Beneficial Effects of Combined Blockade of ACE and AT$_1$ Receptor on Intimal Hyperplasia in Balloon-Injured Rat Artery

Shokei Kim, Yasukatsu Izumi, Yasuhiro Izumiya, Yumei Zhan, Masaru Taniguchi, Hiroshi Iwao

Objective—The present study was undertaken to elucidate the effect of the ACE inhibitor and the angiotensin II type 1 (AT$_1$) receptor antagonist in combination on neointimal hyperplasia after balloon injury.

Methods and Results—Temocapril (an ACE inhibitor), CS-866 (an AT$_1$ receptor antagonist), or their combination was given orally to rats, and their effects were compared on vascular hyperplasia induced by balloon injury. The maximal preventive effect of temocapril and CS-866 alone on neointimal thickening after balloon injury was obtained at a dose of 20 and 10 mg/kg per day, respectively. However, compared with either agent alone, combined temocapril and CS-866 (20 and 10 mg/kg per day, respectively) prevented intimal thickening to a larger extent. Furthermore, compared with either agent alone, combined temocapril and CS-866 prevented vascular smooth muscle cell proliferation in the intima more potently. The increase in platelet-derived growth factor receptor tyrosyl phosphorylation was reduced more potently by the combination of both agents compared with either agent alone. The nonpeptide bradykinin B$_2$ receptor antagonist or the NO synthase inhibitor reduced the prevention of intimal thickening by combined temocapril and CS-866.

Conclusions—Compared with either agent alone, the combination of an ACE inhibitor and an AT$_1$ receptor antagonist is more effective in the prevention of vascular hyperplasia due to bradykinin or NO. (Arterioscler Thromb Vasc Biol. 2002;22:1111-1119)

Key Words: balloon injury † intimal thickening † angiotensin † angiotensin-converting enzyme † combination therapy

Accumulating evidence indicates that the renin-angiotensin system plays an important role in the pathophysiology of vascular thickening and remodeling. 1–3 Either ACE inhibitors4,5 or angiotensin II (Ang II) type 1 (AT$_1$) receptor antagonists6,7 significantly prevent neointimal hyperplasia in the balloon-injured rat artery independent of their hypertensive effect. However, in contrast to the preventive effect of ACE inhibitors on neointimal formation after balloon injury in rats, a multicenter clinical trial (the Multicenter European Research Trial With Cilazapril After Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis [MERCATOR] study) showed that ACE inhibition with cilazapril does not prevent restenosis after percutaneous transluminal coronary angioplasty; this finding does not support the usefulness of the ACE inhibitor in the treatment of vascular restenosis in humans.8 It remains unclear whether the failure of prevention of restenosis by the ACE inhibitor in humans was due to the insufficient dose used or the species difference.

Recently, in hypertensive rats, a combination of low doses of ACE inhibitor and AT$_1$ receptor antagonist has been shown to induce greater reductions in blood pressure and cardiac weight than have been found with monotherapy with the same or higher doses.9,10 In pigs subjected to myocardial ischemia, infarct size is reduced more effectively by an ACE inhibitor and an AT$_1$ receptor antagonist combined than by a monotherapy of either drug alone.11 Very recently, we have also found the beneficial effects of the combination therapy on a rat heart failure model.12 Furthermore, compared with either intervention alone, the combined administration of an ACE inhibitor and an AT$_1$ receptor antagonist decreases blood pressure in normotensive volunteers13,14 and improves cardiac dysfunction in patients with heart failure.15,16 These experimental and clinical findings suggest that compared with either agent alone, an ACE inhibitor and an AT$_1$ receptor antagonist in combination may be more effective in the treatment of hypertension, pathological cardiac hypertrophy, and heart failure. However, the effect of the combination of these 2 drugs on vascular hyperplasia remains to be determined.

Therefore, in the present study, we examined the effect of the combination of an ACE inhibitor and an AT$_1$ receptor
antagonist on neointimal hyperplasia in balloon-injured rat arteries and compared this effect with the effect of either agent alone. We obtained the first evidence that their combination was more effective in the prevention of intimal hyperplasia than was either agent alone.

Methods

Drugs

CS-866, a selective nonpeptide AT1 receptor antagonist, and temocapril hydrochloride, an ACE inhibitor, was purchased from Sankyo Co, Ltd. FR172357, a nonpeptide bradykinin B2 receptor antagonist, was provided as a gift by Fujisawa Pharmaceutical Co, Ltd. These drugs were suspended with 0.5% carboxymethylcellulose. N\textsuperscript{350 to 450}–nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor, was purchased from Sigma Chemical Co.

