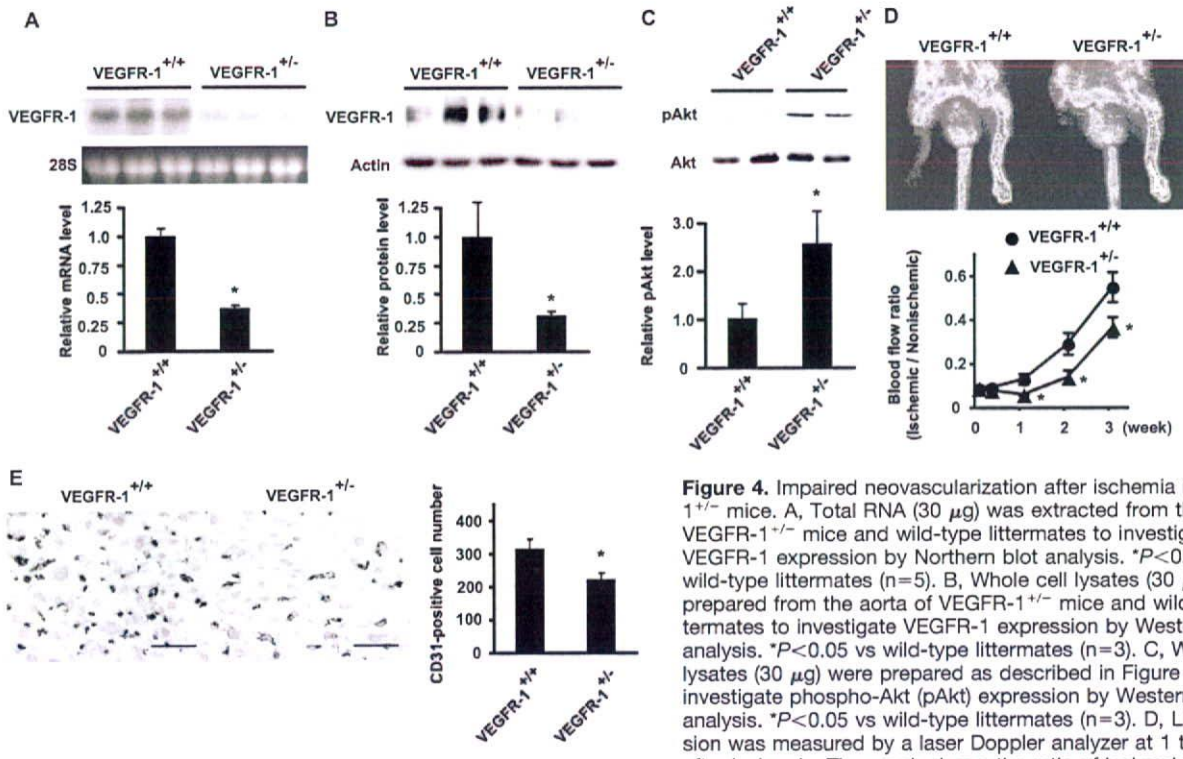

Figure 4. Impaired neovascularization after ischemia in VEGFR-$1^{+/-}$ mice. A, Total RNA (30 μg) was extracted from the lung of VEGFR-$1^{+/-}$ mice and wild-type littermates to investigate VEGFR-1 expression by Northern blot analysis. *$P<0.001$ vs wild-type littermates (n=5). B, Whole cell lysates (30 μg) were prepared from the aorta of VEGFR-$1^{+/-}$ mice and wild-type littermates to investigate VEGFR-1 expression by Western blot analysis. *$P<0.05$ vs wild-type littermates (n=3). C, Whole cell lysates (30 μg) were prepared as described in Figure 4B to investigate phospho-Akt (pAkt) expression by Western blot analysis. *$P<0.05$ vs wild-type littermates (n=3). D, Limb perfusion was measured by a laser Doppler analyzer at 1 to 3 weeks after ischemia. The graph shows the ratio of ischemic (right) to nonischemic limb (left) blood flow. *$P<0.05$ vs wild-type littermates (n=16). E, Immunohistochemistry for CD31 (brown) in ischemic limbs. Scale bar: 50 μm. The number of CD31-positive cells per square millimeter is shown in the graph. *$P<0.05$ vs wild-type littermates (n=4).

positive cells between VEGFR-$1^{+/-}$ mice and their wild-type littermates (Figure 5A). To further test the possible involvement of bone marrow–derived cells, we transplanted wild-type bone marrow cells into VEGFR-$1^{+/-}$ mice or their wild-type littermates. We then produced a hindlimb ischemia model and assessed blood flow recovery and the capillary density of ischemic tissue. Despite the transplantation of wild-type bone marrow, blood flow recovery was still significantly impaired in VEGFR-$1^{+/-}$ mice (Figure 5B). The number of CD31-positive cells was also lower in VEGFR-$1^{+/-}$ mice than in their wild-type littermates (Figure 5C). Thus, it is unlikely that impaired neovascularization in VEGFR-$1^{+/-}$ mice is attributed to reduced migration of bone marrow–derived cells. We could not detect VEGFR-1 expression in muscle cells (supplemental Figure IV). It was noted that the number of endothelial cells double positive for phospho-Akt and CD31 was significantly higher in VEGFR-$1^{+/-}$ mice than in their wild-type littermates (Figure 5D).

Inhibition of Akt Signaling Ameliorates the Impairment of Neovascularization in VEGFR-$1^{+/-}$ Mice

Next, we examined whether an increase of endothelial Akt activity contributed to impaired neovascularization in VEGFR-$1^{+/-}$ mice. Akt1 is the predominant isoform of Akt in endothelial cells and is thought to play an important role in postnatal angiogenesis.[30] It has been reported that the angiogenic response of $Akt1^{-/-}$ mice was enhanced in a tumor angiogenesis model, but was decreased in a hindlimb ischemia model,[30,31] so we thus used $Akt1^{+/-}$ mice for our in vivo experiments. Consistent with the previous reports,[32] phospho-Akt levels were lower in the aorta of $Akt1^{+/-}$ mice compared with wild-type littermates (supplemental Figure V). After creating hindlimb ischemia in VEGFR-$1^{+/-}$ $Akt1^{+/-}$ mice, we examined the extent of blood flow recovery and the capillary density 1 week later. We found that there were no significant differences of blood flow recovery and capillary density between $Akt1^{+/-}$ mice and $Akt1^{+/+}$ mice (Figure 6A and 6B). Decreased VEGFR-1 expression significantly reduced blood flow recovery in $Akt1^{+/+}$ mice, but not in $Akt1^{+/-}$ mice (Figure 6A). Likewise, the capillary density of ischemic tissue was significantly reduced in VEGFR-$1^{+/-}$ $Akt1^{+/+}$ mice compared with wild-type mice, but VEGFR-$1^{+/-}$ $Akt1^{+/-}$ mice had a similar capillary density to that of VEGFR-$1^{+/+}$ $Akt1^{+/-}$ mice (Figure 6B). These results suggest that an increase of endothelial Akt activity may be responsible for impaired neovascularization in VEGFR-$1^{+/-}$ mice.

Discussion

In the present study, we demonstrated that VEGFR-1 modulates postnatal angiogenesis through inhibition of the excessive activation of Akt by VEGF. It has been reported that VEGF and VEGFR-1 can be simultaneously induced by various stimuli, including hypoxia.[33] Thus, the role of VEGFR-1 may vary, depending on the extent of activation of Akt. For example, when overproduction of growth factors such as VEGF and insulin leads to excessive activation of Akt and impairs normal regulation of endothelial proliferation,

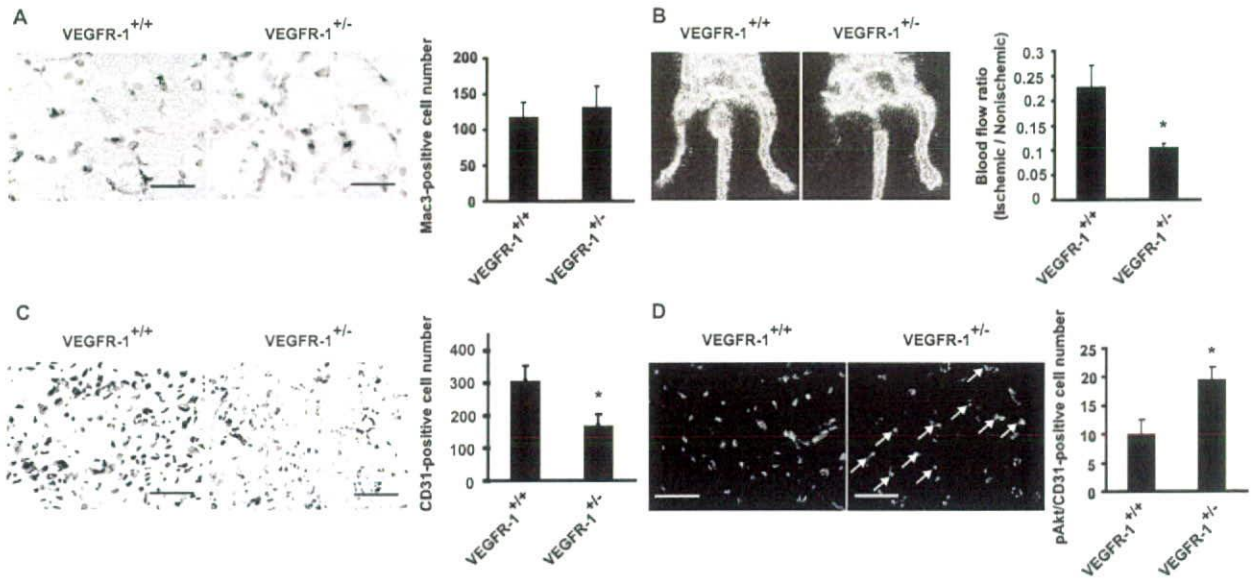

Figure 5. Role of bone marrow–derived cells in impaired neovascularization in VEGFR-1$^{+/-}$ mice. A, Immunohistochemistry for Mac3 (brown) in ischemic limbs. Scale bar: 50 μm. The number of Mac3-positive cells per square millimeter is shown (n=4). B, Wild-type bone marrow cells were transplanted into VEGFR-1$^{+/-}$ mice or their wild-type littermates. Limb perfusion was measured by a laser Doppler analyzer at 1 week after ischemia. *P<0.05 vs wild-type littermates (n=6). C, Immunohistochemistry for CD31 (brown) in ischemic limbs of bone marrow–transplanted mice. Scale bar: 50 μm. *P<0.05 s wild-type littermates (n=6). D, Activation of Akt in endothelial cells of ischemic limbs from VEGFR-1$^{+/-}$ mice. Representative immunostainings for phospho-Akt (red) and CD31 (green) were shown. Arrows indicate phospho-Akt/CD31-positive cells (yellow). Scale bar: 50 μm. The graph shows the ratio of phospho-Akt/CD31-positive cell number to all CD31-positive cell number. *P<0.05 vs wild-type littermates (n=5).

VEGFR-1 may act as a positive regulator of angiogenesis by inhibiting activation of VEGFR-2. Conversely, VEGFR-1 may exert a negative effect on angiogenesis when growth factors appropriately activate the Akt signaling pathway to induce endothelial cell proliferation. These mechanisms may provide an explanation as to why the effects of PlGF on angiogenesis were reported to differ.

