Angiotensin II Type 1 Receptor Blockade Attenuates In-Stent Restenosis by Inhibiting Inflammation and Progenitor Cells

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Abstract—The precise mechanism by which angiotensin II type 1 receptor blocker reduces in-stent restenosis in clinical trials is unclear. We, therefore, investigated the mechanism of in-stent neointima formation. Male cynomolgus monkeys and rabbits were fed a high-cholesterol diet and were allocated to untreated control and type 1 receptor blocker groups. Five days after grouping, multilink stents were implanted in the iliac artery. The type 1 receptor blocker reduced the development of in-stent neointima formation by \(\approx 30\%\) in rabbits and monkeys. To investigate potential mechanisms, we examined the expression of renin–angiotensin system markers, all of which increased in monocytes and smooth muscle-like cells in the neointima and media within 7 days. The type 1 receptor blocker attenuated increased oxidative stress, the enhanced expression of markers of the rennin–angiotensin system and monocyte chemoattractant protein-1, and macrophage infiltration. The effects of type 1 receptor blocker on the differentiation of peripheral blood mononuclear cells into vascular progenitor cells were also examined. Treatment with type 1 receptor blocker suppressed the enhanced differentiation to smooth muscle progenitor cells induced by stenting. The type 1 receptor blocker attenuated in-stent neointima formation by inhibiting redox-sensitive inflammatory changes and by reducing recruitment of the progenitor cells. These potential actions of type 1 receptor blocker on inflammation and progenitor cells constitute a novel mechanism of suppression of in-stent restenosis by type 1 receptor blocker. (Hypertension. 2006;48:1-7.)

Key Words: angiotensin II ■ oxidative stress ■ monocytes

Coronary intervention with metal stent implantation is performed in >1.5 million patients with atherosclerotic lesions worldwide and has become the major revascularization technique. The clinical benefits of this procedure are reduced by in-stent restenosis. In-stent restenosis results exclusively from neointima formation because of proliferation/migration of smooth muscle cells and inflammatory changes in response to stent-associated injury. Recent clinical trials demonstrated great benefits of drug-eluting stents (containing sirolimus, paclitaxel, etc) in preventing restenosis and improving clinical outcomes. However, systemic medical therapies for stent-associated thrombosis and for control of risk factors are essential therapy in addition to drug-eluting stents for the prevention of future coronary events. This notion is supported by recent reports showing multiple atherosclerotic plaque ruptures at sites other than the culprit lesion, as observed in acute coronary syndrome by intravascular ultrasound analysis. The renin–angiotensin system (RAS) has been implicated in the pathogenesis of restenosis and acute coronary syndrome and, thus, may be a potential target for the prevention of in-stent restenosis and atherothrombotic events. Indeed, a recent, single-center VALsartan for Prevention of REstenosis after Stenting of Type B2/C lesions (Val-PREST) trial demonstrated that treatment with angiotensin II type 1 (AT1) receptor blocker (ARB) reduces the incidence of restenosis and revascularization in selected patients with type B2/C lesions. The same group compared valsartan with angiotensin-converting enzyme (ACE) inhibition after bare metal stent implantation in the VALsartan Versus ACE inhibition (VALVACE) trial and reported greater benefits from systemic valsartan treatment than from angiotensin-converting enzyme inhibitors in reducing restenosis.

However, the precise mechanism by which ARB reduces in-stent restenosis in Val-PREST and VALVACE trials is unclear. Although the central role of RAS in the pathogenesis of atherosclerotic vascular disease is evident, the role of RAS in the pathogenesis of in-stent neointimal formation has not been fully addressed. For example, upregulation of ACE is reported in postballoon restenotic samples, but no previous study examined the expression of RAS components (ACE, angiotensin II, AT1, receptor,
and AT$_2$ receptor) and subsequent cellular events after stenting. This point is important because: (1) the mechanism underlying neointimal formation differs considerably between injury methods, and (2) metallic stent implantation now becomes the major revascularization technique. Therefore, the first aim of the present study was to determine the effects of ARB on experimental in-stent restenotic lesions. To gain clinical significance for the results, we used a nonhuman primate model of in-stent neointima formation. We then aimed to investigate the underlying mechanism in a rabbit model. We demonstrate that: (1) increases in local expression of RAS begin at early stages after stenting, and (2) treatment with ARB attenuates in-stent neointima formation associated with reduction in oxidative stress, inflammatory changes, and AT$_1$ receptor expression.

