Synergistic Effects of Calcium-Channel and Angiotensin-Receptor Blockers on Endothelial Function and Inflammatory Responses in a Porcine Drug-Eluting Stent Model

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**Background:** The rate of stent thrombosis is increased in association with drug-eluting stents (DES) due to delayed endothelialization and prolonged inflammation. Clinical studies have shown that either an angiotensin-receptor blocker (ARB) or a calcium-channel blocker (CCB) can improve endothelial dysfunction and inhibit inflammatory reactions in patients with hypertension. The effects of co-administered CCB and ARB on vascular protection after DES implantation, however, remain unknown.

**Methods and Results:** Pigs (n=24) were implanted with coronary stents and randomly assigned to control, CCB, ARB or CCB+ARB groups. Endothelium-mediated vasodilation at the distal edge was significantly impaired compared to the intact site in the control group (P<0.05), but the difference between two sites in the CCB+ARB group was not significant. The combination produced a synergistic effect at the distal edge compared to the ARB, CCB and control groups (P<0.05). The expression of tumor necrosis factor-α and inflammatory cell adhesion were significantly inhibited in the CCB or ARB monotherapy groups compared with the control (P<0.05). The combination of CCB+ARB also improved inflammation.

**Conclusions:** Implanted DES exert adverse effects such as endothelial dysfunction and inflammatory reactions. The administration of either a CCB or an ARB reversed this adverse effect. Furthermore, recovery was synergistically enhanced by a CCB combined with an ARB. *(Circ J 2010; 74: 1704–1710)*

**Key Words:** Endothelial function; Inflammation; Sirolimus-eluting stent; Vasodilation

Drug-eluting stents (DES) that release sirolimus or paclitaxel are routinely used for coronary revascularization because they significantly reduce the rates of restenosis of target lesions compared to bare metal stents. However, DES, however, have limitations such as stent thrombosis due to endothelial dysfunction and prolonged inflammation at the stented site. Late thrombosis is a life-threatening complication that has become a major concern. Therefore, drugs that restore endothelial function and inhibit inflammatory reactions have become the targets of new therapeutic strategies. Calcium-channel blockers (CCB) and angiotensin-receptor blockers (ARB) have anti-inflammatory effects and have improved endothelial dysfunction in both clinical and experimental trials. Few studies, however, have evaluated the effects of drugs on endothelial dysfunction and inflammation after DES implantation. Li et al recently reported that sirolimus-eluting stents (SES) cause coronary vasomotor dysfunction in distal segments of the stent in porcine models within 28 days. The porcine stent model has been helpful in elucidating the responses of damaged blood vessels because this process appears to closely approximate human pathology. The DES porcine model is also useful for studying the effects of various drugs. We examined whether a CCB combined with an ARB would recover the endothelial dysfunction and attenuate inflammatory reactions elicited in porcine coronary arteries implanted with DES.

**Methods**

**Animals**

The Animal Care and Use Committee of Juntendo University approved all experiments. Twenty-four female domestic juvenile pigs (Shiraishi, Tokyo, Japan; aged 8–12 months...
and weighing 25–35 kg) were fed with standard laboratory chow without supplementation, and given daily oral antiplatelet medication (clopidogrel, 75 mg; aspirin, 325 mg) from 1 day before stent implantation until being killed. The pigs were randomized into the following groups: control (n=8), azelnidipine alone (n=4), olmesartan alone, (n=4), or azelnidipine plus olmesartan (n=8). The control group received a placebo. Azelnidipine (1.3 mg/kg) and olmesartan (3.3 mg/kg) were orally administered daily to the monotherapy groups, and half doses (0.65 and 1.65 mg/kg, respectively) were administered to the combination group starting 7 days before stenting and for the next 28 days until death.