Although there is evidence to suggest that VEGFR-1 interacts with the p85 subunit of phosphatidylinositol-3 kinase (PI3K) to regulate its activity,[34–36] VEGFR-1 appears to exert its inhibitory effect on angiogenesis mainly by blocking the activation of Akt mediated by VEGF via VEGFR-2 for the following reasons. First, treatment with VEGF-A increased Akt activity in VEGFR-1–deleted cells, but not in VEGFR-2–deleted cells (Figure 3A and 3B). Second, treatment with a neutralizing anti-VEGF antibody reduced the enhanced activation of Akt in VEGFR-1-deleted cells (Figure 3C). Finally, treatment with PlGF did not provoke any

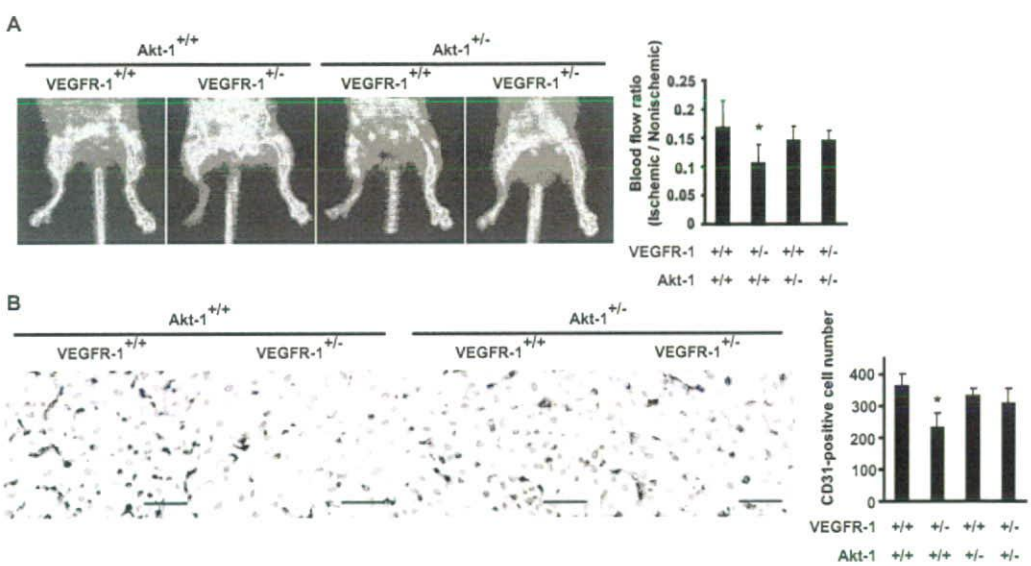

Figure 6. Inhibition of Akt signaling ameliorates the impairment of neovascularization in VEGFR-1$^{+/-}$ mice. A, Limb perfusion was measured by a laser Doppler analyzer at 1 week after creation of ischemia. *P<0.01 vs wild-type littermates (n=14 to 18). B, Immunohistochemistry for CD31 (brown) in ischemic limbs. Scale bar: 50 μm. *P<0.05 vs wild-type littermates (n=6 to 7).

biological response in the presence of anti-VEGF antibody (J. Nishi, T. Minamino, unpublished data, 2007). Our results are consistent with previous studies[37,38] demonstrating that tyrosine phosphorylation of VEGFR-2 was elevated in VEGFR-1–deficient embryonic stem cells, whereas loss of VEGFR-1 led to decreased sprout formation and migration, which resulted in reduced vascular branching. This reduction was restored by blockade of the VEGFR-2 signaling pathway as well as by treatment with soluble VEGFR-1. Although Bussolati et al demonstrated that VEGFR-1 but not VEGFR-2 increases endothelial production of NO, thereby promoting tube formation,[39] cGMP production was significantly decreased in VEGFR-1–deleted endothelial cells (supplemental Figure ID). Moreover, VEGF treatment failed to activate Akt in VEGFR-2-deleted endothelial cells (Figure 3B) and introduction of mutant VEGFR-1 lacking the sites for interaction with PI3K did not mimic the effects of shVEGFR-1 (J. Nishi, T. Minamino, unpublished data, 2007). Taken together, these results suggest that VEGFR-1 acts to provide "fine tuning" of VEGF signaling to achieve the proper formation of blood vessels. The biological consequences of VEGFR-1 deletion appears to be related to loss of its decoy effect, but other mechanisms might be involved such as "cross talk" between VEGFR-1 and VEGFR-2,[8,16,17] direct regulation of the VEGFR-2 signaling pathway by VEGFR-1,[39,40] and some undefined effect of the extracellular domain of membrane-bound VEGFR-1.[41]

We have previously demonstrated that constitutive activation of Akt induced by insulin promotes senescence-like arrest of endothelial cell growth via a p53/p21-dependent pathway.[19] Moreover, tube formation was significantly reduced by overactivation of Akt. Likewise, constitutive activation of Akt has been reported to promote the senescence in other types of cells such as endothelial progenitors and mouse embryonic fibroblasts.[42,43] The study using conditional transgenic mice has demonstrated that sustained activation of Akt in endothelial cells causes increased blood vessel size and generalized edema within 2 weeks and that these changes are reversible.[44] Using the same mouse model, it has been reported that chronic activation of Akt over 8 weeks leads to endothelial cell senescence and loss of endothelium-dependent stroke protection.[45] Recent studies by several groups demonstrated that diabetic state induces activation of the Akt pathway, thereby contributing to the pathology of diabetic complications.[42,46–48] We also detected increased Akt activity in endothelial cells on the surface of coronary atherosclerotic lesions in patients with diabetes.[19] Moreover, accumulating evidence suggests that vascular cell senescence contributes to the pathogenesis of age-associated vascular diseases including diabetic vasculopathy.[49] Thus, these results suggest the potential of the treatment for vascular dysfunction associated with diabetes and aging by modulating Akt activity with a soluble form of VEGFR-1.

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Disclosures

None.

References

1. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005; 438:932–936.
2. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003;9:669–676.
3. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature*. 2005;438:937–945.
4. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 1995;376:66–70.
5. Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in flt-1 knock-out mice. *Development*. 1999;126:3015–3025.
6. Kearney JB, Ambler CA, Monaco KA, Johnson N, Rapoport RG, Bautch VL. Vascular endothelial growth factor receptor Flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division. *Blood*. 2002;99:2397–2407.
7. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A*. 1998;95:9349–9354.
8. Rahimi N, Dayanir V, Lashkari K. Receptor chimeras indicate that the vascular endothelial growth factor receptor-1 (VEGFR-1) modulates mitogenic activity of VEGFR-2 in endothelial cells. *J Biol Chem*. 2000;275: 16986–16992.
9. Keyt BA, Nguyen HV, Berleau LT, Duarte CM, Park J, Chen H, Ferrara N. Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. *J Biol Chem*. 1996;271: 5638–5646.
10. Yang S, Xin X, Zlot C, Ingle G, Fuh G, Li B, Moffat B, de Vos AM, Gerritsen ME. Vascular endothelial cell growth factor-driven endothelial tube formation is mediated by vascular endothelial cell growth factor receptor-2, a kinase insert domain-containing receptor. *Arterioscler Thromb Vasc Biol*. 2001;21:1934–1940.
11. Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, Pelletier N, Ferrara N. Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *J Biol Chem*. 2001;276:3222–3230.
12. Errico M, Riccioni T, Iyer S, Pisano C, Acharya KR, Persico MG, De Falco S. Identification of placenta growth factor determinants for binding and activation of Flt-1 receptor. *J Biol Chem*. 2004;279:43929–43939.
13. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med*. 2002;8:831–840.
14. Adini A, Kornaga T, Firoozbakht F, Benjamin LE. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. *Cancer Res*. 2002;62:2749–2752.
15. Odorisio T, Schietroma C, Zaccaria ML, Cianfarani F, Tiveron C, Tatangelo L, Failla CM, Zambruno G. Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. *J Cell Sci*. 2002;115:2559–2567.
16. Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dom-

browski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med.* 2003;9:936–943.
17. Neagoe PE, Lemieux C, Sirois MG. Vascular endothelial growth factor (VEGF)-A165-induced prostacyclin synthesis requires the activation of VEGF receptor-1 and -2 heterodimer. *J Biol Chem.* 2005;280:9904–9912.
18. Shih SC, Ju M, Liu N, Smith LE. Selective stimulation of VEGFR-1 prevents oxygen-induced retinal vascular degeneration in retinopathy of prematurity. *J Clin Invest.* 2003;112:50–57.
19. Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I. Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *Embo J.* 2004;23:212–220.
20. O'Neill BT, Abel ED. Akt1 in the cardiovascular system: friend or foe? *J Clin Invest.* 2005;115:2059–2064.
21. Clauss M, Weich H, Breier G, Knies U, Rockl W, Waltenberger J, Risau W. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem.* 1996;271:17629–17634.
22. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood.* 1996;87:3336–3343.
23. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, Shibuya M. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. *Blood.* 2001;97:785–791.
24. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med.* 2001;7:1194–1201.
25. Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med.* 2002;8:841–849.
26. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. *Nat Med.* 2006;12:557–567.
27. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med.* 2000;6:389–395.
28. Hiratsuka S, Maru Y, Okada A, Seiki M, Noda T, Shibuya M. Involvement of Flt-1 tyrosine kinase (vascular endothelial growth factor receptor-1) in pathological angiogenesis. *Cancer Res.* 2001;61:1207–1213.
29. Murakami M, Iwai S, Hiratsuka S, Yamauchi M, Nakamura K, Iwakura Y, Shibuya M. Signaling of vascular endothelial growth factor receptor-1 tyrosine kinase promotes rheumatoid arthritis through activation of monocytes/macrophages. *Blood.* 2006;108:1849–1856.
30. Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. *Nat Med.* 2005;11:1188–1196.
31. Ackah E, Yu J, Zoellner S, Iwakiri Y, Skurk C, Shibata R, Ouchi N, Easton RM, Galasso G, Birnbaum MJ, Walsh K, Sessa WC. Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. *J Clin Invest.* 2005;115:2119–2127.
32. Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev.* 2001;15:2203–2208.
33. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med.* 2003;9:677–684.
34. Cunningham SA, Waxham MN, Arrate PM, Brock TA. Interaction of the Flt-1 tyrosine kinase receptor with the p85 subunit of phosphatidylinositol 3-kinase. Mapping of a novel site involved in binding. *J Biol Chem.* 1995;270:20254–20257.
35. Igarashi K, Isohara T, Kato T, Shigeta K, Yamano T, Uno I. Tyrosine 1213 of Flt-1 is a major binding site of Nck and SHP-2. *Biochem Biophys Res Commun.* 1998;246:95–99.
36. Yu Y, Hulmes JD, Herley MT, Whitney RG, Crabb JW, Sato JD. Direct identification of a major autophosphorylation site on vascular endothelial growth factor receptor Flt-1 that mediates phosphatidylinositol 3′-kinase binding. *Biochem J.* 2001;358:465–472.
37. Roberts DM, Kearney JB, Johnson JH, Rosenberg MP, Kumar R, Bautch VL. The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. *Am J Pathol.* 2004;164:1531–1535.
38. Kearney JB, Kappas NC, Ellerstrom C, DiPaola FW, Bautch VL. The VEGF receptor flt-1 (VEGFR-1) is a positive modulator of vascular sprout formation and branching morphogenesis. *Blood.* 2004;103:4527–4535.
39. Bussolati B, Dunk C, Grohman M, Kontos CD, Mason J, Ahmed A. Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide. *Am J Pathol.* 2001;159:993–1008.
40. Zeng H, Dvorak HF, Mukhopadhyay D. Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) peceptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. *J Biol Chem.* 2001;276:26969–26979.
41. Hiratsuka S, Nakao K, Nakamura K, Katsuki M, Maru Y, Shibuya M. Membrane fixation of vascular endothelial growth factor receptor 1 ligand-binding domain is important for vasculogenesis and angiogenesis in mice. *Mol Cell Biol.* 2005;25:346–354.
42. Rosso A, Balsamo A, Gambino R, Dentelli P, Falcioni R, Cassader M, Pegoraro L, Pagano G, Brizzi MF. p53 Mediates the accelerated onset of senescence of endothelial progenitor cells in diabetes. *J Biol Chem.* 2006;281:4339–4347.
43. Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature.* 2005;436:725–730.
44. Phung TL, Ziv K, Dabydeen D, Eyiah-Mensah G, Riveros M, Perruzzi C, Sun J, Monahan-Earley RA, Shiojima I, Nagy JA, Lin MI, Walsh K, Dvorak AM, Briscoe DM, Neeman M, Sessa WC, Dvorak HF, Benjamin LE. Pathological angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. *Cancer Cell.* 2006;10:159–170.
45. Wang C, Kim H, Hiroi Y, Mukai Y, Satoh M, Liao JK. Increase cellular senescence and cerebral infarct size in mice with chronic activation of endothelial protein kinase Akt. *Circulation.* 2006;114:II-160.
46. Hojlund K, Staehr P, Hansen BF, Green KA, Hardie DG, Richter EA, Beck-Nielsen H, Wojtaszewski JF. Increased phosphorylation of skeletal muscle glycogen synthase at NH2-terminal sites during physiological hyperinsulinemia in type 2 diabetes. *Diabetes.* 2003;52:1393–1402.
47. Sheu ML, Ho FM, Yang RS, Chao KF, Lin WW, Lin-Shiau SY, Liu SH. High glucose induces human endothelial cell apoptosis through a phosphoinositide 3-kinase-regulated cyclooxygenase-2 pathway. *Arterioscler Thromb Vasc Biol.* 2005;25:539–545.
48. Clodfelder-Miller B, De Sarno P, Zmijewska AA, Song L, Jope RS. Physiological and pathological changes in glucose regulate brain Akt and glycogen synthase kinase-3. *J Biol Chem.* 2005;280:39723–39731.
49. Minamino T, Komuro I. Vascular cell senescence: contribution to atherosclerosis. *Circ Res.* 2007;100:15–26.