There is accumulating evidence from experimental studies that vascular smooth muscle cells within the neointima of the atherosclerotic vessel wall may originate from bone marrow. Furthermore, a recent study demonstrated that smooth muscle progenitor cells (SMPCs) are present in circulating human blood and that bone marrow-derived smooth muscle cells are highly represented in the intima of human atherosclerotic vessels. Angiotensin II reportedly enhances the proliferation and differentiation of myeloid precursors from CD34$^+$ hematopoietic stem cells through interaction with the AT$_1$ receptor on CD34$^+$ cells. Thus, it is possible that RAS is involved in recruitment and differentiation of bone marrow cells to SMPCs. Therefore, the second aim of this study was to investigate the effects of ARB on the differentiation of peripheral blood mononuclear leukocytes to SMPCs after stenting in rabbits.

Methods

Animal Model of In-Stent Restenosis

The study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the...
Results

**Inhibitory Effects of ARBs on Neointima Formation After Stenting in Monkeys and Rabbits**

As we reported previously,15 significant neointima formation was observed 28 days after stenting in control, untreated monkeys (Figure 1). Treatment with olmesartan or valsartan reduced this neointima formation.

Neointima formation was also examined 28 days after stenting in rabbits (Figure 2). Treatment with olmesartan reduced the degree of neointima formation to a similar extent as seen in monkeys. As expected, serum angiotensin II levels rose on day 28 in the olmesartan group (data not shown).

There were no treatment effects of ARBs on serum cholesterol levels. In monkeys, the total cholesterol levels before and 28 days after stenting were $444 \pm 43$ and $429 \pm 37$ mg/dL in the control group, $469 \pm 30$ and $488 \pm 44$ mg/dL in the olmesartan-treated group, and $469 \pm 30$ and $488 \pm 44$ mg/dL in the valsartan-treated group. In rabbits, the total cholesterol levels before and 28 days after stenting were $919 \pm 81$ and $1072 \pm 93$ mg/dL in the control group and $997 \pm 97$ and $1128 \pm 108$ mg/dL in the olmesartan-treated group. There was no significant treatment effect on body weight among the groups (data not shown).

**Inhibitory Effects of ARB on Markers of RAS (ACE, Angiotensin II, AT$_1$ Receptor, and AT$_2$ Receptor) and Oxidative Stress After Stent Implantation in Rabbits**

To investigate potential mechanisms underlying the beneficial effects of ARBs on in-stent neointima formation, we examined whether markers of RAS are increased after stent implantation in rabbits (Figure I, available online). Immunohistochemical staining revealed that such markers (ACE, angiotensin II, AT$_1$ receptor, and AT$_2$ receptor) increased on day 10 in nearly all of the cells in the neointima (regenerated endothelial cells, monocytes, and smooth muscle-like cells) and in some cells in the media (Figure Ia). Such increased immunoreactivity declined spontaneously. Interestingly, treatment with olmesartan reduced the enhanced expression of AT$_1$ receptor but did not affect the expression of AT$_2$ receptor (online Figure I).

Because oxidative stress plays a central role in vascular pathobiology induced by angiotensin II, we then examined superoxide production by dihydroethidium (DHE) fluorescence on day 10 (Figure Ic). No apparent DHE fluorescence was detected in the nonstented normal artery. The fluorescent signal was markedly increased in the neointima, media, and adventitia of stented arteries from the control group. Treatment with olmesartan reduced the enhanced expression of AT$_1$ receptor but did not affect the expression of AT$_2$ receptor (online Figure I).
Inhibitory Effects of ARB on Inflammatory Changes and Apoptotic Cell Death in Rabbits

As we reported previously, inflammatory changes and apoptotic cell death became evident 7 to 10 days after stent implantation in rabbits (Figure 2a and 2b). Treatment with olmesartan reduced such inflammatory changes and enhanced cell death in the intima after stenting.