**Stent Implantation**

SES (3.0×18 mm, Cordis, Miami, FL, USA) were implanted into the left anterior descending and circumflex arteries of domestic juvenile swine as described by Schwartz et al. This model has been used in several studies and appears to reflect clinical outcomes well.\(^5\)\(^,\)\(^6\) \(^,\)\(^16\) Stent balloons were inflated for 30 s to achieve a 1.1:1–1.2:1 stent-to-artery ratio.

**Preparation**

We killed all 24 pigs and dissected 48 stented coronary arteries. The stented segment in each artery was cut longitudinally, and examined on scanning electron microscopy (SEM). The adjacent segment distal to the stent was also immunostained. Segments located 0 mm distal to the stent edge in each artery were cut and defined as the distal edge of the stent.

**Scanning Electron Microscopy**

Inflammatory cell infiltration was evaluated on SEM at 28 days after stenting. The histological endpoint was the appearance of inflammatory cell adhesion after stenting.\(^17\) Specimens for SEM were washed with phosphate-buffered saline (PBS), placed in osmium tetroxide, washed again with PBS, dehydrated with an ethanol series and dried at critical point before being covered with gold and palladium. The numbers of inflammatory cells were counted in all specimens.\(^18\)

**Immunohistochemistry of Tumor Necrosis Factor (TNF)-α and Endothelial Nitric Oxide Synthase (eNOS)**

Specimens were immunocytochemically labeled as previously described.\(^19\)\(^,\)\(^20\) Rings distal to the stent edge were fixed in 10% neutral buffered formalin and embedded in paraffin to detect eNOS and TNF-α expression. Sections were incubated overnight at 4°C with a primary goat monoclonal antibody to eNOS (10 μg/ml; BD Transduction Laboratories, Lexington, KY, USA) and a mouse monoclonal antibody to TNF-α (7.5 μg/ml; R&D Systems, Minneapolis, MN, USA). The sections were incubated with biotinylated secondary antibodies (including goat antimouse or goat) diluted 1:400 for 30 min at room temperature, followed by streptavidin–horseradish peroxidase for 30 min at room temperature. Immunoreactivity was visualized using a 3-amino-9-ethylcarbazole substrate. The total numbers of TNF-α–positive cells per field and total numbers of eNOS per circle were counted under high-power magnification (×200) using a KS-400 image analyzing system (Carl Zeiss Imaging Solutions, Hallbergmoos, Germany) and the proportions (%) were calculated.\(^21\) All sections from each animal were evaluated.

**Measurement of Changes in Force**

Porcine coronary artery segments were transferred to the laboratory in cold Krebs-HEPES (25 mmol/L) buffer (pH 7.4) immediately after dissection and all samples were investigated on the same day. After removing surrounding connective tissue and fat, vessel segments were cut into rings of approximately 3 mm while taking care not to contact the endothelial surface to preserve functional endothelium. Changes in force were measured in oxygenated Krebs bicarbonate buffer (37°C) using an adaptation of described methods.\(^22\)\(^,\)\(^23\) Briefly, the arterial rings were mounted on wire hooks attached to force displacement transducers (Nihon Kohden, Tokyo, Japan) to determine changes in isometric force on a polygraph (Rikadenki, Tokyo, Japan). The rings were incubated in 10-ml baths individually kept at a temperature of 37°C for 120 min at an optimal passive tension of 5 g in Krebs bicarbonate buffer (pH 7.4) containing the following: 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.5 mmol/L CaCl\(_2\), 25 mmol/L NaHCO\(_3\), 1.1 mmol/L MgSO\(_4\), 1.2 mmol/L KH\(_2\)PO\(_4\), and 5.6 mmol/L glucose, and bubbled with 21% O\(_2\)–5% CO\(_2\) (balanced N\(_2\)). The rings were contracted with Krebs bicarbonate buffer containing KCl instead of NaCl to generate maximal force and enhance the reproducibility of subsequent contractions. The rings were then washed with fresh Krebs bicarbonate buffer and re-equilibrated for 30 min. The rings were initially contracted with U-46619 (30 mmol/L) and after a steady-state level of contraction was reached, endothelium-dependent relaxation was evaluated by the cumulative application of bradykinin (BK; 0.1 mmol/L–1 μmol/L). Relaxation was expressed as % change in the steady-state level of contraction induced by U-46619.