Liver X Receptor Activator Downregulates Angiotensin II Type 1 Receptor Expression Through Dephosphorylation of Sp1

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Abstract—Atherosclerosis is considered to be a combined disorder of lipid metabolism and chronic inflammation. Recent studies have reported that liver X receptors (LXRs) are involved in lipid metabolism and inflammation and that LXR agonists inhibit atherogenesis. In contrast, angiotensin II is well known to accelerate atherogenesis through activation of the angiotensin II type 1 receptor (AT1R). To better understand the mechanism of LXR on the prevention of atherogenesis, we examined whether activation of LXR affects AT1R expression in vascular smooth muscle cells. T0901317, a synthetic LXR ligand, decreased AT1R mRNA and protein expression with a peak reduction at 6 hours and 12 hours of incubation, respectively. A well-established ligand of LXR, 22-(R)-hydroxycholesterol, also suppressed AT1R expression. The downregulation of AT1R by T0901317 required de novo protein synthesis. AT1R gene promoter activity measured by luciferase assay revealed that the DNA segment between −61 bp and +25 bp was sufficient for downregulation. Luciferase construct with a mutation in Sp1 binding site located in this segment lost its response to T0901317. T0901317 decreased Sp1 serine phosphorylation. Although preincubation of vascular smooth muscle cells with T0901317 for 30 minutes had no effect on angiotensin II–induced extracellular signal–regulated kinase phosphorylation, phosphorylation of extracellular signal–regulated kinase by angiotensin II was markedly suppressed after 6 hours of preincubation. These results indicate that the suppression of AT1R may be one of the important mechanisms by which LXR ligands exert antiatherogenic effects. (***Hypertension*. 2008;51:1631-1636.**)

Key Words: liver X receptor ■ angiotensin II type 1 receptor ■ Sp1

The liver X receptors (LXRs) are member of the nuclear hormone receptor superfamily.[1] Their endogenous ligands are oxidized cholesterol derivatives, such as oxysterols[1] and glucose.[2] LXRs regulate the expression of genes involved in lipid and glucose metabolism. In lipid metabolism, LXRs are known to regulate genes involved in "reverse cholesterol transport," which includes cholesterol efflux, transport, and excretion. ATP binding cassette A1 is involved in cholesterol efflux, and ABCG5 and ABCG8 are involved in cholesterol transport.[3,4] Expression of these ABC proteins is increased by LXR agonists. In mice, LXR agonists were reported to promote biliary and fecal excretion of cholesterol.[5] It was reported recently that glucose directly activated LXRs[2] and regulated the transcription of genes such as GLUT4.[6] In addition, LXR agonists were reported to negatively regulate the expression of inflammatory cytokines[7] and prevent the formation of atherosclerotic lesions in atherosclerosis-prone mice.[8,9]

The effects of angiotensin II (Ang II) are mediated by Ang II receptors, and so far 2 isoforms, type 1 receptor (AT1R) and type 2 receptor, have been identified.[10] AT1R mediates most of the traditional effects of Ang II, such as vasoconstriction and cell proliferation. It is well known that Ang II enhances atherogenesis,[11,12] and an AT1R antagonist attenuated atherogenesis in animal models.[13–15]

Emerging evidence has suggested that Ang II is critically involved in various aspects of inflammation.[16] In line with this notion, Ang II was reported to activate nuclear factor-κB, a transcription factor involved in the regulation of many inflammation-related genes, in vascular smooth muscle cells (VSMCs) through AT1R.[17] Intriguingly, an LXR agonist inhibited nuclear factor-κB activation.[7] Therefore, it may be possible that the LXR and Ang II/AT1R pathways functionally antagonize in terms of the inflammatory response.

A recent study showed that GW3965, a synthetic LXR agonist, increased murine renin gene expression.[18] However, the effect of LXR activation on AT1R expression has not been determined. In the present study, we tested whether LXRs are involved in the regulation of AT1R gene expression.

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Materials and Methods

Materials

DMEM was purchased from GIBCO BRL. FBS was purchased from JRH Biosciences. BSA, T0901317, 22-(R)-hydroxycholesterol (22-R-HC), 22-(S)-hydroxycholesterol (22-S-HC), cycloheximide (CHX), actinomycin D (ActD), trichostatin A (TSA), PD123319, and mouse monoclonal anti-α-tubulin were purchased from Sigma Chemical Co. Rabbit polyclonal antibodies against AT1R[19,20] and p16 were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against Sp1 and phosphoserine were purchased from Upstate, Inc, and Chemicon International, Inc, respectively. Rabbit polyclonal antibodies against extracellular signal–regulated kinase (ERK) and phosphorylated ERK (pERK) were purchased from Cell Signaling Co. Horseradish peroxidase–conjugated secondary antibodies (antirabbit and antimouse IgG) were purchased from Vector Laboratories, Inc. Losartan was kindly provided by Merck Co. [α-^{32}P]dCTP was purchased from Perkin-Elmer Life Sciences. Luciferase assay system was purchased from Promega Biosciences, Inc. Other chemical reagents were purchased from Wako Pure Chemicals unless mentioned specifically.

Cell Culture

VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats by an explant method and maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 95% air-5% CO_2. At passage 2, >95% of cells were positive for α-smooth muscle actin. VSMCs were cultured until grown to confluence. The medium was changed to DMEM with 0.1% BSA, and the cells were cultured for an additional 2 days. Then, the VSMCs were used in the experiment. Cells between passages 4 and 13 were used.

Northern Blot Analysis

Northern blot analysis was performed as described previously.[21] ActD (5 μg/mL) was used to examine the stability of AT1R mRNA. ActD was added after 6 hours of stimulation with T0901317 (10 μmol/L). In a control experiment, only ActD was added. Cells were harvested after 3, 6, 12, and 24 hours of ActD supplementation, and the expression level of AT1R mRNA was examined by Northern blot analysis.

Measurement of AT1R Gene Promoter Activity

Five deletion mutants of the AT1A gene promoter were prepared by digestion with restriction endonucleases and ligated to the luciferase gene. The AT1R promoter-luciferase construct with mutation in the GC-box–related sequence (wild-type: TGCAGAGCAGCGACGC-CCCCTAGGC; mutant: TGCAGAGCAGCGACGTTTCCTAGGC) was a generous gift from Dr Akira Sugawara (Tohoku University, Sendai, Japan).[22]

Confluent VSMCs were split by trypsin/EDTA solution, and cells were prepared in a 6-cm tissue culture dish. At 80% confluence, 5 μg of AT1 promoter-luciferase fusion DNA and 2 μg of β-galactosidase gene (LacZ) were introduced to VSMCs by the DEAE-dextran method according to the manufacturer's instruction (Promega Corporation). AT1R promoter/luciferase DNA construct with a mutation in the GC box (Sp1 binding site) was also introduced to VSMCs with the LacZ expression plasmid. VSMCs were cultured in DMEM with 10% FBS for 18 hours, washed twice with PBS, cultured in DMEM with 0.1% BSA for 24 hours, and then stimulated with T0901317 (10 μmol/L) for 12 hours. The luciferase activity was measured as described previously.[21]

Western Blot Analysis

Western Blot analysis was performed as described previously.[21]

Immunoprecipitation

VSMCs were lysed in a Nonidet P-40 lysis buffer (0.5% Nonidet P-40; 10 mmol/L of Tris-HCl [pH 7.5]; 150 mmol/L of NaCl; 2.5 mmol/L of KCl; 20 mmol/L of β-glycerol phosphate; 50 mmol/L of NaF; 1 mmol/L of Na_3VO_4; 1% aprotinin; 0.5% leupeptin; and 1 mmol/L of dithiothreitol), and the lysates were subjected to immunoprecipitation with an anti-Sp1 antibody as described previously.[23] Western blot analysis was performed with the antiphosphoserine antibody as described previously.[23] The intensity of the bands was quantified with a MacBAS bioimaging analyzer (Fujifilm).

Statistical Analysis

Statistical analysis was performed with either 1-way ANOVA or 2-way ANOVA and Fisher's test, if appropriate. Statistical significance was designated as $P<0.05$. Values are expressed as means±SEMs.

Results

LXR Agonist Reduced the Expression of AT1R mRNA and Protein

VMSCs were incubated with T0901317 (10 μmol/L) for various periods, and expression of AT1R mRNA was examined. The expression of AT1R mRNA was reduced with a peak at 6 hours of incubation (Figure 1A). The downregulation was transient, and the expression level resumed to the control level at 12 hours. We tested whether a second challenge with T0901317 affected the AT1R mRNA expression. The medium was replaced with fresh serum-free medium containing 10 μmol/L of T0901317 at 6 hours of stimulation, and VSMCs were further incubated for 6 hours. In this case, AT1R mRNA was still downregulated at 12 hours (Figure S1, available online at http://hyper.ahajournals.org). AT1R mRNA was reduced in a dose-dependent manner at 6 hours of incubation with T0901317 (Figure 1B). T0901317 reduced the AT1R protein level with a peak reduction at 6 hours of incubation (Figure 1C). The expression level of AT1R protein in VSMCs without T0901317 was quite stable during this incubation period (data not shown). T0901317 suppressed AT1R expression in a dose-dependent manner (Figure 1D). Janowski et al[24] had reported that 22-R-HC is a potent agonist of LXR. To confirm whether LXR mediates AT1R downregulation, we examined the effect of 22-R-HC (10 μmol/L, 6 hours) and its S enantiomer, 22-S-HC (10 μmol/L, 6 hours). 22-R-HC decreased the expression of AT1R. However, 22-S-HC, which is not an agonist of LXR, did not change the expression of AT1R (Figure 2A). Even the higher concentrations of 22-S-HC (30 to 50 μmol/L, 6 hours) did not affect AT1R mRNA expression (Figure 2B). These data suggest that AT1R is downregulated by the specific effect of LXR activation.

