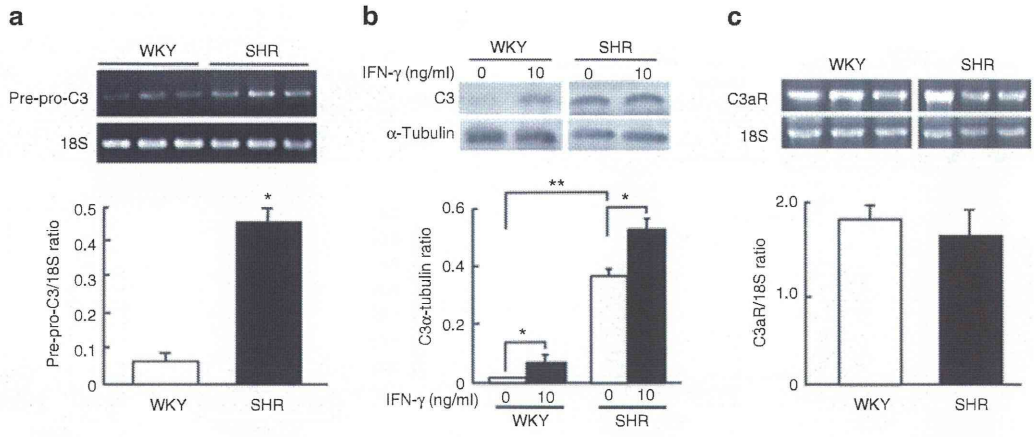

Figure 1 | Expression of complement 3 (C3) and the C3a receptor in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. Total RNA was extracted from quiescent VSMCs of 3-week-old SHR and WKY rats. Semiquantitative determination of the abundance 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.

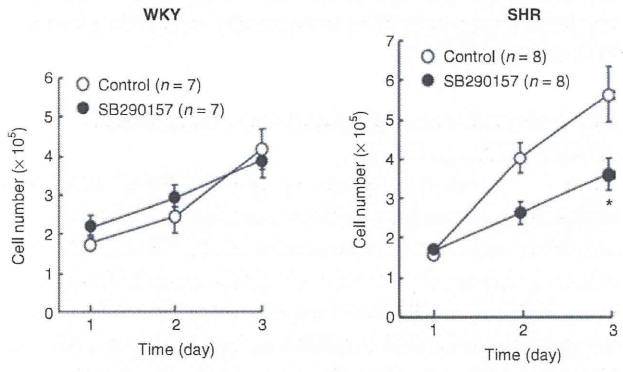

Figure 2 | Effects of the complement 3a receptor inhibitor, SB290157, on proliferation of vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. VSMCs were inoculated at 5×10^4 cells/cm^2 in DMEM containing 5% calf serum without (control) or with 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.

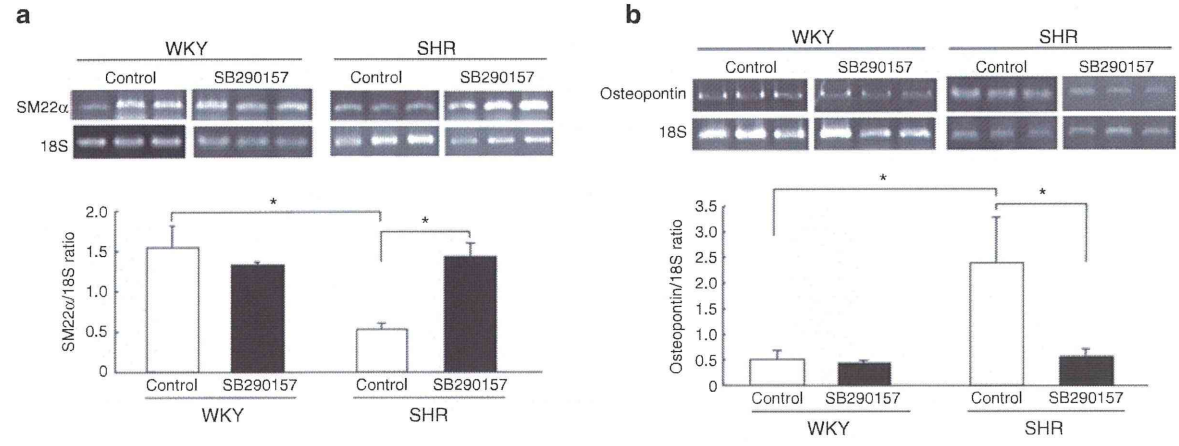

Figure 3 | Effects of the complement 3a receptor inhibitor, SB290157, on expression of the mRNAs of the phenotypic markers, (**a**) SM22α and (**b**) osteopontin, in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. Quiescent VSMCs from 3-week-old 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.

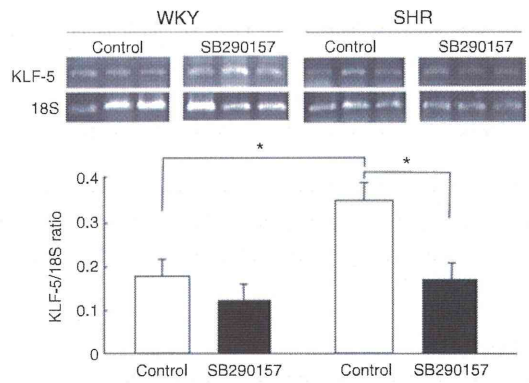

Figure 4 | Effects of the complement 3a receptor inhibitor, SB290157, on expression of Kruppel-like factor 5 (KLF-5) mRNAs in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. Quiescent VSMCs from 3-week-old SHR and WKY rats were incubated without (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,

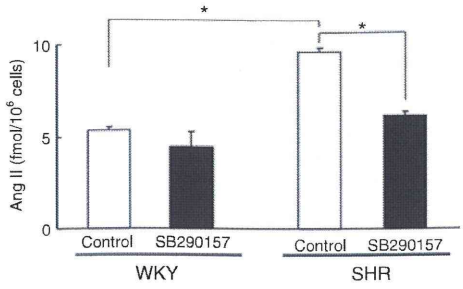

Figure 5 | Effects of the complement 3a receptor inhibitor, SB290157, on production of angiotensin II (Ang II) in conditioned medium of vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and Wistar–Kyoto (WKY) rats. VSMCs (10^6) were incubated first for 24 h with serum-free 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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