**Statistical Analysis**

All data are presented as mean±SD, with n representing the number of examined vessels. Non-normally distributed variables were compared between groups using the Mann–Whitney U-test, and normally distributed variables were com-

### Table. Mean Changes in Blood Pressure vs Time

<table>
<thead>
<tr>
<th></th>
<th>Control (n=8)</th>
<th>CCB (n=4)</th>
<th>ARB (n=4)</th>
<th>CCB + ARB (n=8)</th>
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<tbody>
<tr>
<td><strong>Day –7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>104.8±18.4</td>
<td>111.6±37.4</td>
<td>112.7±10.1</td>
<td>116.6±16.7</td>
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<tr>
<td>DBP (mmHg)</td>
<td>86.8±20.4</td>
<td>73.0±21.9</td>
<td>70.0±12.6</td>
<td>79.6±21.5</td>
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<tr>
<td><strong>Day 28</strong></td>
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<td></td>
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</tr>
<tr>
<td>SBP (mmHg)</td>
<td>125.5±18.3</td>
<td>126±9.8</td>
<td>114±22.3</td>
<td>112.8±14.3</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>90.8±15.4</td>
<td>73.3±21.4</td>
<td>84.8±12.1</td>
<td>93.4±8.9</td>
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</tbody>
</table>

No significant differences in SBP and DBP at either day –7 or day 28 among the 4 groups: control, CCB, ARB, CCB + ARB.

CCB, calcium-channel blocker; ARB, angiotensin-receptor blocker; SBP, systolic blood pressure; DBP, diastolic blood pressure.
Figure 1. Scanning electron microscopy. Globular cells are inflammatory monocytes and lymphocytes. Vessels from: (A) control (×2000), (B) calcium-channel blocker (CCB; ×2000), (C) angiotensin-receptor blocker (ARB; ×2000) and (D) combination (×2000) groups at 28 days after drug-eluting stent implantation. (E) No. inflammatory cells adherent to the endothelium in 10 sites from control, CCB, ARB and CCB+ARB groups. *P<0.05 vs control; **P<0.05 for the comparison between combination vs CCB or ARB groups.

Figure 2. Immunostaining for tumor necrosis factor (TNF)-α. Photomicrographs of porcine coronary arteries were immunostained for TNF-α (magnification ×200) in (A) control, (B) calcium-channel blocker (CCB), (C) angiotensin-receptor blocker (ARB) and (D) CCB+ARB groups at 28 days after insertion of drug-eluting stent. Positive cells are stained dark brown. (E) Proportion of areas positive for TNF-α. *P<0.05 vs control.
pared using Student’s unpaired t-test. Blood pressure at days –7 and 28 were compared among groups using two-way analysis of variance. Differences with P<0.05 were considered statistically significant.

Results
Each group of animals was matched for age and weight at the start of the experiment. Forty-eight stents were implanted without complication into 24 animals, all of which survived without any adverse events. Coronary angiography before death confirmed that the stent sites were free of obstruction.

Scanning Electron Microscopy
SEM indicated leukocytes attached to the luminal surface as described.24,25 Figures 1A–D shows SEM findings of vessels from the control (n=16), CCB (n=8), ARB (n=8) and combination (n=16) groups. Globular cells are inflammatory cells. Figure 1E shows that the mean of inflammatory cells counted in 10 sites at the edges of stents was reduced in the CCB and ARB groups, and further decreased in the combination group (45.8±4.6 in the control group vs 28.2±2.5 in the CCB group, 25.8±2.3 in the ARB group, and 10.5±1.3 in the combination group, P<0.05, respectively). In addition, the combination group had significantly less inflammatory cells than either the CCB group or ARB group (P<0.05, respectively). There was no significant difference between the CCB and ARB groups.