T0 Inhibits AT1R Expression at the Transcriptional Level

Deletion mutants of AT1 promoter/luciferase fusion DNA were used to determine the specific promoter region responsible for T0901317-induced AT1R suppression. Luciferase activity was suppressed in all of the DNA constructs (Figure 3A). The DNA construct with mutation in Sp1 binding site (AT1R promoter region from −58 to −34 bp) showed reduced basal luciferase activity compared with wild type (−61 bp) luciferase construct (data not shown), as reported previously, and stimulation with T0901317 (10 μmol/L) did not affect the luciferase activity in the Sp1 mutant luciferase construct (Figure 3A). Therefore, we suppose that Sp1 is a positive regulatory element in the AT1R gene promoter and

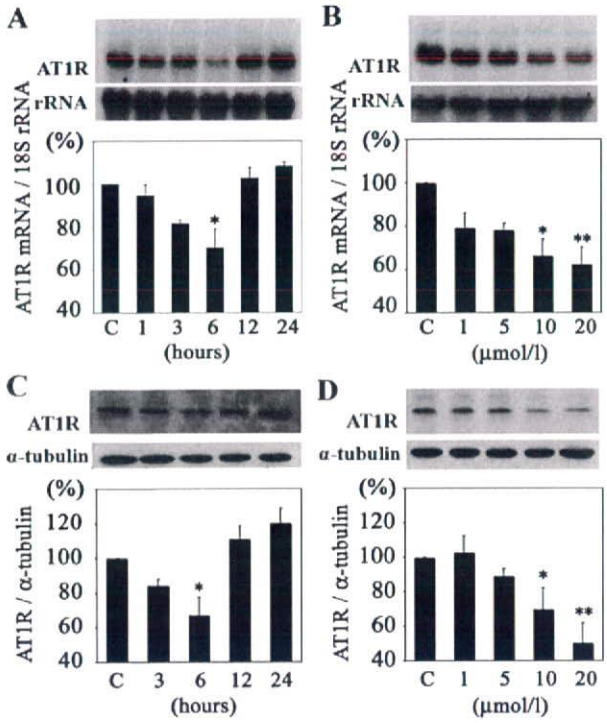

Figure 1. T0901317 suppressed AT1R expression in VSMCs. A, VSMCs were incubated with T0901317 (10 μmol/L) for various periods as indicated in the figure. Total RNA was isolated, and expression of AT1R mRNA and 18S rRNA (rRNA) was determined by Northern blot analysis. Radioactivity of AT1R mRNA was measured with an imaging analyzer and was normalized by radioactivity of rRNA. The ratio of AT1R mRNA to rRNA is shown in the bar graph. B, VSMCs were incubated with T0901317 at concentrations varying from 1 to 20 μmol/L for 6 hours. The expression of AT1R mRNA was determined and analyzed as described above. C, VSMCs were incubated with T0901317 (10 μmol/L) for various periods as indicated. Expression of AT1R protein and α-tubulin was detected by Western blot analysis. The density of the specific band was scanned and quantified with an imaging analyzer. The ratio of AT1R to α-tubulin is shown in the bar graph. D, VSMCs were incubated with T0901317 at various concentrations from 1 to 20 μmol/L for 6 hours. The expression of AT1R protein was determined and analyzed as described above. Values (means±SEMs) are expressed as a percent of control culture (100%; n=6). *$P<0.05$ vs control; **$P<0.01$ vs control. C indicates control.

that T0901317 induced suppression of AT1R gene expression by inhibiting Sp1 function.

T0901317 did not affect the degradation rate of AT1R mRNA (Figure 3B). These data suggest that T0901317 inhibits AT1R gene transcription and does not affect AT1R mRNA stability.

De Novo Protein Synthesis Is Required for T0901317-Induced Downregulation of AT1R Expression

We used CHX (10 μg/mL, 1 hour), a protein synthesis inhibitor, to examine whether T0901317-induced downregulation of AT1R expression depended on de novo protein synthesis. Incubation with CHX alone did not affect AT1R mRNA expression. CHX, however, inhibited the T0901317-induced AT1R mRNA downregulation (Figure 4A). These

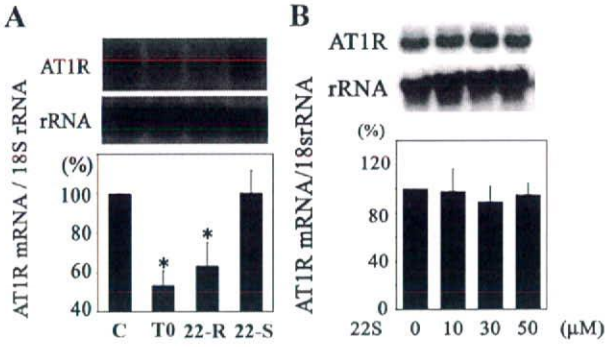

Figure 2. Downregulation of AT1R by 22-R-hydroxycholesterol (HC) but not by 22-S-HC. A, VSMCs were incubated with T0901317 (10 μmol/L), 22-R-HC (10 μmol/L), or 22-S-HC (10 μmol/L) for 6 hours. Expression of AT1R mRNA was determined as described in the legend to Figure 1A. Values (means±SEMs) are expressed as a percentage of control culture (100%; n=5). *$P<0.05$ vs control. B, VSMCs were incubated various concentration of 22-S-HC indicated in the figure for 6 hours (n=3). Expression of AT1R mRNA was determined as described in the legend to Figure 1A.

data suggest that downregulation of AT1R mRNA by LXR requires de novo protein synthesis.

Histone Deacetylase Activity Is Not Involved in T0901317-Induced Downregulation

It has been reported that the recruitment of histone deacetylase (HDAC) is necessary for gene regulation by a LXR agonist.[25] We used TSA (1 μmol/L), an HDAC inhibitor, to examine the involvement of HDAC in the process. Preincubation with TSA for 24 hours had no effect on T0901317-induced AT1R mRNA suppression (Figure 4B). It is, therefore, suggested that HDAC is not required for T0901317-induced AT1R downregulation.

T0901317 Increased p16 Expression and Suppressed Sp1 Phosphorylation

Previously, Wang et al[26] reported that p16 inhibited Sp1-mediated gene transcription by suppression of cyclin A expression and phosphorylation of Sp1 at the serine residue. We examined the effect of T0901317 (10 μmol/L) on p16 expression and the Sp1 phosphorylation level. T0901317 increased p16 expression and decreased the phosphorylation level of Sp1 at the serine residue. (Figure 4C and 4D).

T0901317-Induced AT1R Downregulation Reduced Cellular Response to Angiotensin II

It is well known that Ang II induces phosphorylation of ERK in VSMCs through AT1R.[27] First, we confirmed the receptor isoform responsible for Ang II-induced ERK activation. Losartan, an AT1R antagonist, but not PD123319, an Ang II type 2 receptor–specific antagonist, inhibited Ang II-induced ERK phosphorylation, indicating that AT1R is responsible (Figure 5A). Next we determined whether downregulation of AT1R gene expression leads to a reduction of functional response of VSMCs to Ang II stimulation. VSMCs were pretreated with T0901317 (10 μmol/L) for 30 minutes and 3, 6, and 12 hours and then stimulated with Ang II (100 nmol/L) for 5 minutes. The phosphorylation of ERK was examined by Western blot analysis. Ang II-induced ERK phosphorylation

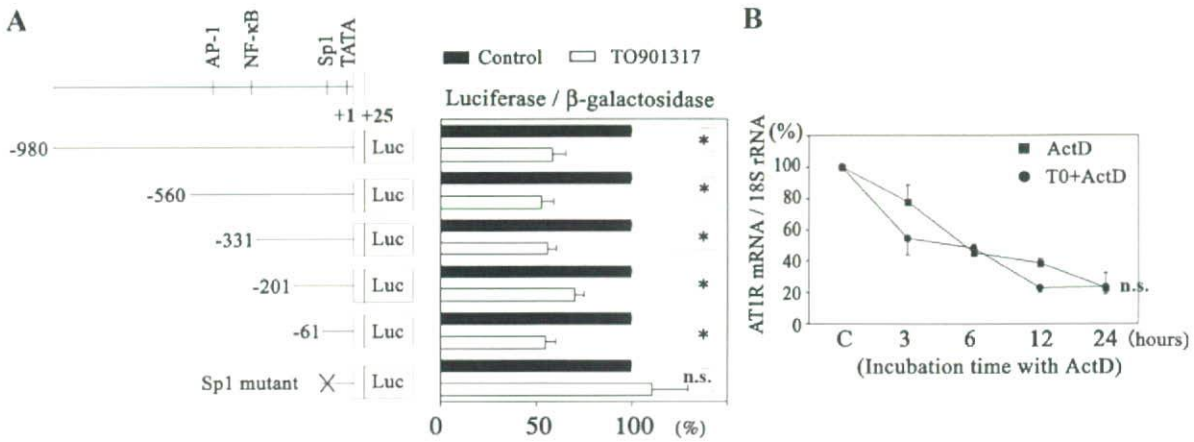

Figure 3. Effect of T0901317 on AT1R gene promoter activity and AT1R mRNA stability. A, The scheme of deletion mutants of AT1R promoter/luciferase fusion DNA construct is indicated on the left side. Relative luciferase activity normalized by β-galactosidase activity is indicated by the bar graph on the right. Relative luciferase activity of unstimulated VSMCs in each construct was set as 100%. Black and open bars indicate the relative luciferase activity of unstimulated and T0901317 (10 μmol/L)-stimulated VSMCs, respectively, which are transfected with the same construct indicated on the left. Values (means±SEMs) are expressed as a percentage of control culture (n=6). *$P<0.05$ vs unstimulated cells. n.s. indicates not significant. B, Total RNA was isolated at the indicated time after ActD (5 μg/mL) supplementation, and expression levels of AT1R mRNA and rRNA were determined with the method described in the legend to Figure 1A. Expression level of AT1R mRNA was normalized with that of rRNA. The normalized AT1R mRNA expression before the addition of ActD in each group was set as 100% (n=4).

was not affected by 30 minutes of preincubation with T0901317, suggesting that T0901317 had no direct effect on Ang II signaling. ERK phosphorylation was remarkably reduced after 6 to 12 hours of preincubation with T0901317 (Figure 5B) when AT1R expression is maximally suppressed (Figure 1A). However, phorbol ester (100 nM) increased ERK phosphorylation after incubation with T0901317 for 6 to 12 hours, suggesting that the ERK activation pathway may not be affected by T0901317 (data not shown). Thus, downregulation of AT1R consequently resulted in the attenuation of the cellular response to Ang II.

Discussion

In the present study, we demonstrated that T0901317, a synthetic LXR agonist, suppressed the expression of AT1R at mRNA and protein levels and that cellular response to Ang II was reduced by AT1R suppression. The results of the luciferase assay suggest that the AT1R promoter region that contains the Sp1 binding site is essential for T0901317-induced AT1R suppression. This is the first study reporting the effect of LXR activation on AT1R expression and its molecular mechanism. We also showed for the first time that LXR agonists upregulated p16 and induced dephosphorylation of Sp1, which may inhibit AT1R gene expression.

