo, Japan) (40 mg kg^{-1} per day) for 2 weeks.

Histological examination

At 2 weeks after the start of simvastatin treatment, hearts were removed from mice and embedded in paraffin. Paraffin-embedded sections obtained from each segment were stained with hematoxylin-eosin stain and Masson's trichrome. The fibrous area was determined on the basis of the representative Masson's trichrome-stained area using Photoshop CS3 extended (Adobe, Tokyo, Japan). Vessel formation was immunostained with α-smooth muscle actin (α-SMA) (DAKO, Tokyo, Japan) and isolectin B_4 antibodies. Biotinylated isolectin B_4 (Vector Laboratories, Burlingame, CA, USA) and streptavidin-FITC (Vector Laboratories) were used for microvessel immunostaining. Nuclei were stained with Hoechst 33342 (Sigma-Aldrich, St Louis, MO, USA). Myocyte number was determined counting the nuclei of isolectin B_4- and α-SMA-negative cells. Apoptotic cells were determined by terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) staining using an apoptosis detection kit (Takara, Shiga, Japan).

RNA extraction and PCR

Total RNA was extracted from mouse hearts using a Trizol reagent (Invitrogen, CA, USA). Total RNA (1 μg) was reverse transcribed into complementary DNA with random 9-mers using a Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio, Ohtsu, Japan). Assay-on-demand primers and probes (c-Kit: Mm00445212_m1, Sca-1: Mm00726565_s1 and TaqMan Rodent GAPDH control reagents) were purchased from Applied Biosystems. Messenger RNA (mRNA) was quantified with an ABI Prism 7300 (Applied Biosystems, Carlsbad, CA, USA). Each sample (each reaction, 5 μl complementary DNA; total volume, 25 μl) was run in triplicate. Cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After confirming that the efficiencies of the target and the endogenous control amplifications were approximately equal, relative gene expression was analyzed using the comparative ΔΔCt method with GAPDH as the endogenous control.

Measurement of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$)

Mice were placed in metabolic cages to collect urine for 24 h after the infusion of Ang II with high-salt diet and oral treatment of simvastatin for 2 weeks. Urinary 8-iso-$PGF_{2\alpha}$ was measured by enzyme-linked immunoassay (Oxford Biomedical Research, Oxford, UK).

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test and Welch's *t*-test. Data were expressed as the mean ± standard error of the mean (s.e.m.). Differences were considered significant at the level of $P<0.05$.

RESULTS

Effects of simvastatin on cardiac fibrosis

Ang II/high salt loading induced marked cardiac fibrosis in the left ventricle (Figure 1) and around coronary arteries (Figure 2a) in AM$^{+/-}$ mice but not in wild-type mice. Cardiac fibrosis was quantitatively analyzed by measuring the extent of perivascular fibrosis around coronary arteries. The area of perivascular fibrosis was significantly higher ($P<0.05$) in hearts from AM$^{+/-}$ mice. This effect was significantly ($P<0.05$) inhibited by simvastatin treatment (Figure 2b).

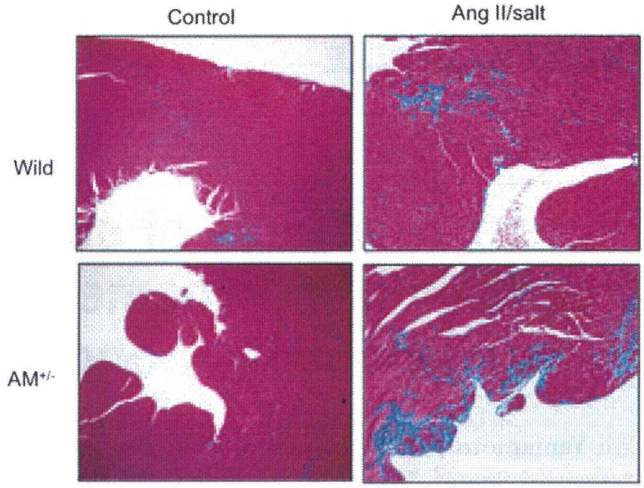

Figure 1 Cardiac fibrosis in hearts from adrenomedullin heterozygous knockout (AM$^{+/-}$) mice infused with angiotensin II (Ang II) and submitted to salt loading. Seven-week-old wild-type or AM$^{+/-}$ mice were infused with Ang II (640 ng kg^{-1} min^{-1}) on an 8% high-salt diet. Hearts were removed from mice and embedded in paraffin and were stained with hematoxylin-eosin and Mason's trichrome stains.

