Important Role of Nitric Oxide in the Effect of Angiotensin-Converting Enzyme Inhibitor Imidapril on Vascular Injury

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Abstract—To examine the possible role of the bradykinin-NO system in the action of ACE inhibitors, we studied the effects of imidapril, an ACE inhibitor, on inflammatory vascular injury by using AT1a-receptor–deficient (AT1aKO) mice. A polyethylene cuff was placed around the femoral artery of AT1aKO mice and wild-type (WT; C57BL/6J) mice. Neointimal area in cross sections of the artery was measured 14 days after cuff placement. A low dose of imidapril (1 mg/kg per day), which did not affect blood pressure, was administered by gavage. Expression of monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)-α was detected by immunohistochemical staining and reverse transcriptase–polymerase chain reaction (RT-PCR) 7 days after the operation. Neointimal formation, vascular smooth muscle cell proliferation, and expression of MCP-1 and TNF-α were attenuated in the injured artery in AT1aKO mice compared with those in WT mice. Imidapril inhibited neointimal formation, DNA synthesis of vascular smooth muscle cells, and expression of MCP-1 and TNF-α in AT1aKO mice as well as in WT mice. In addition, imidapril increased tissue cGMP content after cuff placement. These inhibitory effects of imidapril were significantly reduced or abolished by a bradykinin receptor antagonist, Hoechst 140, or an NO synthase inhibitor, L-NAME, both in WT and AT1aKO mice. Treatment with imidapril did not change AT2 receptor and ACE expression detected by RT-PCR in the injured artery. These results indicate that not only blockade of angiotensin II production but also activation of the bradykinin-NO system plays an important role in the beneficial effects of imidapril on vascular remodeling. (Hypertension. 2003;42:542-547.)

Key Words: nitric oxide ■ angiotensin ■ vasculature ■ arteries

Recent evidence has revealed that angiotensin (Ang) II plays important roles not only in blood pressure control but also in cardiovascular remodeling, insulin resistance, and regulation of water retention.1,2 Arterial neointimal thickening is a critical process in the development of atherosclerosis, bypass graft failure, and restenosis after angioplasty. Since the AT1 receptor is a major subtype of Ang II receptors, direct vascular effects caused by Ang II, such as vasoconstriction, inflammation, vascular remodeling, and thrombosis, are mediated by the AT1 receptor.2–4 Thus, blockade of AT1 receptor function appears to be important for the treatment of cardiovascular disease. ACE inhibitors and AT1 receptor blockers (ARB) are widely used to inhibit the function of the renin-angiotensin system in clinical practice. ARB specifically block Ang II binding to the AT1 receptor and thereby Ang II may preferentially stimulate unbound the AT1 receptor, another Ang II receptor subtype, which could act antagonistically against the function of the AT1 receptor.5,6 In contrast, blockade of Ang II production by ACE inhibitors is not complete, since Ang II can also be produced by chymase in humans.7,8 However, ACE inhibitors increase the bradykinin level through the inhibition of kininase and thus activate the kallikrein-kinin system.9 The increase in bradykinin elevates prostacyclin production, eNOS activity, and nitric oxide (NO) production.10,11 Thus, the increase in bradykinin induces various actions, such as vasodilatation, anticoagulation, hypotension, and cardiovascular inflammatory changes.11

In the present study, to explore the implication of bradykinin and NO in the action of ACE inhibitors on vascular remodeling, we examined the effect of imidapril, an ACE inhibitor, on inflammatory vascular injury induced by polyethylene-cuff placement. To confirm that ACE inhibitors have beneficial actions independent of blockade of Ang II production, we used AT1a-receptor–deficient mice and examined the effect of imidapril on vascular remodeling.