It was reported that LXRs regulate gene transcription by 2 mechanisms. One is a DNA-dependent pathway that involves binding of liganded LXR to LXR response element of target genes after the formation of heterodimer with the retinoid X receptor.[1] The other is an LXR response element–independent pathway that involves interference with other transcription factor pathways.[28] Several studies reported various indirect transcriptional regulations by LXR.[29,30] Transcription factors such as AP-1,[31] nuclear factor-κB,[28] c-Jun, and c-Fos[32] are inhibited by LXR. The AT1R gene promoter region does not contain the consensus sequence of LXR response element.[33] It is, therefore, suggested that the effect of LXR on AT1R downregulation may be mediated by the inhibition of other transcription factors.

Based on the deletion and mutation analysis of AT1R gene promoter, it was suggested that the Sp1 binding site located between −58 and −34 bp is crucial for T0901317-induced AT1R suppression. The basal luciferase activity of Sp1 mutant was 50% to 70% of wild-type (−61 bp) luciferase construct (data not shown), which is consistent with the previous study.[22] Therefore, the Sp1 site is a positive regulatory element of the AT1R gene, and T0901317 may suppress AT1R gene expression by inhibiting Sp1 function. Wang et al[26] reported the interaction between Sp1 and cyclinA in Sp1-mediated gene transcription. In this report, they concluded that p16, a cyclin-dependent kinase inhibitor, induced cyclinA/cyclin-dependent kinase downregulation, which resulted in the attenuation of phosphorylation of Sp1, and consequently suppressed Sp1-mediated gene transcription. Our results that showed increased p16 expression and a decreased phosphorylation level of Sp1 at the serine residue by T0901317 are consistent with this previous report. It is reported that T0901317 decreased platelet-derived growth factor–induced expression of cyclinA and D1 and inhibited VSMC proliferation.[34] However, it has not been reported that LXR ligand upregulates p16 as far as we know. Because T0901317-induced AT1R suppression requires de novo protein synthesis, it is suggested that upregulation of p16 expression and resultant inhibition of Sp1 phosphorylation may be responsible for T0901317-induced AT1R downregulation.

A recent report showed that the LXR ligand inhibited cytokine-induced clearance of nuclear receptor corepressor complexes from the C-reactive protein gene promoter.[25] It may be possible that recruitment of nuclear receptor corepressor complexes and HDAC is involved in LXR-induced

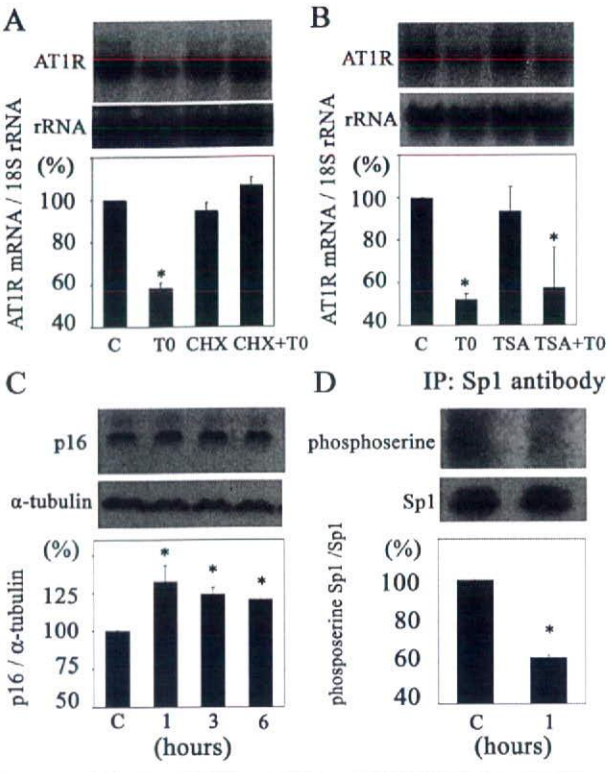

Figure 4. Effects of CHX and TSA on T0901317-induced downregulation of AT1R and effects of T0901317 on p16 expression and phosphorylation level of Sp1. A, VSMCs were incubated with or without CHX (10 μg/mL) for 1 hour and then incubated with T0901317 (10 μmol/L) for 6 hours. Expression of AT1R mRNA was determined as described in the legend to Figure 1A. Values (means±SEMs) are expressed as a percentage of control culture (100%; n=5). *P<0.05 vs control. B, VSMCs were incubated with TSA (1 μmol/L) for 24 hours and then incubated with T0901317 (10 μmol/L) for 6 hours. Expression of AT1R mRNA was determined as described in the legend to Figure 1A. Values (means±SEMs) are expressed as a percent of control culture (100%; n=5). *P<0.05 vs control or TSA. C, VSMCs were incubated with T0901317 (10 μmol/L) for various periods, as indicated. The expressions of p16 protein and α-tubulin were determined and analyzed as described in the legend to Figure 1C. Values (means±SEMs) are expressed as a percentage of control culture (100%; n=6). *P<0.05 vs control. D, VSMCs were incubated with T0901317 for 1 hour. The cell lysates were subjected to immunoprecipitation with an anti-Sp1 antibody, followed by Western blot analysis with an antiphosphoserine antibody (top). The membrane was stripped and reprobed with an anti-Sp1 antibody (bottom). *P<0.05 vs control. C indicates control.

AT1R downregulation. However, in our study, we found that TSA did not affect the T0901317-induced AT1R suppression, which may exclude the possible involvement of HDAC in LXR agonist-induced AT1R downregulation.

The expression of AT1R returned to the control level after 12 hours of stimulation with T0901317. However, replacement of the medium with a fresh serum-free medium containing 10 μmol/L of T0901317 at 6 hours of stimulation resulted in the suppression of AT1R at 12 hours. These data suggested that recovery of the AT1R expression at 12 hours was because of degradation or metabolism of the T0901317 rather than the desensitization of AT1R gene expression to T0901317. We also showed that T0901317 reduced Ang II-induced ERK phosphorylation. Intriguingly, ERK phosphorylation was still suppressed after 12 hours of stimulation when the expression of the AT1R level returned to the control level. This may suggest that AT1R protein in the surface of VSMCs had not been fully recovered, although the mRNA or protein level of AT1R was recovered.

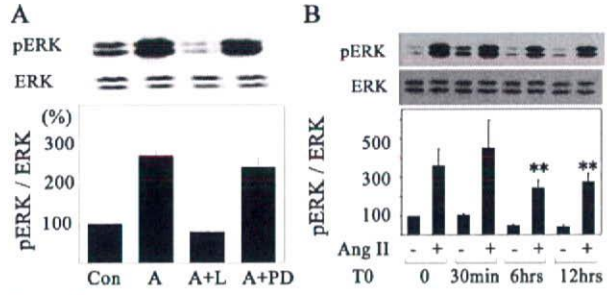

Figure 5. Reduction of Ang II-induced ERK phosphorylation by T0901317. A, VSMCs were pretreated with either losartan (10 μmol/L) or PD123319 (10 μmol/L) for 30 minutes and then stimulated with Ang II (100 nmol/L) for 5 minutes (n=3). B, VSMCs were pretreated with T0901317 (T0: 10 μmol/L) for 30 minutes and 3, 6, and 12 hours and then stimulated with Ang II (100 nmol/L) for 5 minutes. pERK and ERK protein were detected by Western blot analysis. The density of the specific band was scanned and quantified with an imaging analyzer. The ratio of pERK to ERK is shown in the bar graph. Values (means±SEMs) are expressed as a percentage of control culture (100%; n=5). ##P<0.01 vs control; **P<0.01 vs Ang II without T0901317.

LXR activators are reported to prevent the development and progression of atherosclerosis in animal models.[8,9] The important molecular mechanisms involve the reduction of inflammatory responses, such as cytokine production and improvement of glucose and lipid metabolism. Joseph et al[7] reported that LXR activators inhibit inflammation by downregulating the expression of inducible NO synthase, cyclooxygenase-2, and interleukin-6. In contrast, the enhanced Ang II signaling pathway causes atherosclerosis,[11] which results from activation of inflammatory responses, such as cytokine production, matrix deposition, and induction of adhesion molecules. Therefore, it may be possible that the antiatherosclerotic effects of the LXR activator involve AT1R downregulation.

Perspectives

Our results showing that LXRs agonists downregulate AT1R expression and attenuate the cellular response to Ang II indicate another anti-inflammatory property of LXR activators through inhibition of Ang II signaling. Ang II plays an important role in various pathological conditions and is effective through Ang II receptors. The cellular response to Ang II depends on the expression level of AT1R; thus, downregulation of AT1R can be one way to avoid the vicious effect of Ang II. Because LXRs are expressed ubiquitously, activation of LXRs may be a novel and an effective therapy to attenuate pathological effects of Ang II.

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Disclosures

None.