Effects of simvastatin on expression of c-Kit and Sca-1 mRNA

Figures 3a and b show effects of simvastatin on mRNA expression of c-Kit and Sca-1 in hearts from wild or AM$^{+/-}$ mice treated with or without Ang II/high salt. The abundance of c-Kit and Sca-1 mRNAs in hearts from Ang II/salt-loading AM$^{+/-}$ mice was significantly ($P<0.05$) lower than in hearts from wild-type mice. Treatment with simvastatin significantly ($P<0.05$) increased the abundance of c-Kit as well as Sca-1 mRNA in hearts from Ang II/salt-loading AM$^{+/-}$ mice.

Effects of simvastatin on oxidative stress in AM$^{+/-}$ mice

Figure 3c shows the effects of simvastatin on expression of the oxidative stress marker 8-iso-$PGF_{2\alpha}$ in urine from wild or AM$^{+/-}$ mice treated with or without Ang II/high salt. The excretion of 8-iso-$PGF_{2\alpha}$ in urine from Ang II/salt-loading AM$^{+/-}$ mice was higher (but not significantly) than in urine from Ang II/salt-loading wild-type mice. Treatment with simvastatin decreased urinary excretion of 8-iso-$PGF_{2\alpha}$ in Ang II/salt-loading AM$^{+/-}$ mice.

Angiogenic effects of simvastatin

Figure 4 shows immunohistochemical staining for isolectin B_4 and α-SMA in the left ventricles of hearts from wild-type and AM$^{+/-}$ mice treated with Ang II/high salt and simvastatin. The capillary density of the ventricle was significantly ($P<0.05$) lower in hearts from Ang II/salt-loading AM$^{+/-}$ mice than in Ang II/salt-loading wild-type mice. Treatment with simvastatin significantly increased the number of isolectin B_4-positive ($P<0.05$) and α-SMA-positive ($P<0.01$) capillaries in the ventricles of hearts from Ang II/salt-loading AM$^{+/-}$ mice.

Anti-apoptotic effects of simvastatin

Figure 5 shows TUNEL staining in the left ventricles of hearts from wild-type and AM$^{+/-}$ mice treated with Ang II/high salt and