Inhibitory Effects of ARB on Expression of Proinflammatory Factors and NADPH Oxidase Subunits

Treatment with olmesartan reduced the increased mRNA levels of monocyte chemoattractant protein (MCP)-1, interleukin (IL)-1β, tumor necrosis factor-α, p22phox, and gp91phox in rabbits (Figure 3a). Olmesartan did not affect the increased levels of IL-6 and transforming growth factor-β. Immunohistochemical staining performed 10 days after stenting revealed increased immunoreactive platelet-derived growth factor (PDGF)-β and MCP-1 in cells in the neointima and in smooth muscle cells in the media. This was attenuated by olmesartan treatment (Figure 3b). Treatment with olmesartan did not affect neovascularization in the neointima and adventitia or re-endothelialization 28 days after stenting (data not shown).

Effects of ARB on Transdifferentiation of Mononuclear Cells to Vascular Progenitor Cells

To investigate the potential contribution of vascular progenitor cells, peripheral blood mononuclear cells (MNCs) were isolated and cultured to stimulate the differentiation into SMPCs or endothelial progenitor cells (EPCs), as described previously. The cells cultured in the PDGF-BB–enriched and basic fibroblast growth factor–enriched medium exhibited a hill and valley morphology that is characteristic of smooth muscle cells within 2 weeks. The smooth muscle cell phenotype was confirmed by immunostaining with antibodies specific for smooth muscle cell markers: SMPCs expressed α-SMA, myosin, and calponin, which were all detected in human coronary artery smooth muscle cells and were not detected in MNCs and Cos-7 cells (data not shown). Expression of α-SMA gene in SMPCs was also confirmed by PCR analysis (data not shown). As reported, the expression of inflammatory markers (MCP-1, IL-1β, etc) was greater in SMPCs than in cultured rabbit aortic smooth muscle cells (data not shown). The cells cultured in the vascular endothelial growth factor–enriched medium exhibited the typical cobblestone morphology of EPCs. The EPCs stained positively for von Willebrand factor and VE-cadherin and incorporated acetylated low-density lipoprotein (data not shown).

Analysis of colony-forming areas showed that the degree of transformation to SMPC was greater in MNCs from animals fed a high-cholesterol diet than in those from untreated, normal animals (Figure 4A). The transformation to SMPCs further enhanced in MNCs from animals that underwent stenting. Treatment of rabbits with olmesartan for 5 to 7 days suppressed the increased transformation to SMPCs induced by stenting. In contrast, there were no differences in the degree of transformation to EPCs among the groups. Immunohisto-
chemical staining was then performed to examine the presence of AT1 receptor and AT2 receptor. Both receptors were found in SMPCs and EPCs (Figure 4B). We considered the possibility that AT1 signals might be involved in increasing the transformation capacity of MNCs and, therefore, examined the effects of in vitro administration of angiotensin II or olmesartan on the transformation of MNCs. Angiotensin II did not enhance transdifferentiation, and olmesartan did not suppress transdifferentiation in vitro (Figure 4C).

**Plasma ARB levels and Arterial Blood Pressure**

The maximum drug concentration (C_{max}) levels of olmesartan at 15 mg/kg per day and valsartan at 50 mg/kg per day were 107±17 and 300±24 ng/mL, respectively. The C_{max} level of olmesartan at 3 mg/kg per day was 537±24 ng/mL in rabbits. Therefore, the dose of olmesartan used in rabbits is within a clinically relevant dose range. The C_{max} levels after oral administration of olmesartan at 5, 10, and 20 mg/body in hypertensive subjects are reported to be 149±21, 273±17, and 470±23 ng/mL (n=6, each), respectively. The C_{max} values after oral administration of valsartan at 80 and 160 mg/body in hypertensive subjects are reported to be 2830±920 and 5260±2300 ng/mL, respectively, according to the manufacturer’s interview form. Thus, the doses of olmesartan and valsartan used in the present study are within or below the clinically relevant dose range.

Treatment with olmesartan showed no effect on systolic and diastolic arterial pressure. Systolic and diastolic pressure were 94±2 and 59±5 mm Hg in the control group and 92±2 and 52±6 mm Hg in the olmesartan group.