References

1. Peet DJ, Janowski BA, Mangelsdorf DJ. The LXRs: a new class of oxysterol receptors. *Curr Opin Genet Dev*. 1998;8:571–575.
2. Mitro N, Mak PA, Vargas L, Godio C, Hampton E, Molteni V, Kreusch A, Saez E. The nuclear receptor LXR is a glucose sensor. *Nature*. 2007;445:219–223.
3. Naik SU, Wang X, Da Silva JS, Jaye M, Macphee CH, Reilly MP, Billheimer JT, Rothblat GH, Rader DJ. Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. *Circulation*. 2006; 113:90–97.
4. Laffitte BA, Repa JJ, Joseph SB, Wilpitz DC, Kast HR, Mangelsdorf DJ, Tontonoz P. LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc Natl Acad Sci U S A*. 2001;98:507–512.
5. Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, Tran J, Tippin TK, Wang X, Lusis AJ, Hsueh WA, Law RE, Collins JL, Willson TM, Tontonoz P. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci U S A*. 2002;99:7604–7609.
6. Laffitte BA, Chao LC, Li J, Walczak R, Hummasti S, Joseph SB, Castrillo A, Wilpitz DC, Mangelsdorf DJ, Collins JL, Saez E, Tontonoz P. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. *Proc Natl Acad Sci U S A*. 2003;100:5419–5424.
7. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med*. 2003;9:213–219.
8. Tangirala RK, Bischoff ED, Joseph SB, Wagner BL, Walczak R, Laffitte BA, Daige CL, Thomas D, Heyman RA, Mangelsdorf DJ, Wang X, Lusis AJ, Tontonoz P, Schulman IG. Identification of macrophage liver X receptors as inhibitors of atherosclerosis. *Proc Natl Acad Sci U S A*. 2002;99:11896–11901.
9. Levin N, Bischoff ED, Daige CL, Thomas D, Vu CT, Heyman RA, Tangirala RK, Schulman IG. Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists. *Arterioscler Thromb Vasc Biol*. 2005;25:135–142.
10. Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL, Timmermans PBMWM. Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun*. 1989;165:196–203.
11. Daugherty A, Cassis L. Chronic angiotensin II infusion promotes atherogenesis in low density lipoprotein receptor−/− mice. *Ann N Y Acad Sci*. 1999;892:108–118.
12. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest*. 2000;105:1605–1612.
13. Hayek T, Attias J, Coleman R, Brodsky S, Smith J, Breslow JL, Keidar S. The angiotensin-converting enzyme inhibitor, fosinopril, and the angiotensin II receptor antagonist, losartan, inhibit LDL oxidation and attenuate atherosclerosis independent of lowering blood pressure in apolipoprotein E deficient mice. *Cardiovasc Res*. 1999;44:579–587.
14. Keidar S, Attias J, Smith J, Breslow JL, Hayek T. The angiotensin-II receptor antagonist, losartan, inhibits LDL lipid peroxidation and atherosclerosis in apolipoprotein E-deficient mice. *Biochem Biophys Res Commun*. 1997;236:622–625.
15. Vaughan DE. AT(1) receptor blockade and atherosclerosis: hopeful insights into vascular protection. *Circulation*. 2000;101:1496–1497.
16. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292:C82–C97.
17. Sahar S, Reddy MA, Wong C, Meng L, Wang M, Natarajan R. Cooperation of SRC-1 and p300 with NF-kappaB and CREB in angiotensin II-induced IL-6 expression in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2007;27:1528–1534.
18. Morello F, de Boer RA, Steffensen KR, Gnecchi M, Chisholm JW, Boomsma F, Anderson LM, Lawn RM, Gustafsson JK, Lopez-Ilasaca M, Pratt RE, Dzau VJ. Liver X receptors alpha and beta regulate renin expression in vivo. *J Clin Invest*. 2005;115:1913–1922.
19. Calegari VC, Bezerra RM, Torsoni MA, Torsoni AS, Franchini KG, Saad MJ, Velloso LA. Suppressor of cytokine signaling 3 is induced by angiotensin II in heart and isolated cardiomyocytes, and participates in desensitization. *Endocrinology*. 2003;144:4586–4596.
20. Fraccarollo D, Galuppo P, Hildemann S, Christ M, Ertl G, Bauersachs J. Additive improvement of left ventricular remodeling and neurohormonal activation by aldosterone receptor blockade with eplerenone and ACE inhibition in rats with myocardial infarction. *J Am Coll Cardiol*. 2003; 42:1666–1673.
21. Imayama I, Ichiki T, Inanaga K, Ohtsubo H, Fukuyama K, Ono H, Hashiguchi Y, Sunagawa K. Telmisartan downregulates angiotensin II type 1 receptor through activation of peroxisome proliferator-activated receptor gamma. *Cardiovasc Res*. 2006;72:184–190.
22. Sugawara A, Takeuchi K, Uruno A, Ikeda Y, Arima S, Kudo M, Sato K, Taniyama Y, Ito S. Transcriptional suppression of type 1 angiotensin II receptor gene expression by peroxisome proliferator-activated receptor-gamma in vascular smooth muscle cells. *Endocrinology*. 2001; 142:3125–3134.
23. Ichiki T, Tokunou T, Fukuyama K, Iino N, Masuda S, Takeshita A. Cyclic AMP response element-binding protein mediates reactive oxygen species-induced c-fos expression. *Hypertension*. 2003;42:177–183.
24. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*. 1996;383:728–731.
25. Ghisletti S, Huang W, Ogawa S, Pascual G, Lin ME, Willson TM, Rosenfeld MG, Glass CK. Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma. *Mol Cell*. 2007;25:57–70.
26. Wang CH, Chang HC, Hung WC. p16 inhibits matrix metalloproteinase-2 expression via suppression of Sp1-mediated gene transcription. *J Cell Physiol*. 2006;208:246–252.
27. Eguchi S, Matsumoto T, Motley ED, Utsunomiya H, Inagami T. Identification of an essential signaling cascade for mitogen-activated protein kinase activation by angiotensin II in cultured rat vascular smooth muscle cells. Possible requirement of Gq-mediated p21ras activation coupled to a Ca2+/calmodulin-sensitive tyrosine kinase. *J Biol Chem*. 1996;271: 14169–14175.
28. Terasaka N, Hiroshima A, Ariga A, Honzumi S, Koieyama T, Inaba T, Fujiwara T. Liver X receptor agonists inhibit tissue factor expression in macrophages. *Febs J*. 2005;272:1546–1556.
29. Grempler R, Gunther S, Steffensen KR, Nilsson M, Barthel A, Schmoll D, Walther R. Evidence for an indirect transcriptional regulation of glucose-6-phosphatase gene expression by liver X receptors. *Biochem Biophys Res Commun*. 2005;338:981–986.
30. Blaschke F, Takata Y, Caglayan E, Collins A, Tontonoz P, Hsueh WA, Tangirala RK. A nuclear receptor corepressor-dependent pathway mediates suppression of cytokine-induced C-reactive protein gene expression by liver X receptor. *Circ Res*. 2006;99:e88–99.
31. Schmuth M, Elias PM, Hanley K, Lau P, Moser A, Willson TM, Bikle DD, Feingold KR. The effect of LXR activators on AP-1 proteins in keratinocytes. *J Invest Dermatol*. 2004;123:41–48.
32. Ogawa D, Stone JF, Takata Y, Blaschke F, Chu VH, Towler DA, Law RE, Hsueh WA, Bruemmer D. Liver x receptor agonists inhibit cytokine-induced osteopontin expression in macrophages through interference with activator protein-1 signaling pathways. *Circ Res*. 2005;96:e59–e67.
33. Takeuchi K, Alexander RW, Nakamura Y, Tsujino T, Murphy TJ. Molecular structure and transcriptional function of the rat vascular AT1a angiotensin receptor gene. *Circ Res*. 1993;73:612–621.
34. Blaschke F, Leppanen O, Takata Y, Caglayan E, Liu J, Fishbein MC, Kappert K, Nakayama KI, Collins AR, Fleck E, Hsueh WA, Law RE, Bruemmer D. Liver X receptor agonists suppress vascular smooth muscle cell proliferation and inhibit neointima formation in balloon-injured rat carotid arteries. *Circ Res*. 2004;95:e110–e123.

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Effects of Valsartan or Amlodipine on Endothelial Function and Oxidative Stress after One Year Follow-up in Patients with Essential Hypertension

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Endothelial function is impaired in hypertensive patients. Decreased nitric oxide production and increased oxidative stress are involved in this abnormality. The aim of the present study was to evaluate whether endothelial function and oxidative stress differ following long-term antihypertensive treatment with an angiotensin type 1 receptor blocker, valsartan, or a calcium channel blocker, amlodipine, in patients with essential hypertension. Hypertensive patients were treated with valsartan (80–160 mg/day) or amlodipine (5–10 mg/day) for one year (n = 9 for each). The baseline blood pressure was similar between groups, and the magnitude of the decreases in blood pressure did not differ during treatment at three months, six months, or one year. Endothelial function and oxidative stress markers were examined before and after treatment. Endothelial function, assessed by flow-mediated vasodilation, was significantly improved in hypertensive patients treated with valsartan (5.8 ± 1.2 to 10.7 ± 1.4 %, $p < 0.01$) but not in those treated with amlodipine. The percent increase in vasodilation induced by sublingual nitroglycerin did not differ between the two groups. As markers of oxidative stress, urinary excretion of 8-isoprostane and 8-hydroxy-2′-deoxyguanosine was significantly reduced in patients treated with valsartan, but not in those treated with amlodipine. These findings suggest that the treatment of hypertensive patients with valsartan for at least one year improves endothelial function in association with reduced oxidative stress. The improved endothelial function and reduced oxidative stress might be involved in the benefits of anti-hypertensive treatment beyond simply lowering blood pressure, although the effects of treatment with valsartan or amlodipine over a much longer period are unknown.

Keywords blood pressure, endothelium, angiotensin receptors, calcium channel blockers

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Introduction

Valsartan, an angiotensin type 1 (AT1) receptor blocker, and amlodipine, a long-acting dihydropyridine calcium channel blocker (CCB), are widely used for the treatment of hypertension. Large clinical trials have confirmed their usefulness for preventing cardiovascular events mainly by lowering blood pressure (1,2). These relatively new classes of antihypertensive drugs are expected to have some beneficial effects beyond their blood pressure-lowering actions due to their nitric oxide (NO)-producing, anti-oxidant, and anti-inflammatory effects, which were established in experimental animal studies (3,4).

Endothelial dysfunction occurs at the early stage of hypertension and leads to hypertensive vascular diseases (5,6). The precise mechanisms involved are complicated, but involve reduced NO production and increased generation of reactive oxygen species (ROS; 7,8). The effects of antihypertensive drugs on endothelial function have been studied clinically with a relatively small population (9–12). Some studies demonstrated that inhibitors of the renin-angiotensin system, such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ARBs), improve impaired endothelial function in the forearm in hypertensive patients (9,10,12). To the best of our knowledge, however, there are no studies comparing the effects of valsartan and amlodipine on endothelial function together with their anti-oxidant effects in hypertensive patients. Therefore, the aim of the present study was to examine whether long-term treatment with valsartan or amlodipine improves endothelial function and reduces ROS generation in hypertensive Japanese patients.

Methods

Patients and Design

Twenty-five patients with hypertension participated in this study. The definitions used for hypertension were those of the Japanese Society of Hypertension: systolic blood pressure of at least 140 mmHg and/or diastolic blood pressure of at least 90 mmHg. Valsartan (80 mg) or amlodipine (5 mg) was administered. The study was an open trial study. After confirming the blood pressure level and the absence of side effects, some patients received higher doses of each drug (160 mg [valsartan] or 10 mg [amlodipine]) to achieve an optimal blood pressure level. The patients were observed at 30-day intervals during the study. None of the patients were diabetic or current smokers. Baseline total cholesterol, triglyceride, and high-density lipoprotein cholesterol levels are shown in Table 1. The study was approved by the Ethics Committee of Kyushu University, and written informed consent was obtained from each patient.

Measurement of Flow-Mediated Vasodilation (FMD)

Imaging studies of the right brachial artery were performed using a Toshiba Power Vision 8000 ultrasound machine equipped with a 11-MHz linear array transducer. The brachial artery diameter was measured in response to increased flow (flow-mediated vasodilation, FMD) and in response to nitroglycerin spray (400 μg; 13,14). Increased flow was achieved by inflating a pneumatic tourniquet placed around the forearm to a pressure of 50 mmHg above the systolic pressure for 5 minutes followed by rapid cuff deflation. Blood flow velocity was measured using the Doppler echo method. All ultrasound

Table 1
Patient characteristics

	Valsartan	Amlodipine
Number of patients	9	9
Age (y)	57 ± 3	66 ± 3
Male/female	2/7	4/5
Height (cm)	157 ± 3	156 ± 3
Weight (kg)	63 ± 4	60 ± 4
BMI (kg/m2)	25.7 ± 1.5	24.5 ± 1.1
FBS (mg/dL)	91.7 ± 2.1	93.8 ± 3.1
HbA1c (%)	4.98 ± 0.04	4.99 ± 0.12
Total cholesterol (mg/dL)	212.6 ± 16.3	203.9 ± 10.4
Triglyceride (mg/dL)	144.6 ± 31.5	97.3 ± 6.9
HDL-C (mg/dL)	59.7 ± 5.0	59.8 ± 5.1

Values are mean ± SE. Abbreviations: BMI = body mass index, FBS = fasting blood sugar.

measures were performed by one experienced well-trained ultrasonographer who was blind to the treatment. The intra-observer variability was 0.79 ± 0.23%.

Measurement of Oxidative Stress Markers

Serum concentrations of cholesterol, triglycerides, HDL-cholesterol, glucose, etc., were measured before and during the treatment as needed. The urinary concentrations of 8-iso-prostane and 8-hydroxy-2′-deoxyguanosine (8-OHdG) were assayed before and after 12 months of treatment using an enzyme-linked immunosorbent assay (SRL, Tokyo, Japan). Plasma concentrations of renin, angiotensin II, aldosterone, and brain natriuretic peptide were measured using an enzyme-linked immunosorbent assay (SRL).

Statistical Analysis

Baseline values were examined using an unpaired Student's *t*-test, and the basal values before and after treatment were compared using a paired *t*-test. Changes in blood pressure and FMD were analyzed using a two-way analysis of variance. Data are expressed as mean ± SE. A p value of less than 0.05 was considered significant.