Experimental Protocol

All procedures were in accordance with institutional guidelines for animal research. Ten- to 12-week-old male Sprague-Dawley rats (Clea Japan, Tokyo, Japan), weighing 350 to 450 g, were used in the present study. In the first series of experiments, temocapril (10, 20, or 40 mg/kg per day), CS-866 (5, 10, or 20 mg/kg per day), combined temocapril (20 mg/kg per day) and CS-866 (10 mg/kg per day), or amlodipine (10 mg/kg per day), was given orally to rats by gastric gavage once a day from 3 days after balloon injury, and arterial bromodeoxyuridine (BrdU) labeling was given orally to rats by gastric gavage once a day from 3 days before balloon injury until the end of the experiments. Balloon injury of rat left common carotid artery was carried out by 3 passages of a Fogarty 2F balloon catheter (Baxter Healthcare), as previously described. At 10 days after the initiation of drug treatment, systolic blood pressure of conscious rats was measured by the tail-cuff method, at 3 to 5 hours after oral dosing. Arterial intimal and medial area was measured at 14 days after balloon injury, and arterial bromodeoxyuridine (BrdU) labeling index, proliferating cell nuclear antigen (PCNA) contents, and platelet-derived growth factor (PDGF)-\beta receptor tyrosyl phosphorylation were estimated at 7 days.

In a second series of experiments, rats were divided into 4 groups, including (1) vehicle treatment, (2) combined temocapril (20 mg/kg per day) and CS-866 (10 mg/kg per day), (3) combined temocapril (20 mg/kg per day), CS-866 (10 mg/kg per day), and FR172357 (30 mg/kg per day), and (4) combined temocapril (20 mg/kg per day), CS-866 (10 mg/kg per day), and L-NAME (30 mg/kg per day). All drugs were given orally to rats by gastric gavage once a day from 3 days before balloon injury until the end of the experiments, except for L-NAME, which was given as the drinking water. Balloon injury of rat left common carotid artery was carried out, as described above, and arterial intimal and medial areas were measured at 14 days after balloon injury.

Measurement of Intimal and Medial Areas

For the estimation of intimal and medial areas, 14 days after balloon injury, rats were anesthetized with ether, and their carotid arteries were fixed by perfusion of 10% (wt/vol) formaldehyde for 15 minutes under constant pressure, removed, embedded in paraffin, and sectioned at 3-\textmu m thickness. Cross sections were stained with elastica van Gieson and with hematoxylin and eosin. The intimal and medial areas of each cross section were measured with a light microscope connected to the image analyzer to calculate the ratio of intimal/medial areas.

BrdU Immunohistochemistry and Morphometric Analyses

To investigate the effects on cell proliferation after balloon injury, the percentages of arterial BrdU-positive cells were measured 7 days after injury. In brief, for BrdU immunohistochemistry, rats were injected intraperitoneally with 100 mg/kg BrdU at 24 hours and at 1 hour before euthanasia. The left carotid arteries were fixed by perfusion of 10% (wt/vol) formaldehyde for 15 minutes, removed, embedded in paraffin, and sectioned at 3-\textmu m thickness. BrdU immunohistochemistry was performed with a mouse anti-BrdU monoclonal antibody (Amersham) and LSAB2 kit (DAKO Japan Co, Ltd). The BrdU labeling index was calculated as the number of labeled nuclei per total nuclei.

Immunoprecipitation and Western Blot Analysis

The method of Western blot analysis and immunoprecipitation has been described in detail in our previous reports. In brief, pooled arterial tissues were homogenized in lysis buffer, sonicated, and centrifuged. For estimation of PDGF-\beta receptor tyrosyl phosphorylation, arterial protein extracts (250 \mu g) were preabsorbed with protein A–sepharose and incubated with rabbit polyclonal anti–PDGF-\beta receptor antibody (Santa Cruz Biotechnology, Inc), as previously described. The immunocomplexes were precipitated with protein A–sepharose, boiled in Laemmli sample buffer, and centrifuged, and the resulting supernatants were electrophoresed on 8% SDS-polyacrylamide gel, transferred to Hybond–polyvinylidene difluoride membranes (Amersham Life Sciences), and immunoblotted with mouse monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology). Immunocomplexes were visualized by using an enhanced chemiluminescence method (ECL, Amersham). The densities were measured by using the public domain National Institutes of Health IMAGE program. After the previous antibody was stripped off, the membranes were again immunoblotted with the above-mentioned anti–PDGF-\beta receptor antibody, as described above.