Methods

Animals
Adult male AT1aKO mice (based on C57BL/6J strain and donated by Tanabe Seiyaku Co, Ltd, Osaka, Japan) and wild-type mice (C57BL/
6J; age, 10 to 12 weeks; weight, 25 to 30 g) were used in this study. The mice were housed in a room in which lighting was controlled (12 hours on, 12 hours off) and room temperature was kept at 25°C. They were given a standard diet (MF, Oriental Yeast Co, Ltd) and water ad libitum. The experimental protocol was approved by the Animal Studies Committee of Ehime University. Inflammatory vascular injury was induced by polyethylene cuff placement around the femoral artery according to methods described previously.12,13 and morphometric analysis to measure neointimal area was performed as previously described.13 Imidapril (donated by Tanabe Seiyaku Co, Ltd) was administered by gavage at a dose of 1 mg/kg per day in 0.5% carboxymethylcellulose sodium solution from the day of cuff placement. TA606 (donated by Tanabe Seiyaku Co, Ltd) was administered intraperitoneally with an osmotic minipump from the day of cuff placement. L-NAME (20 mg/kg per day; Funakoshi) was also administered orally, and Hoechst 140 (100 μg/kg per day; Peptide Institute, Inc) was administered intraperitoneally with an osmotic minipump from the day of cuff placement. Blood pressure was measured by the indirect tail-cuff method with a blood pressure monitor (MK-1030, Muromachi Kikai Co, Ltd).

Immunohistochemical Staining
Monocytic chemoattractant protein-1 (MCP-1) and tumor necrosis factor (TNF-α) were stained by the streptavidin-biotin-peroxidase method with the use of formalin-fixed, paraffin-embedded sections, as described previously.5,13 Proliferating cell nuclear antigen (PCNA) was stained with the use of an MOM immunodetection kit (Vector Laboratories, Inc) with anti-PCNA monoclonal antibody (Vector Laboratories, Inc). PCNA labeling index was calculated as the percentage of PCNA-positive nuclei in total cell nuclei.

Reverse Transcription–Polymerase Chain Reaction
Cuffed arteries at 7 days after cuff placement and control intact arteries were pooled (n = 8, 10 for each group, respectively). RNA was prepared with the use of TRIzol reagent (GIBCO-BRL), and RT-PCR was performed as described previously.5,13 PCR primers were designed for MCP-1, TNF-α, AT1 receptor, AT2 receptor, ACE, and GAPDH, as reported previously.5,11 PCR primers for AT1 receptor are 5'-GATAGGACATCCAGAAAAC-3' (forward) and 5'-ATGAGGACATCCAGAAAAC-3' (reverse).

Western Blot Analysis
Total proteins were prepared from pooled arteries at 7 days after cuff placement (n = 6 to 8 for each group), and Western blotting was performed as previously described.5

Determination of Tissue cGMP Content
Artery samples were obtained at 7 days after cuff placement, quickly frozen in the liquid nitrogen, and stored at −80°C until use. Pooled samples (n = 5) were homogenized in 6% (wt/vol) tetrachloroacetic acid solution and centrifuged, and the supernatant was used to measure cGMP with an RIA kit (Amersham Pharmacia Biotech), according to the manufacturer’s protocol.

Statistical Analysis
Values are expressed as mean ± SEM in the text and figures. The data were analyzed by means of 2-way ANOVA. If a statistically significant effect was found, post hoc analysis was performed to detect the difference between the groups. A value of *P < 0.05 was considered statistically significant.

Results

Effect of the ACE Inhibitor Imidapril on Neointimal Formation and Cell Proliferation After Cuff Placement in WT and AT1aKO Mice
As previously reported, polyethylene cuff placement induced neointimal formation in mice.5,13,14 In AT1a-receptor–deficient (AT1aKO) mice, neointimal formation 14 days after operation was significantly less than that in wild-type (WT) mice (Figure 1). Imidapril inhibited neointimal formation by ≈60% in WT mice at a dose of 1 mg/kg per day, which did not change blood pressure (data not shown). The inhibitory effect of imidapril on neointimal formation was smaller but significant in AT1aKO mice (Figure 1). PCNA labeling index, a marker of cell proliferation, was also significantly lower in AT1aKO mice and was decreased by imidapril in the intima and media of both strains (Figure 1). To assess the possible involvement of blockade of AT1b receptor in the inhibitory action of imidapril, we examined the effect of the AT1b-receptor blocker TA606 on neointima formation after cuff placement in AT1aKO mice (Figure 2). Treatment with TA606 at a dose of 1 mg/kg per day significantly reduced neointimal formation in WT mice without affecting the blood pressure, but it showed no significant effect in AT1aKO mice. After cuff placement, expression of the AT1a receptor, AT1b receptor, AT2 receptor, and ACE was increased (Figures 3A and 3B). The increases in ACE and AT2 receptor were not significantly different in WT and AT1aKO mice (Figures 3A and 3B). On the other hand, expression of AT1b receptor was very low in femoral artery and not changed after cuff placement (Figure 3A).