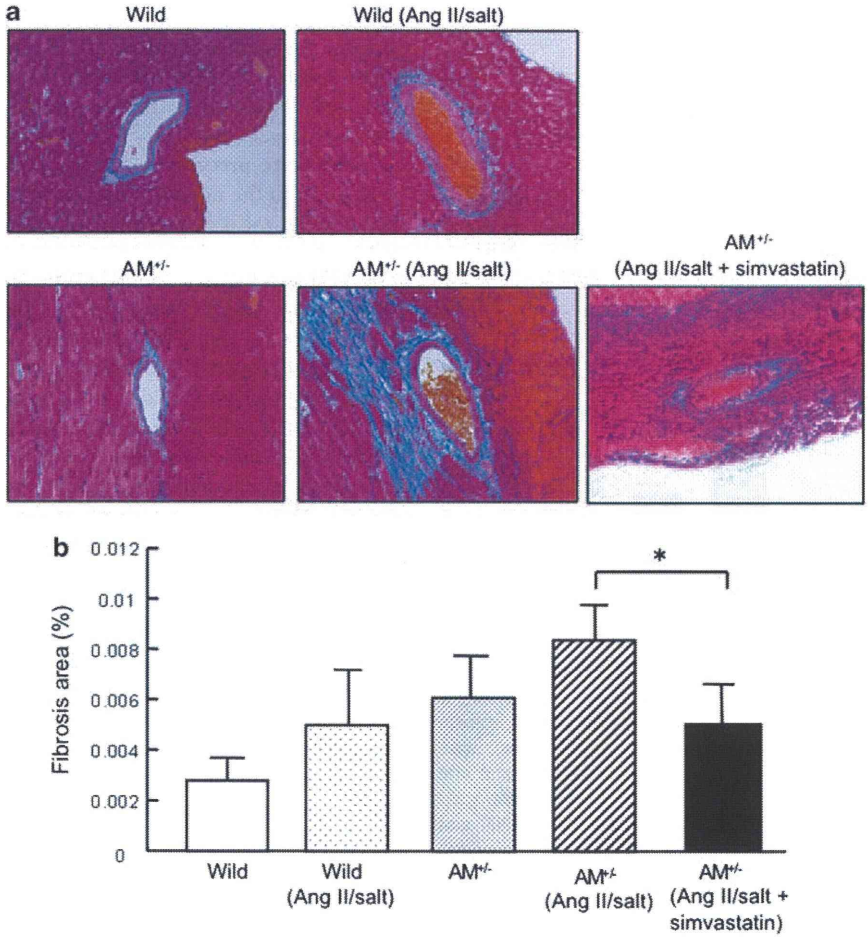

Figure 2 (**a**) Cardiac fibrosis around coronary arteries in left ventricles from adrenomedullin heterozygous knockout (AM$^{+/-}$) mice treated with angiotensin II (Ang II) and salt loading. Seven-week-old wild-type or AM$^{+/-}$ mice were infused with Ang II (640 ng kg^{-1} min^{-1}) on an 8% high-salt diet with oral simvastatin treatment (40 mg kg^{-1} per day). (**b**) The fibrous area was identified as the Masson's trichrome-stained area on each NIH image. Data are the mean ± s.e.m. (n=4). *P<0.05 between indicated columns.

simvastatin. The percentage of apoptotic cells in the ventricle was significantly (P<0.05) higher in hearts from AM$^{+/-}$ mice as compared with wild-type mice. Treatment with simvastatin significantly (P<0.05) decreased the percentage of apoptotic cells in the ventricles of hearts from Ang II/salt-loading AM$^{+/-}$ mice. Treatment with simvastatin did not appreciably affect the number of myocytes in the ventricles of hearts from AM$^{+/-}$ mice treated with Ang II/high salt.

DISCUSSION

To investigate mechanisms of the cardioprotective effects of statin, we examined effects of simvastatin on fibrosis, apoptosis and angiogenesis in hearts from AM$^{+/-}$ mice treated with Ang II and high salt. AM$^{+/-}$ mice showed marked cardiac fibrosis in heart muscle with Ang II/salt-induced oxidative stress. Treatment with simvastatin significantly repaired the cardiac fibrosis and decreased levels of 8-iso-$PGF_{2\alpha}$ in hearts from Ang II/salt-loading AM$^{+/-}$ mice, suggesting that simvastatin inhibited cardiac fibrosis through its antioxidative actions.

Moreover, treatment with simvastatin significantly increased capillary density, as determined by immunostaining with isolectin B_4 and α-SMA in hearts from Ang II/salt-loading AM$^{+/-}$ mice, suggesting that simvastatin strongly increased mature capillary arteries comprising endothelium and vascular smooth muscle. We recently demonstrated that atorvastatin induces proangiogenic effects, with increase in the expression of angiogenic factors, including vascular endothelial growth factor, interluekin-8, angiopoietins and eNOS, in the ischemic hindlimbs of rats.[19] Statins have been reported to increase HO-1 production and, thus, inhibit oxidation *in vivo*.[12] Hsu *et al.*[20] demonstrated that oral administration of statins increases HO-1 production in the mouse liver, lung, brain and heart, and suggested that the antioxidative effects of statins in cardiovascular tissues are strongly induced by HO-1. Moreover, we demonstrated that a statin strongly potentiates colony formation among endothelial progenitor cells during angiogenesis and repairs the endothelium in oxidative hypertensive rats with increases in HO-1 *in vivo*.[19] It is possible that the proangiogenic effects of simvastatin also protected against cardiac fibrosis. Suzuki *et al.*[21] recently demonstrated that pravastatin mobilizes bone marrow progenitor cells in hibernating hearts and increases the number of myocytes that reenter the growth and mitotic phases of the cell cycle. In this experiments, simvastatin did not increase the number of myocytes; however, simvastatin decreased the number of apoptotic cells in hearts from Ang II/salt-loading AM$^{+/-}$ mice, indicating that the cardioprotective effects of simvastatin are associated with anti-apoptotic effects.

The presence of cardiac stem cells in the heart has recently been reported. Beltrami *et al.*[22] first reported the discovery of a distinct