Results

Study Population Characteristics

The clinical characteristics of the hypertensive patients (n = 18, 7 men and 11 women; mean age: 61 ± 2 years; ages between 39–76 years) are summarized in Table 1. Body mass index, fasting blood sugar, and lipid profiles did not differ between the valsartan- and amlodipine-treated groups. Plasma hormone profiles are shown in Table 2. Plasma brain natriuretic peptide levels were significantly lower in the valsartan-treated group than in the amlodipine-treated group at 6 and 12 months.

Table 2
Plasma hormone and inflammatory markers before and during the treatment

	Valsartan				Amlodipine			
	Baseline	6-m	12-m	*p* value	Baseline	6-m	12-m	*p* value
Renin (pg/mL)	4.4 ± 0.6	9.1 ± 1.7	9.9 ± 1.6	0.024	6.8 ± 0.8	7.7 ± 2.4	7.3 ± 1.6	0.921
Ang II (pg/mL)	9.0 ± 2.6	11.2 ± 2.4	10.0 ± 2.2	0.808	6.0 ± 0.9	7.8 ± 1.1	6.6 ± 1.3	0.548
Aldosterone (pg/mL)	124.9 ± 15.6	88.9 ± 13.9	97.2 ± 11.6	0.176	105.0 ± 12.5	114.9 ± 15.3	108.2 ± 16.7	0.896
Noradrenaline (pg/mL)	418.4 ± 41.2	487.0 ± 34.9	500.9 ± 72.6	0.499	549.8 ± 53.2	654.6 ± 80.0	555.0 ± 74.6	0.510
Adrenaline (pg/mL)	31.7 ± 6.7	34.9 ± 10.3	34.2 ± 10.1	0.966	50.7 ± 7.9	39.3 ± 8.3	43.2 ± 8.2	0.609
BNP (pg/mL)	23.4 ± 6.0	15.6 ± 4.0*	13.0 ± 2.9*	0.250	48.7 ± 15.4	43.8 ± 12.0	51.3 ± 15.6	0.934
hs-CRP (μg/mL)	641 ± 368	611 ± 248	541 ± 268	0.971	620 ± 134	1355 ± 600	1178 ± 517	0.513

p value shows intra-group comparison.*$p < 0.05$ vs. amlodipine at the same month.
Abbreviations: Ang II = angiotensin II, BNP = brain natriuretic peptide, hs-CRP = high sensitivity C-reactive protein.

Blood Pressure and Heart Rate Changes During the Treatment

Time courses of blood pressure and heart rate are shown in Figure 1. The blood pressure reduction following treatment did not differ between groups. Heart rate did not change during either treatment (see Figure 2).

Effect of Valsartan or Amlodipine on FMD

Baseline brachial arterial diameter before treatment did not differ between the valsartan- and amlodipine-treated groups (3.24 ± 0.32 vs. 3.35 ± 0.23 mm). The baseline diameter after the treatment also did not differ between the two groups (4.03 ± 0.15 vs. 4.43 ± 0.30 mm). The baseline diameter in both groups tended to increase, although the increase was not statistically significant. The percent change in FMD did not differ between the groups before the treatment. Treatment with valsartan for 12 months, however, increased the percent change in

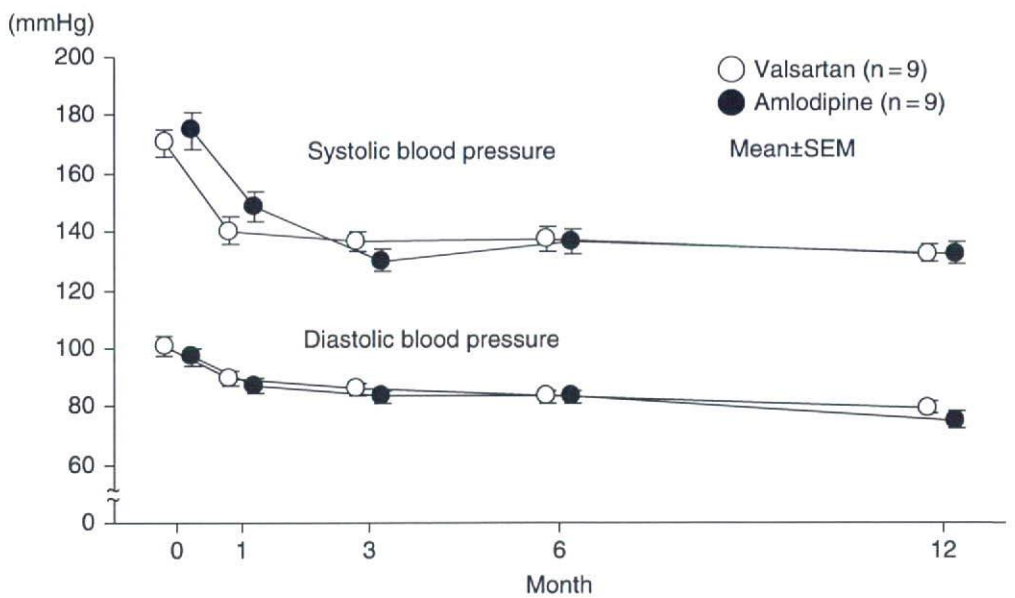

Figure 1. Time course of systolic and diastolic blood pressure in hypertensive patients treated with valsartan (open circle) or amlodipine (closed circle) (n = 9 for each).

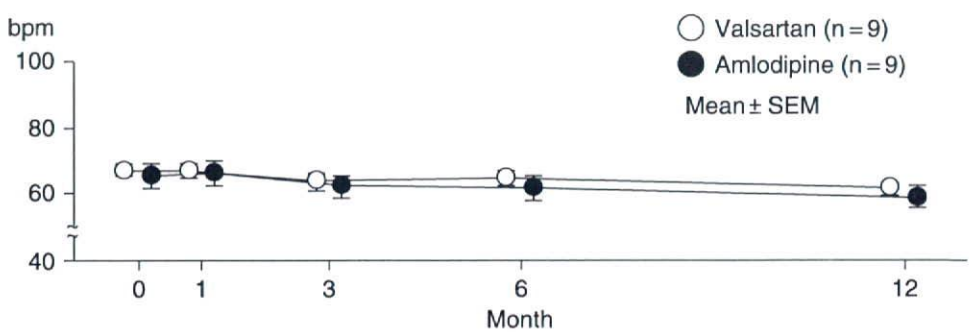

Figure 2. Time course of heart rate in hypertensive patients treated with valsartan (open circle) or amlodipine (closed circle) (n = 9 for each).

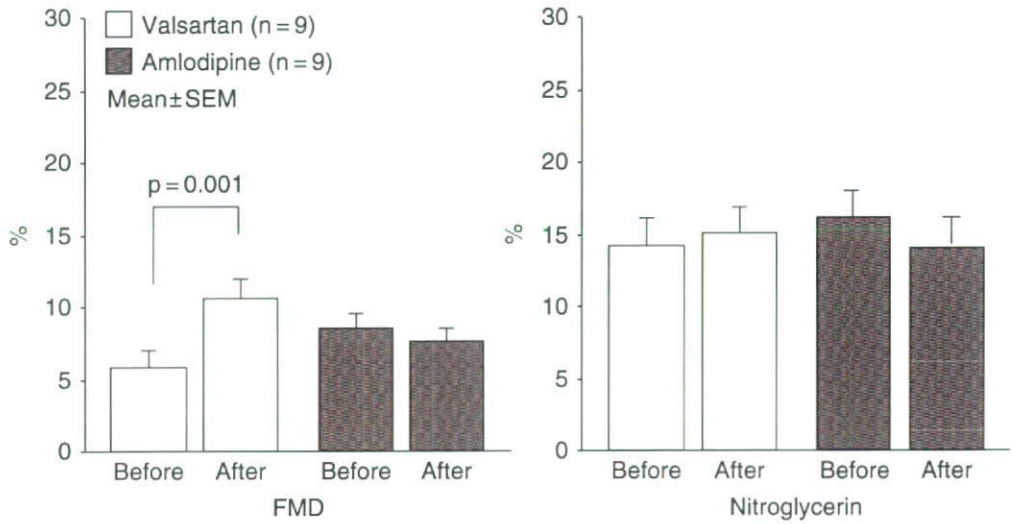

Figure 3. Percent change in the brachial diameter in response to FMD (left panel) or nitroglycerin (right panel) before and after the treatment with valsartan (open bar) or amlodipine (shadow bar).

FMD, but treatment with amlodipine did not (see Figure 3). The percent change in Doppler flow velocity evoked by reactive hyperemia did not differ between groups or before and after each treatment (before and after valsartan: 236 ± 18 vs. 213 ± 17%; before and after amlodipine: 183 ± 9 vs. 197 ± 12%). The percent change in the brachial artery diameter evoked by nitroglycerin did not differ between before and after either treatment (see Figure 3).

Effect of Valsartan or Amlodipine on Oxidative Stress Markers

Urinary levels of 8-isoprostane and OHdG were significantly decreased in patients treated with valsartan, but not in patients treated with amlodipine (see Figure 4). The baseline values of these markers did not differ between the valsartan- and amlodipine-treated groups.

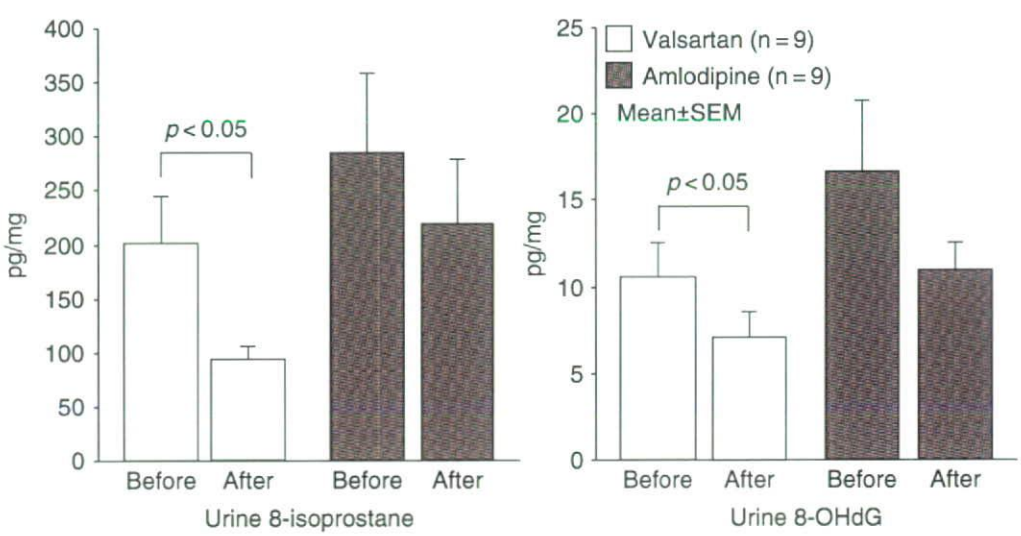

Figure 4. Urinary concentration of 8-isoprostane or 8-OHdG before and after treatment with valsartan (open bar) or amlodipine (shadow bar).

Discussion

The major findings of the present study were that treatment of patients with hypertension for 12 months with valsartan, but not amlodipine, increased the percent change in FMD in association with reduced urinary levels of 8-isoprostane and OHdG. These results suggest that valsartan, an AT1 receptor blocker, improves endothelial function (probably due to reduced oxidative stress) in hypertensive patients.