Arterial PCNA levels were determined by Western blot analysis with mouse monoclonal anti-PCNA antibody (SC-56, Santa Cruz Biotechnology, Inc).

Statistical Analysis

All data are presented as mean±SEM. Statistical significance was determined by 1-way ANOVA, followed by Duncan multiple range comparison test with the use of SuperANOVA (Abacus Concepts, Inc). Differences were considered statistically significant at a value of \(P<0.05\).

Results

Effects of Temocapril, CS-866, and Their Combination on Arterial Intimal and Medial Areas at 14 Days After Balloon Injury

Ten days after the initiation of the drug treatment, blood pressures for each group of rats were measured. Compared with the vehicle-treated group (124±3 mm Hg), blood pressure was significantly reduced (\(P<0.01\)) by temocapril at 10, 20, and 40 mg/kg (99±2, 87±1, and 80±3 mm Hg, respectively), by CS-866 at 5, 10, and 20 mg/kg (96±1, 84±2, and 81±2 mm Hg, respectively), by combined temocapril at 20 mg/kg and CS-866 at 10 mg/kg (81±4 mm Hg), and by amlodipine (85±3 mm Hg); there were 7 in each group. However, there was no significant difference in blood pressure levels among the groups treated with temocapril (20 or 40 mg/kg), CS-866 (10 or 20 mg/kg), combined temocapril and CS-866, and amlodipine. As shown in Figure 1, temocapril at 20 or 40 mg/kg and CS-866 at 10 or 20 mg/kg significantly and comparably prevented intimal thickening at 14 days after balloon injury. Thus, the maximal preventive effect of temocapril and CS-866 on arterial intimal thickening was obtained at doses of 20 and 10 mg/kg, respectively. On the other hand, the inhibitory effect of combined temocapril (20 mg/kg per day) and CS-866 (10 mg/kg per day) on intimal thickening was significantly greater than the maximal inhibitory effect of temocapril or CS-866 alone. Although the hypotensive effect of amlodipine was comparable to that of
combined temocapril (20 mg/kg per day) and CS-866 (10 mg/kg per day), amiodipine did not significantly prevent arterial intimal thickening after balloon injury (Figure 1). Arterial medial area at 14 days after injury was not altered by temocapril or CS-866 alone at any doses, by their combination, or by amiodipine (data not shown).

Effects on Cell Proliferation After Balloon Injury
To elucidate the mechanism underlying the greater preventive effect of the combination therapy compared with the monotherapy, the effects of combined temocapril (20 mg/kg per day) and CS-866 (10 mg/kg per day) on arterial cell proliferation were compared with those of temocapril (40 mg/kg per day) or CS-866 (20 mg/kg per day) alone. As shown in Figure 2, the intimal BrdU labeling index in the artery at 7 days after injury was 39±3%. Temocapril (40 mg/kg per day) or CS-866 (20 mg/kg per day) alone reduced the intimal BrdU labeling index (P<0.01). However, compared with each agent alone, their combination decreased the intimal BrdU labeling index to a greater extent (P<0.05).

Arterial PCNA levels were dramatically increased after balloon injury and increased by 5.9-fold at 7 days after injury (see online Figure IA, which can be accessed at http://atvb.ahajournals.org). Arterial PCNA levels at 7 days after injury were prevented by 40 mg/kg temocapril per day or 20 mg/kg CS-866 per day (P<0.05). However, compared with monotherapy, their combination more potently suppressed arterial PCNA expression (P<0.05; see online Figure IB).
Effects on Arterial PDGF-β Receptor Tyrosine Phosphorylation

As shown in Figure 3A, arterial PDGF-β receptor tyrosyl phosphorylation was significantly enhanced at 7 days after balloon injury compared with the basal value (noninjured artery). Temocapril (40 mg/kg per day) and CS-866 (20 mg/kg per day) suppressed arterial PDGF-β receptor tyrosyl phosphorylation to a comparable degree at 7 days after injury (*P<0.05). The suppressive effect of the combination of temocapril (20 mg/kg per day) and CS-866 (10 mg/kg per day) was larger (*P<0.05) than that of temocapril (40 mg/kg per day) or CS-866 (20 mg/kg per day) alone. On the other hand, arterial PDGF-β receptor protein levels at 7 days after balloon injury were not significantly different from the basal value (noninjured artery) and were not affected by temocapril, CS-866, or their combination (Figure 3B).