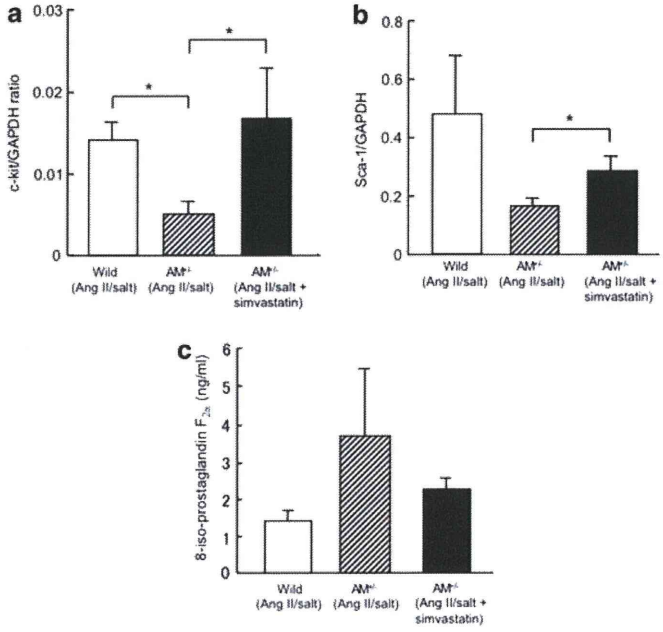

Figure 3 Effects of simvastatin on mRNA expression of the oxidative stress marker 8-iso-$PGF_{2\alpha}$, and CSC markers c-Kit and Sca-1 in the hearts of wild or adrenomedullin heterozygous knockout ($AM^{+/-}$) mice treated with angiotensin (Ang) II and high salt loading. Seven-week-old wild-type or $AM^{+/-}$ mice were infused with Ang II (640 ng kg^{-1} min^{-1}) on an 8% high-salt diet and oral treatment with simvastatin (40 mg kg^{-1} per day). Total RNA was extracted from mouse hearts. Expression of c-Kit (**a**) and Sca-1 (**b**) mRNA was evaluated by real-time PCR analysis. Relative gene expression was analyzed by the comparative ΔΔCt method with GAPDH. (**c**) Levels of the oxidative stress marker, 8-iso-$PGF_{2\alpha}$, in mouse urine were measured by ELISA. Data are the mean ± s.e.m. (n=4). *P<0.05 between indicated columns.

resident population of cardiac stem cells that express c-Kit, the receptor for stem cell factor. C-Kit, the transmembrane tyrosine kinase receptor for stem cell factor, is required for melanocyte and mast cell development, hematopoiesis and the differentiation of spermatogonial stem cells.[23] c-Kit is also transiently expressed in cardiomyocyte precursors during development and in a rare cell population in the normal adult heart. Li *et al.*[24] demonstrated that in the heart, c-Kit is expressed not only by cardiac stem cells but also by cardiomyocytes. Expression is observed immediately after birth and terminates a few days later and, thus, coincides with the onset of cardiomyocyte terminal differentiation. c-Kit expression in the heart has also been implicated in mediating repair and remodeling after myocardial infarction as well as in the maintenance of cardiac function.[25] Thus, c-Kit is a marker of stem and progenitor cells as well as the regeneration of damaged heart tissue. We previously examined the effects of an Ang II-receptor blocker on the expression of c-Kit in hearts from hypertensive rats. Expression of c-Kit mRNA was significantly lower than in normotensive rats. The Ang II-receptor blocker significantly increased expression of c-Kit through antioxidative mechanisms.[26] In this experiments, the abundance of c-Kit and Sca-1 mRNA in hearts from $AM^{+/-}$ mice treated with Ang II/high salt was significantly lower than in heart tissue from wild-type mice. Treatment with simvastatin significantly increased the abundance of c-Kit and Sca-1 mRNA in hearts from $AM^{+/-}$ mice treated with Ang II/high salt. These findings suggest that simvastatin inhibited cardiac fibrosis through the regeneration of damaged heart and/or activation of cardiac stem cells through antioxidative actions in Ang II/salt-loading $AM^{+/-}$ mice.

In conclusion, statins have potent cardioprotective effects that may be associated with anti-fibrotic, proangiogenic and anti-apoptotic effects in Ang II/salt-loading $AM^{+/-}$ mice.