We evaluated the endothelial function of the brachial artery (conduit artery, not resistance artery) by FMD in the present study. This method is rapidly achieving widespread use because it is non-invasive (13,14). The extent of the change in dilatation, however, is small, and endothelial function evaluated using this method is based on the assumption that increases in flow are due to reactive hyperemia because the stimuli are the same. Guidelines or statements regarding the implications and limitations for assessing endothelial function were recently proposed (13,14). In addition, we measured urinary excretion of 8-isoprostane and 8-OHdG, which are relatively sensitive markers of ROS generation (15–17). An evaluation of oxidative stress levels in humans is somewhat difficult due to variation in the measurements. Therefore, the validity of these measurements and whether they truly reflect oxidative stress, particularly in outpatients, is unknown. Urinary excretion levels of 8-isoprostane and 8-OHdG were reduced after treatment with valsartan, but not after treatment with amlodipine, at least at the one-year follow-up. These results suggest that oxidative stress was reduced by the treatment with valsartan in our hypertensive patients.

Endothelial function is determined by the balance between NO and superoxide production (6). Bioavailable NO is scavenged by superoxide (6). An important source of superoxide production is NAD(P)H oxidase, which is activated by AT1 receptor stimulation (18). Thus, it is possible that ARB improves endothelial dysfunction by this mechanism. There are few reports, however, describing this action of ARB in humans (12,19–21). In particular, to the best of our knowledge, there are no reports describing valsartan-induced improvement of endothelial dysfunction in hypertensive patients. Our observations suggest that valsartan increased FMD in hypertensive patients and reduced oxidative stress markers.

Endothelial dysfunction in hypertension precedes the development of atherosclerotic processes in the vasculature, which lead to the hypertensive vascular diseases and organ damage (5,6). Although strict long-term treatment with antihypertensive drugs is the most important therapy, evidence from large clinical trials suggests that both ARB and CCB are widely used for the treatment of hypertension without consideration of their effects beyond lowering blood pressure (1,2). In particular, both classes of drugs improve endothelial dysfunction (12,22). ARBs, such as losartan, irbesartan, and candesartan, ameliorate endothelial dysfunction in hypertensive patients (12,19–21). CCBs, such as long-acting nifedipine, amlodipine, and efonidipine, also improve endothelial dysfunction (10,22–25), although there is some controversy (26). We do not have a clear explanation for our finding that amlodipine did not improve endothelial dysfunction, because it has antioxidant effects in vitro (4).

In addition to ARB and CCB, some studies show effects of various antihypertensive drugs on endothelial function, although there are differences among drugs in their effects on endothelial function (23–26). Acute blood pressure reduction itself does not improve endothelial function (25), suggesting that long-term treatment with antihypertensive drugs is needed to improve endothelial dysfunction. It also suggests that some classes of drugs have beneficial effects on endothelial function beyond lowering blood pressure, although

this is difficult to demonstrate in humans. In general, inhibitors of the renin-angiotensin system, such as angiotensin-converting enzyme inhibitors or ARBs, are superior to other treatments (9,24,27). β-blockers or diuretics do not effectively improve endothelial function (24,26). Indeed, angiotensin II increases oxidative stress in human forearm vessels (28). Caution is necessary, however, in interpreting the results of those studies, as differences in the methods used, the duration of the treatment, and the study population must be considered.

There are some limitations of the present study. The number of study subjects was small due to difficulties enrolling patients according to the guidelines established by the Ethics Committee of our University. In addition, some patients dropped out from the study due to the necessity of a 12-month follow-up and the repetitive nature of the protocol. Another limitation was that the clinical characteristics varied among the patients enrolled in the present study. This was an open trial study, and each drug was administered in the order in which the patients were enrolled.

In conclusion, the present study demonstrates that valsartan improves endothelial function in hypertensive patients in association with a reduction in oxidative stress markers after a one-year follow-up. Amlodipine, however, did not have this effect in the patients we studied. More long-term follow-up and large multicenter clinical trials are necessary to confirm the improvement of endothelial dysfunction and the prevention of future cardiovascular events.

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References

1. Julius S, Kjeldsen SE, Weber M, Brunner HR, Ekman S, Hansson L, Hua T, Laragh J, McInnes GT, Mitchell L, Plat F, Schork, A, Smith B, Zanchetti A. Outcomes in hypertensive patients at high cardiovascular risk treated with regimens based on valsartan or amlodipine: The VALUE randomized trial. *Lancet*. 2004;363:2022–2031.
2. The ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group. Major outcomes in high-risk hypertensive patients randomized to angiotensin converting enzyme inhibitor or calcium channel blocker vs. diuretic. The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). *JAMA*. 2002;288:2981–2997.
3. Sironi L, Gelosa P, Guerrini U, Banfi C, Crippa V, Brioschi M, Gianazza E, Nobili E, Gianella A, de Gasparo M, Tremoli E. Anti-inflammatory effects of AT1 receptor blockade provide end-organ protection in stroke-prone rats independently from blood pressure fall. *J Pharmacol Exp Ther*. 2004;311:989–995.
4. Hirooka Y, Kimura Y, Nozoe M, Sagara Y, Ito K, Sunagawa K. Amlodipine-induced reduction of oxidative stress in the brain is associated with sympatho-inhibitory effects in stroke-prone spontaneously hypertensive rats. *Hypertens Res*. 2006;29:49–56.
5. Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, Ferraro A, Chello M, Mastroroberto P, Verdecchia P, Schillaci G. Prognostic significance of endothelial function in hypertensive patients. *Circulation*. 2001;104:191–196.
6. Brunner H, Cockcroft JR, Deanfield J, Donald A, Ferrannini E, Halcox J, Kiowski W, Lüscher TF, Mancia G, Natali A, Oliver JJ, Pessina AC, Rizzoni D, Rossi GP, Salvetti A, Spieker LE, Taddei S, Webb DJ. Endothelial function and dysfunction, part II: Association with cardiovascular risk factors and diseases. A statement by the working group on

endothelin and endothelial factors of the European Society of Hypertension. *J Hypertens*. 2005;23:233–246.
7. Panza J, Casino PR, Kilcoyne CM, Quyyumi AA. Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation*. 1993;87:1468–1474.
8. Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation*. 1998;97:2222–2229.
9. Hirooka Y, Imaizumi T, Masaki H, Ando S, Harada S, Momohara M, Takeshita A. Captopril improves impaired endothelium-dependent vasodilation in hypertensive patients. *Hypertension*. 1992;20:175–180.
10. Taddei S, Virdis A, Ghiadoni L, Matti P, Salvetti A. Effects of angiotensin converting enzyme inhibition on endothelium-dependent vasodilatation in essential hypertensive patients. *J Hypertens*. 1998;16:447–456.
11. Taddei S, Virdis A, Ghiadoni L, Magagna A, Favilla S, Pompella A, Salvetti A. Restoration of nitric oxide availability after calcium antagonist treatment in essential hypertension. *Hypertension*. 2001;37:943–948.
12. Ghiadoni L, Virdis A, Magagna A, Taddei S, Salvetti A. Effect of the angiotensin II type 1 receptor blocker candesartan on endothelial function in patients with essential hypertension. *Hypertension*. 2000;35:501–506.
13. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery. *J Am Coll Cardiol*. 2002;39:257–265.
14. Deanfield J, Donald A, Ferri C, Giannattasio C, Halcox J, Halligan S, Lerman A, Mancia G, Oliver JJ, Pessina AC, Rizzoni D, Rossi GP, Salvetti A, Schiffrin EL, Taddei S, Webb DJ. Endothelial function dysfunction, part I: Methodological issues for assessment in the different vascular beds: A statement by the working group on endothelin and endothelial factors of the European Society of Hypertension. *J Hypertens*. 2005;23:7–17.
15. Hozawa A, Ebihara S, Ohmori K, Kuriyama S, Ugajin T, Koizumi Y, Suzuki Y, Matsui T, Arai H, Tsubono Y, Sasaki H, Tsuji I. Increased plasma 8-isoprostane levels in hypertensive subjects: The Tsurugaya Project. *Hypertens Res*. 2004;27:557–561.
16. Schwedhelm E, Bartling A, Lenzen H, Tsikas D, Maas R, Brümmer J, Gutzki F-M, Chem I, Berger J, Frölich JC, Böger RH. Urinary 8-iso-prostaglandin $F_{2\alpha}$ as a risk marker in patients with coronary heart disease: A matched case-control study. *Circulation*. 2004;109: 843–848.
17. Oshima T, Ozono R, Yano Y, Higashi Y, Teragawa H, Miho N, Ishida T, Ishida M, Yoshizumi M, Kambe M. Beneficial effect of T-type calcium channel blockers on endothelial function in patients with essential hypertension. *Hypertens Res*. 2005;28:889–894.
18. Cai H, Griendling KK, Harrison DG. The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. *Trends Pharmacol Sci*. 2003;24:471–478.
19. Schiffrin EL, Park JB, Intengan HD, Touyz RM. Correction of arterial structure and endothelial dysfunction in human essential hypertension by the angiotensin receptor antagonist losartan. *Circulation*. 2000;101:1653–1659.
20. Koh KK, Ahn JY, Han SH, Kim DS, Jin DK, Kim HS, Shin M-S, Ahn TH, Choi IS, Shin EK. Pleiotropic effects of angiotensin II receptor blocker in hypertensive patients. *J Am Coll Cardiol*. 2003;42:905–910.
21. Koh KK, Han SH, Chung W-J, Ahn JY, Jin DK, Kim HS, Park GS, Kang WC, Ahn TH, Shin EK. Comparison of effects of losartan, irbesartan, and candesartan on flow-mediated brachial artery dilation and inflammatory and thrombolytic markers in patients with systemic hypertension. *Am J Cardiol*. 2004;93:1432–1435.
22. On Y-K, Kim C-H, Oh B-H, Lee M-M, Park Y-B. Effects of angiotensin converting enzyme inhibitor and calcium antagonist on endothelial function in patients with essential hypertension. *Hypertens Res*. 2002;25:365–371.

23. Muiesan ML, Salvetti M, Monteduro C, Rizzoni D, Zulli R, Corbellini C, Brun C, Agabiti-Rosei E. Effect of treatment of flow-dependent vasodilation of the brachial artery in essential hypertension. *Hypertension*. 1999;33:575–580.
24. Ghiadoni L, Magagna A, Versari D, Kardasz I, Hunag Y, Taddei S, Salvetti A. Different effect of antihypertensive drugs on conduit artery endothelial function. *Hypertension*. 2003;41:1281–1286.
25. Ghiadoni L, Huang Y, Magagna A, Buralli S, Taddei S, Salvetti A. Effect of acute blood pressure reduction on endothelial function in the brachial artery of patients with essential hypertension. *J Hypertens*. 2001;19:547–551.
26. Higashi Y, Sasaki S, Nakagawa K, Ueda T, Yoshimizu A, Kurisu S, Matsuura H, Kajiyama G, Oshima T. A comparison of angiotensin-converting enzyme inhibitors, calcium antagonists, beta-blockers and diuretic agents on reactive hyperemia in patients with essential hypertension: A multicenter study. *J Am Coll Cardiol*. 2000;35:284–291.
27. Iwatsubo H, Nagano M, Sakai T, Kumamoto K, Morita R, Higaki J, Ogihara T, Hata T. Converting enzyme inhibitor improves forearm reactive hyperemia in essential hypertension. *Hypertension*. 1997;29:286–290.
28. Hirooka Y, Eshima K, Setoguchi S, Kishi T, Egashira K, Takeshita A. Vitamin C improves attenuated angiotensin II-induced endothelium-dependent vasodilation in human forearm vessels. *Hypertens Res*. 2003;26:953–959.