Effect of Imidapril on Inflammatory Response Induced by Cuff Placement in WT and AT1aKO Mice
The inflammatory response, such as expression of MCP-1 and TNF-α, was determined by RT-PCR, Western blot, and
immunohistochemical staining. RT-PCR and Western blot with the use of pooled artery samples showed that the expression of both MCP-1 and TNF-α was increased after cuff placement in both WT and AT1 aKO mice; however, this increase was smaller in AT1 aKO mice (Figures 4A and 4B). Imidapril inhibited the expression of MCP-1 and TNF-α not only in WT mice but also in AT1 aKO mice (Figures 4A and 4B). Consistent with these results, immunohistochemical staining showed similar results (Figures 5 through 8).

Effects of Hoechst140 and L-NAME on Inhibitory Action of Imidapril on Vascular Injury After Cuff Placement

To examine the potential roles of bradykinin and NO in the vascular protective effect of imidapril, a bradykinin B2 receptor blocker, Hoechst140, or an NO synthase inhibitor, L-NAME, was administered with imidapril. As shown in Figure 9, treatment with Hoechst140 antagonized the inhibitory effect of imidapril on neointimal formation after cuff placement in WT mice. Hoechst140 caused 80% recovery of neointimal formation inhibited by imidapril (Figure 9). Similar antagonism by L-NAME was also observed (Figure 9). In AT1 aKO mice, Hoechst140 or L-NAME totally abolished the inhibitory action of imidapril on neointimal formation after cuff placement (Figure 9). This dose of either Hoechst140 or L-NAME did not significantly affect the area of neointima in WT as well as AT1 aKO mice.

Treatment with Hoechst140 or L-NAME attenuated the effect of imidapril on expression of MCP-1 and TNF-α after cuff placement. RT-PCR and/or Western blotting showed that recovery of MCP-1 and TNF-α was inhibited by imidapril in both WT and AT1 aKO mice (Figure 4). These effects of Hoechst140 and L-NAME were also observed in immunohistochemical studies (Figures 5 through 8).

Figure 10 shows the content of cGMP in the femoral artery after cuff placement. cGMP content was slightly decreased after cuff placement and significantly increased by the treatment with imidapril. This increase in cGMP content by imidapril was attenuated by L-NAME (Figure 10).

Discussion

In the present study, to examine the potential role of the bradykinin-NO system in the effects of ACE inhibitors on neointimal formation and inflammatory responses in vascular injury induced by cuff placement, we studied the effects of imidapril on vascular remodeling by using AT1 aKO mice, in which the actions of major part of AT1 receptor are genetically omitted. Imidapril inhibited cell proliferation and neo-
intimal formation as well as inflammatory responses, such as expression of MCP-1 and TNF-α, in the injured artery. However, these inhibitory actions of imidapril were observed not only in WT mice but also in AT1aKO mice. Moreover, the inhibitory actions of imidapril were significantly attenuated by Hoechst140 and L-NAME, inhibitors of the bradykinin B2 receptor and NO synthase, respectively. These results indicate that the inhibitory effect of imidapril on vascular injury was at least partly mediated through stimulation of the bradykinin-NO system.

Ang II plays an important role in hemodynamic control, cardiovascular function, atherosclerotic changes, and insulin resistance. Major functions of Ang II, such as vasoconstriction, cell growth, antiapoptosis, and induction of inflammatory factors, are mediated through the AT1 receptor. One of the advantages of ACE inhibitors appears to be the increase in bradykinin level followed by stimulation of NO synthesis. In our study, imidapril inhibited neointimal formation, proliferation of vascular smooth muscle cells, and inflammatory responses induced by cuff placement. These effects of imidapril appear to be independent of its hemodynamic action, because the dose of imidapril used in our study did not affect the systemic blood pressure (data not shown). As previously described, the inhibitory action of ACE inhibitor, imidapril, appears to be caused mainly by a reduction of AT1-receptor–mediated signaling through the blockade of Ang II production. In fact, neointimal formation and perivascular inflammation were significantly unmarked in