**Discussion**

We have demonstrated for the first time that oral treatment with 2 types of ARBs (valsartan and olmesartan) attenuated in-stent neointimal formation in nonhuman primates (cynomolgus monkeys), supporting the conclusions of the VALEST and VALVACE trials,12,13 which involved a relatively small number of patients. Although it is uncertain which animal model is most appropriate for the evaluation of in-stent neointima formation (restenotic changes), a nonhuman primate model may have an advantage over nonprimate models, because vascular inflammatory and proliferative responses to injury in nonhuman primates are more similar to those in humans than are other, nonprimate models.
Hence, the use of nonhuman primates may work for evaluation of the efficacy of ARB on in-stent neointima formation in clinically relevant conditions.

To obtain mechanistic insight into the beneficial effects of ARB, we first examined the time course of local expression of RAS components in rabbits (Figure 2). We found that expression of all of the components (ACE, angiotensin II, AT1 receptor, and AT2 receptor) increased, mainly in cells composed of neointima (monocytes and smooth muscle cells), at early stages (7 to 10 days after stenting), and persisted until 28 days after stenting. This RAS activation colocalized with increased NADPH oxidase–dependent DHE fluorescence (generation of superoxide anion) and was associated with increased levels of NADPH oxidase subunit mRNAs, consistent with previous reports showing that increased reactive oxygen species can be detected in activated smooth muscle cells after balloon injury.21,22 These in vivo observations are also consistent with previously published in vitro data suggesting that proliferation and migration of smooth muscle cells are critically mediated by oxidative stress via AT1-mediated activation of NADPH oxidases.23–29 Interestingly, treatment with ARB not only attenuated the levels of oxidative stress markers but also reduced the level of immunoreactive AT1. These data suggest the presence of a positive feedback loop in which activation of AT1 further enhances expression and activity of the AT1 receptor in vivo, as seen in the present study.

It is known that oxidative stress–induced inflammatory and proliferative processes are central to neointima formation after vascular injury.24,25 We and others have demonstrated that increased monocyte-mediated inflammation or MCP-1 expression is associated with greater neointima formation after stenting.26,27 and anti–MCP-1 gene therapy15,28–30 or administration of blocking antibody against the MCP-1 receptor31 markedly reduces neointima formation after vascular injury. However, no previous study examined whether or not those inflammatory and proliferative changes after stenting depend on the AT1 receptor. In the present study, we, therefore, examined the effects of ARB on monocyte recruitment and MCP-1 expression after stenting and found that ARB reduced monocyte/macrophage recruitment, as well as MCP-1 immunoreactivity and gene expression. Furthermore, ARB inhibition increased the expression of growth-promoting factors, such as PDGF and IL-1β. These data suggest that the beneficial effects of ARB may be attributed to the inhibition of oxidative stress–induced inflammatory and proliferative changes.

Recent studies have shown that peripheral blood contains bone marrow–derived progenitor cells, which contribute to neointima formation after injury.16,18,32 However, the role of RAS in the recruitment/differentiation of progenitor cells into the neointimal cells after stenting has not been addressed. Here we found that differentiation to SMPCs increased in MNCs from rabbits fed a high-cholesterol diet and was further enhanced in those rabbits that had also undergone stenting. Differentiation into EPCs was not affected by either the diet or stenting. In vivo treatment with ARB suppressed the increased differentiation into SMPCs induced by diet or stenting. In contrast, in vitro treatment with angiotensin II or ARB did not affect the capacity to differentiate into SMPCs or EPCs. Therefore, the capacity to recruit or form SMPCs from MNCs after stenting might be determined by an AT1 receptor–mediated pathway in vivo and, thus, contribute to in-stent neointima formation.

It must be mentioned that ARB did not significantly reduce arterial blood pressure in rabbits. Although arterial pressure was not measured in monkeys, the dose of ARB used in the present study is reported to show no effects on arterial blood pressure in monkeys.33 Plasma ARB level was within or below the clinical range. Furthermore, ARB did not affect serum lipid levels. Therefore, the beneficial effect of ARB on in-stent neointimal formation is likely to be independent of its effects on arterial blood pressure or serum lipid.

**Perspectives**

This study provides experimental evidence suggesting that oral treatment with ARB at a clinical dose range attenuates in-stent neointima formation in rabbits and nonhuman primates. The beneficial effects were associated with reduced local oxidative stress, reduced expression of MCP-1 and other inflammation-promoting factors, and reduced recruitment/differentiation of SMPCs, suggesting that ARB is of potential clinical benefit in patients who have undergone vascular interventions.


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