Effects of FR172357 and L-NAME on Intimal Thickening in Balloon-Injured Arteries

In preliminary experiments, we examined the effect of FR172357 on the prevention of intimal thickening by temocapril and found that FR172357 significantly lessened the inhibitory effect of intimal thickening by temocapril (ratio of intimal/medial areas 0.95 ± 0.05 versus 0.73 ± 0.05, respectively; *P<0.05; n=7). Our preliminary data regarding the contribution of bradykinin in the prevention of intimal hyperplasia by ACE inhibition is in good agreement with the previous report by Farhy et al.5 Therefore, in the present study, we examined the possible role of bradykinin in the beneficial effects of combination of temocapril and CS-866 on intimal hyperplasia. The Table shows blood pressure at 10 days and the serum creatinine level at 14 days after the initiation of drug treatment. FR172357 or L-NAME treatment did not significantly affect the blood pressure of rats treated with temocapril and CS-866 combined. Serum creatinine levels were not significantly altered by the combination therapy of temocapril and CS-866. As shown by the ratio of intimal/medial areas in Figure 4, FR172357 (0.75 ± 0.05) and L-NAME (0.89 ± 0.07) significantly reversed the prevention of intimal hyperplasia by combined temocapril and CS-866 (0.52 ± 0.06).

Discussion

A growing body of evidence supports the idea that there are many differences between the pharmacological actions of ACE inhibitors and AT<sub>1</sub> receptor antagonists. The ACE inhibitor is well known to increase bradykinin accumulation, leading to the increase in NO release.5 On the other hand, the AT<sub>1</sub> receptor antagonist, but not the ACE inhibitor, may stimulate the Ang II type 2 receptor via the increase in circulating Ang II.27 These findings suggest that the combination of the ACE inhibitor and the AT<sub>1</sub> receptor antagonist may exert differential pharmacological action from either agent alone. However, there is no available information on the effect of the combination of the ACE inhibitor and the AT<sub>1</sub> receptor

Blood Pressure and Serum Creatinine in Rats Treated With Temocapril, CS-866, FR172357, or L-NAME

<table>
<thead>
<tr>
<th></th>
<th>Ve</th>
<th>Te+CS</th>
<th>Te+CS+FR</th>
<th>Te+CS+L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure, mm Hg</td>
<td>119 ± 4</td>
<td>81 ± 5</td>
<td>86 ± 4*</td>
<td>90 ± 3*</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>0.653 ± 0.027</td>
<td>0.717 ± 0.060</td>
<td>0.752 ± 0.045</td>
<td>0.682 ± 0.066</td>
</tr>
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Values are means ± SEM (n=7). Blood pressure and serum creatinine were determined at 10 and 14 days, respectively, after the start of drug treatment. Rats were treated with Ve; combined temocapril (20 mg · kg<sup>-1</sup> · d<sup>-1</sup>) and CS-866 (10 mg · kg<sup>-1</sup> · d<sup>-1</sup>) (Te+CS); combined temocapril (20 mg · kg<sup>-1</sup> · d<sup>-1</sup>), CS-866 (10 mg · kg<sup>-1</sup> · d<sup>-1</sup>), and FR172357 (Te+CS+FR); or combined temocapril (20 mg · kg<sup>-1</sup> · d<sup>-1</sup>), CS-866 (10 mg · kg<sup>-1</sup> · d<sup>-1</sup>) and L-NAME (Te+CS+L-NAME).

*P<0.01 vs Ve.
antagonist on vascular intimal thickening. These findings encouraged us to compare the effects of combination therapy on intimal hyperplasia after balloon injury with the effects of monotherapies.

In the present study, temocapril, CS-866, or their combination significantly prevented neointimal thickening in the balloon-injured rat artery, in contrast to no significant effect of the calcium channel blocker, indicating that this effect of these drugs and their combination was independent of their blood pressure–lowering effect. As shown in Figure 1, the maximal preventive effect of temocapril and CS-866 was observed at a dose of 20 and 10 mg/kg per day, respectively, and there was no significant difference in the preventive effect between the 2 drugs. Of note are the observations that compared with each agent alone, combined temocapril and CS-866 (20 and 10 mg/kg per day, respectively) prevented intimal thickening to a greater extent. Thus, our present work provided the evidence that compared with each agent alone, a combination of the ACE inhibitor and AT1 receptor antagonist was more effective in the prevention of neointimal thickening, supporting the notion that the combination therapy of these agents may be useful in the treatment of vascular diseases. To further elucidate the effect of combination therapy, the effects on vascular smooth muscle cell (VSMC) proliferation were examined. The BrdU labeling index and Western blot analysis on PCNA content clearly demonstrated that compared with each agent alone, combination therapy more potently suppressed VSMC proliferation after balloon injury. These observations show that greater prevention of intimal thickening by the combination therapy compared with monotherapy was due to greater inhibition of VSMC proliferation.