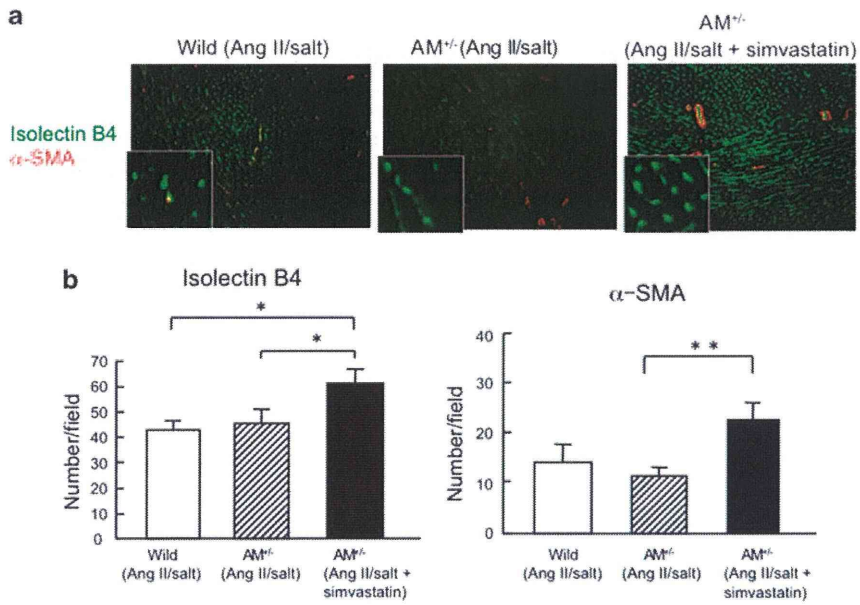

Figure 4 Angiogenic effects of simvastatin on hearts from wild or adrenomedullin heterozygous knockout ($AM^{+/-}$) mice treated with or without angiotensin (Ang) II and high salt loading. Seven-week-old wild-type or $AM^{+/-}$ mice were infused with or without Ang II (640 ng kg^{-1} min^{-1}) on an 8% high-salt diet with or without oral treatment with simvastatin (40 mg kg^{-1} per day). After 2 weeks, hearts were removed from mice and embedded in paraffin. (**a**) Vessels were immunostained with α-smooth muscle actin (α-SMA) and isolectin B_4 antibodies. High magnification insets in each image. (**b**) Number of capillaries in each field (×200). Scale bar indicates 100 μm. Data are expressed as mean ± s.e.m. (n=4). Data are the mean ± s.e.m. (n=4). *P<0.05, **P<0.01 between indicated columns.

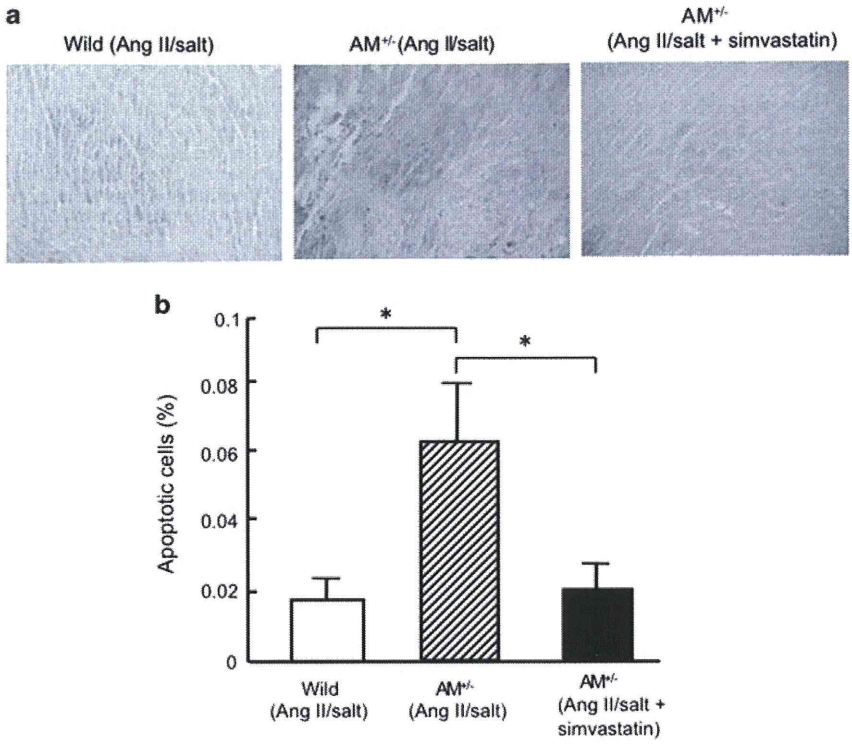

Figure 5 Anti-apoptotic effects of simvastatin on hearts from wild or adrenomedullin heterozygous knockout (AM$^{+/-}$) mice treated with or without angiotensin (Ang) II and high salt loading. Seven-week-old wild type or AM$^{+/-}$ mice were infused with or without Ang II (640 ng kg^{-1} min^{-1}) on an 8% high-salt diet with or without oral treatment with simvastatin (40 mg kg^{-1} per day). After 2 weeks, hearts were removed from mice and embedded in paraffin. (**a**) Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) staining (brown). (**b**) Apoptotic cell percentage was calculated as the number of TUNEL-positive cells divided by the total number of nuclei (×200). Data are the mean ± s.e.m. (n=8). *P<0.05 between indicated columns.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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