Figure 5. Effects of imidapril, Hoechst140, and L-NAME on immunohistochemical detection of MCP-1 in injured artery after cuff placement in WT mice. Immunohistochemical staining of MCP-1 was performed 7 days after cuff placement as described in the Methods section. A, Control; B, imidapril (1 mg/kg per day); C, imidapril + Hoechst140 (100 μg/kg per day); D, imidapril + L-NAME (20 mg/kg per day). Magnification ×200.
AT1αKO mice. However, imidapril inhibited neointimal formation, DNA synthesis, and inflammatory responses also in AT1aKO mice. These observations indicate that the effects of imidapril are due to not only the inhibition of Ang II production but also other factors. Previous studies have suggested that the bradykinin-NO system plays an important role in the action of ACE inhibitors on cardiovascular remodeling.17,18 Thus, we examined the involvement of the bradykinin-NO system in the action of imidapril by using AT1aKO mice.

In our study, the inhibitory effects of imidapril on neointimal formation and perivascular inflammation were attenuated ~80% by a bradykinin-receptor antagonist, Hoechst 140, in WT mice (Figure 9). This result indicates that bradykinin was at least partly involved in the action of imidapril on vascular remodeling after cuff placement. It is reported that NO is an important mediator of the vasoactive action of bradykinin.9,10 In our study, administration of an NO-synthase inhibitor, L-NAME, also attenuated the action of imidapril to a similar extent to Hoechst 140 (Figure 9). Moreover, the content of cGMP, an intracellular messenger of NO, in the femoral artery was significantly increased by treatment with imidapril, and this increase of cGMP was inhibited with L-NAME (Figure 10). These results suggest the involvement of the bradykinin-NO system in the action of imidapril on cuff-induced vascular injury.
Previous studies demonstrated that vascular remodeling including the proliferation of vascular smooth muscle cells is mediated through the AT$_1$ receptor. Interestingly, Hoechst140 and L-NAME almost completely abolished the inhibitory action of imidapril on neointimal formation and perivascular inflammation in AT$_1$αKO mice (Figures 5 through 9). This observation strongly supports the idea that the effects of imidapril on vascular remodeling are mediated not only through blockade of the Ang II receptor but also through activation of the bradykinin-NO system.

We have previously reported that the AT$_2$ receptor is upregulated in injured arteries and is involved in vascular remodeling.\textsuperscript{5,13} Since AT$_1$αKO mice have still the AT$_1$,β and AT$_2$ receptors, it might be possible that Hoechst140 and/or L-NAME modulated the effects of the AT$_1$,β and/or AT$_2$ receptors. In fact, previous reports suggested the interaction between AT$_2$–receptor–mediated signaling and the bradykinin-NO system.\textsuperscript{19,20} However, it is not likely in the present study, since the action of imidapril was completely abolished by Hoechst140 or L-NAME in AT$_1$αKO mice (Figure 9). Interestingly, the inhibitory effect of imidapril on neointimal formation tended to be not completely recovered by Hoechst140 or L-NAME (Figure 9). AT$_1$,β receptor also appeared not to play an important role in this injury model, since its expression in the femoral artery was very weak and not changed after cuff placement both in WT and AT$_1$αKO mice (Figure 3A), and the subpressor dose of TA606 reduced neointimal formation in WT mice but not in AT$_1$αKO mice (Figure 2).

The results in the present study indicate that not only blockade of the Ang II production but also stimulation of the bradykinin-NO system plays an important role in the beneficial effects of ACE inhibitors on vascular remodeling.

**Perspectives**

We examined the possible involvement of bradykinin and NO in the effect of ACE inhibitors on vascular remodeling. We demonstrated that treatment of mice with imidapril attenuated neointimal formation, cell proliferation, and the expression of MCP-1 and TNF-α and an increase in tissue cGMP content without affecting blood pressure. These inhibitory actions of imidapril were observed even in AT$_1$α-receptor–deficient mice. The effects of imidapril were almost totally abolished by Hoechst140 or L-NAME in AT$_1$α-receptor null mice. These results indicate that activation of the bradykinin-NO system is important in the action of imidapril on vascular remodeling.

**Acknowledgments**

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, the Cardiovascular Research Foundation, the Japan Research Foundation for Clinical Pharmacology, the Tokyo Biochemical Research Foundation, and the Smoking Research Foundation.

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Hypertension. 2003;42:542-547; originally published online September 8, 2003; doi: 10.1161/01.HYP.0000092440.52239.39

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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