Accumulating evidence indicates that PDGF plays a central role in vascular diseases, via VSMC proliferation and migration. The action of PDGF is mediated by 2 receptors, including PDGF-β and α receptors. Particularly, the PDGF-β receptor, which is activated by the PDGF B chain but not by the PDGF A chain, is abundant in VSMCs and is reported to be significantly activated in balloon-injured arteries, as shown by the increase in its tyrosyl phosphorylation. PDGF B chain antibody significantly inhibits neointimal thickening after balloon injury. These findings support the important role of PDGF-β receptor activation in intimal thickening after balloon injury. Furthermore, recently, Ang II has been shown to induce PDGF-β receptor tyrosyl phosphorylation in cultured VSMCs, and the AT1 receptor antagonist has been shown to inhibit PDGF-β receptor tyrosyl phosphorylation in balloon-injured arteries. Therefore, in the present study, we examined the effect of combination therapy on PDGF-β receptor activation compared with the monotherapy. Interestingly, compared with the monotherapy, the combination therapy more potently inhibited PDGF-β receptor tyrosyl phosphorylation. These findings suggest that the larger suppression of VSMC proliferation by combination therapy compared with monotherapy may be at least in part mediated by greater suppression of PDGF-β receptor activation.

A previous report has shown that the preventive effect of the ACE inhibitor on intimal thickening after balloon injury is partially mediated by bradykinin or NO. Furthermore, in pigs, bradykinin has been reported to be involved in the reduction of infarct size after myocardial ischemia by combined treatment with ACE inhibitors and AT1 receptor antagonists. Therefore, it is conceivable that the beneficial effects of the combination seen in the present study might be partially mediated by bradykinin or NO accumulation. To elucidate this possibility, we examined the effect of the bradykinin B2 receptor antagonist FR172357 and the NO synthase inhibitor L-NAME on the inhibitory effects of intimal thickening by the combination of temocapril and CS-866. As shown in the Table and Figure 4, without affecting blood pressure, FR172357 or L-NAME treatment significantly reversed the prevention of intimal thickening by the combination therapy, providing the first evidence that the beneficial effects of the combination of the ACE inhibitor and the AT1 receptor antagonist on vascular hyperplasia are at least in part mediated by bradykinin and NO. However, it cannot be excluded that more potent inhibition of Ang II–mediated AT1 receptor activation.
activation might also contribute to the beneficial effects of the combination therapy, inasmuch as the increase in circulating Ang II induced by the AT1 receptor antagonist may interfere with the binding of the AT1 receptor antagonist to the AT1 receptor. Further study is needed to elucidate the detailed mechanism of the beneficial effects of the combination of the ACE inhibitor and the AT1 receptor antagonist on intimal hyperplasia.

In conclusion, our present work provided the first evidence that the combination of the ACE inhibitor and the AT1 receptor antagonist prevented intimal thickening after balloon injury more potently than did monotherapy with either agent alone. More beneficial effects of the combination therapy on intimal thickening were mediated by greater suppression of VSMC proliferation due to PDGF-β receptor activation. Furthermore, either bradykinin or NO contributes to the beneficial effects of the combination of the ACE inhibitor and the AT1 receptor antagonist. However, because the systemic combination therapy of these drugs is reported to cause adverse effects, such as dehydration and renal dysfunction in rats,5,34 local application of these drugs to injured vessels seems to be preferable.

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References


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(A) Non-injury vs. injury, 7 day

* P<0.01 vs. cont

PCNA levels

cont 1 day 3 day 7 day

(B) Injury, non-injury, and combined treatment

† P<0.05, * P<0.01 vs. Ve

P<0.05

PCNA levels

Ve Te(40) CS(20) Te(20) + CS(10)
(A) * P<0.01 vs. cont

(B) † P<0.05, * P<0.01 vs. Ve

P<0.05