Immunostaining of TNF-α and eNOS
Figures 2A–D shows immunostaining of TNF-α in vessels from the control (n=16), CCB (n=8), ARB (n=8) and combination (n=16) groups, respectively, at 28 days after stenting. Cells positive for TNF-α were stained brown in Figure 2E. Areas of TNF-α-positive cells at the distal edges of stents (control, 66.1±7.5%) were reduced in the CCB, ARB (n=8) and combined (n=16) groups (39.3±24.6%, 28.9±21.8% and 31.2±24%, respectively; all P<0.05). In addition, the combination group showed no significant difference compared with either the CCB group or ARB group. There was no significant difference between the CCB and ARB groups.

Figures 3A–D shows immunostaining for eNOS in vessels from the control (n=16), CCB (n=8), ARB (n=8) and combination (n=16) groups, respectively, at 28 days after stenting. Areas of TNF-α-positive cells at the distal edges of stents (control, 66.1±7.5%) were reduced in the CCB, ARB and combination groups (39.3±24.6%, 28.9±21.8% and 31.2±24%, respectively; all P<0.05). In addition, the combination group showed no significant difference compared with either the CCB group or ARB group. There was no significant difference between the CCB and ARB groups.

Endothelium-Dependent Vasodilatation
Endothelium-dependent relaxation at the stent edge (n=16) was significantly reduced compared to intact sites (n=16) in the control group (Figure 4A; P<0.05). In contrast, relaxation was not reduced at the edge of the stent in the CCB+ARB group (n=16; Figure 4B). Figure 4C shows that reduced endothelium-dependent relaxation at the edge of the stent was recovered in the CCB or ARB groups (P<0.05) and further recovered in the combination group (P<0.05). Figure 4D also shows that decreased endothelium-dependent vasodilation at intact sites was recovered in the CCB or ARB groups (P<0.05), and further recovered in the CCB+ARB groups (P<0.05).
Figure 4. Endothelium-dependent vasodilation. Vasodilation tested at distal edge of stents and at intact sites in (A) control and (B) calcium-channel blocker + angiotensin-receptor blocker (CCB+ARB) groups. *P<0.05 vs intact sites. (C, D) Vasodilation tested at distal edge of stents and at intact sites in control, CCB, ARB and CCB+ARB groups. Data given as dose–response curves for relaxation induced by bradykinin. Data expressed as mean±SD. *P<0.05 vs control; †P<0.05 vs control; ‡P<0.05 vs control; §P<0.05 vs ARB; ¶P<0.05 vs CCB.
Discussion

We showed that endothelial dysfunction and prolonged inflammation developed at the distal site of the porcine coronary artery after DES implantation, which is consistent with published findings. This is a critical issue because endothelial dysfunction and prolonged inflammation are associated not only with late stent thrombosis but also with long-term clinical events. Furthermore, no medical treatment can currently prevent vascular dysfunction at the edges of implanted DES. The present study showed that olmesartan or and azelnidipine improved endothelial dysfunction evaluated using an organ chamber and decreased inflammation assessed according to TNF-α and eNOS expression. To the best of our knowledge, this is the first evidence supporting the notion that drugs can prevent vascular failure after implantation with a DES. Impaired endothelial function was synergistically recovered by CCB co-administered with an ARB.

We focused on TNF-α and eNOS because these factors play important roles in inflammatory reaction and endothelial function. A theory of endothelial injury and endothelial dysfunction leading to atherosclerosis has been proposed and multifactorial causes of endothelial dysfunction have been postulated. Nitric oxide (NO) is a critical factor in vascular physiological changes produced by eNOS that causes smooth muscle relaxation and plays an important role in angiogenesis. Normal endothelial cells constitutively express eNOS, which, when inhibited under pathological conditions, can reduce NO bioavailability. Inflammatory responses after stent implantation play a key role in vascular lesion formation early in atherosclerosis and re-stenosis. Stent implantation in coronary porcine arteries is associated with the early activation of TNF-α expression. Inhibition of TNF-α activation with a soluble receptor accelerates re-endothelialization at sites of balloon angioplasty.

Because endothelial dysfunction and inflammation play a crucial role in long-term prognosis, their respective recovery and inhibition are potential targets for treating adverse cardiovascular diseases. Angiotensin II activates inflammation and reduced endothelial function, and the renin–angiotensin system is activated after vascular injury. These findings suggest that inhibition of the renin–angiotensin system would ameliorate inflammatory reactions and endothelial dysfunction. Thus, the present findings that olmesartan improved endothelial dysfunction and inhibited inflammation support this theory. Furthermore, experimental and clinical data have provided evidence that ARB attenuate inflammatory reactions and improve endothelial function.

Ma et al reported that azelnidipine enhances basal NO production by endothelial NO synthesis in human umbilical vein endothelial cells and reduces MCP-1 expression associated with intimal hyperplasia attenuation in the cynomolgus monkey stent model fed with a high-cholesterol diet. The present findings are consistent with these results, but the present study differed from others in terms of methodology and procedure; we implanted DES and directly measured vaso-motor functions.

A combination of an ARB and a CCB recovered endothelial dysfunction and eNOS expression more effectively than monotherapy through unknown mechanisms. Two possible explanations for the synergistic mechanism are as follows. Previous studies indicate that Ca2+ is necessary for inflammatory progression caused by AT1 receptors and that CCB exert inhibitory actions by blocking AT1 receptor-mediated signaling through Ca2+ mobilization. Furthermore, a threshold level of intracellular Ca2+ is required for activating subsequent intracellular signaling molecules. Even at a non-effective dose, each drug reduces the Ca2+ concentration that is elevated with angiotensin II but not to a level below this intracellular Ca2+ threshold. When they are combined, the intracellular Ca2+ level might be lowered to below this threshold. Thus, combined therapy appears to synergistically affect inflammation, but further studies are necessary to address endothelial function.

Clinical Implications

Agents such as olmesartan or/and azelnidipine, which might prevent inflammatory response and recover endothelial function at distal sites of vascular injury, could help to prevent the progression of coronary artery disease. This has important clinical implications because the DES that are currently used to prevent re-stenosis are effective only against localized lesions and cannot be expected to prevent atherosclerotic progression. The outcome of long-term treatment with olmesartan or/and azelnidipine and their effects on the progression of atherosclerotic lesions throughout the coronary tree should be investigated in detail.

Study Limitations

Human coronary arteries critically differ from porcine coronary arteries. Stents are implanted into normal non-atherosclerotic arteries of the porcine model whereas they are deployed on extant atherosclerotic plaque in humans. Similar reductions, however, of re-stenosis in native porcine coronary arteries by stents eluting paclitaxel and sirolimus have been achieved in clinical trials. Furthermore, the present model has been used in prior studies and it appears to reflect the outcomes of clinical trials well. Thus, normal porcine coronary arteries might be a viable substitute for atherosclerotic lesions in humans. Long-term studies are required to evaluate whether the beneficial effects of olmesartan or/and azelnidipine exceed the period of stent-related drug release and whether these drugs cannot yet confirm that olmesartan or/and azelnidipine help prevent thrombosis, but their beneficial effects in the present study suggest that this approach warrants further testing in a long-term human study.

Previous studies have shown that differences among pigs are undetectable, so we could not determine an appropriate sample size. Our experience, however, indicates that the sample size used in the present study is appropriate.

In conclusion, the present results showed that CCB or/and ARB protect vascular function and that CCB and ARB co-administration might have synergistic, protective effects after implantation with SES. The present results also extend the notion that a combination of CCB and ARB could be more clinically effective in terms of long-term outcomes after implantation with a drug-eluting stent.

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References


