Background: Sirolimus-eluting stents (SES) are widely used in coronary artery disease as revascularization therapy. Although endothelial dysfunction induced by implanted SES can become a major clinical concern, therapeutic strategies to overcome this disorder remain unclear. The aim of the present study was therefore to identify effective therapies in a clinically relevant animal model.

Methods and Results: Twenty-one pigs were randomized to control, candesartan (CAN) and candesartan plus pioglitazone (CAN+PIO) groups. Drugs were administered orally for 7 days before SES implantation until the time of death. Forty-two SES were used in porcine coronary arteries. Early inflammatory cell adhesion in SES evaluated on scanning electron microscopy at 3 days was significantly suppressed in the CAN and CAN+PIO groups compared with controls. Bradykinin-induced endothelium-dependent relaxation at an adjacent segment distal to the SES evaluated using organ chambers was reduced compared with intact segments in control coronaries at 28 days. Endothelial dysfunction was reversed by CAN and even more obviously improved in the CAN+PIO group.

Conclusions: Candesartan protected against vascular inflammation and restored endothelial function after SES implantation. The combination of candesartan and pioglitazone was more effective than candesartan monotherapy and might confer vascular protection when administered before SES implantation. (Circ J 2011; 75: 1098–1106)

Key Words: Angiotensin II type 1 receptor blocker; Endothelial dysfunction; Inflammation; Sirolimus-eluting stent; Thiazolidinedione

D rug-eluting stents are widely used to treat coronary artery disease. Sirolimus-eluting stents (SES) have reduced rates of re-stenosis and target lesion revascularization compared with bare metal stents.¹,² Late stent thrombosis (LST), however, has recently become a major concern among patients implanted with SES because it is correlated with fatal cardiovascular events.³,⁴ One powerful histological predictor of stent thrombosis is endothelial coverage that arises through implanted coronary SES disrupting the endothelial layer, which results in the thrombogenic metallic surface of the SES being exposed to the bloodstream.⁵ In addition, impaired endothelial recovery can also have adverse long-term outcomes such as LST and constric-

tive vascular remodeling.⁶,⁷ Reports have also shown that SES cause delayed neointimalization and endothelial vaso-
motor dysfunction of coronary artery segments distal to implanted segments in porcine as well as in human coronary arteries.⁸–¹¹ Treatment strategies for endothelial dysfunction induced by SES, however, are not currently established.

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The renin–angiotensin system has important modulatory activities in the atherogenic process, and angiotensin II induces endothelial dysfunction and augments vascular inflammation.¹² Therapy with angiotensin II receptor blockers (ARB) is an effective approach to treating vascular dysfunction.¹³,¹⁴ Other studies have also shown that the ARB candesartan improves endothelial dysfunction and coronary vasomotion.¹⁵,¹⁶ Pioglitazone is a peroxisome proliferator-activated...
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Candesartan + pioglitazone (N = 7) (1.5 + 5 mg/kg, p.o.)
Starting 7 days before stenting and continuing until euthanasia.
Oral antiplatelet medication (clopidogrel, 75 mg; aspirin, 325 mg)

Figure 1. Flow of study. eNOS, endothelial nitric oxide synthase; LAD, left ascending coronary artery; LCX, left circumflex artery; SES, sirolimus-eluting stent; TNF-α, tumor necrosis factor-α.

Methods

Animals

The Animal Care and Use Committee of Juntendo University approved all experiments. Twenty-one female domestic pigs (Shiraishi, Tokyo, Japan; aged 8–12 months and weighing 25–35 kg) received standard laboratory chow without supplementation, and daily oral antiplatelet medication (clopidogrel, 75 mg; aspirin, 325 mg) from 7 days before stent implantation until death. The pigs were randomized to the following groups: placebo (control, n=7), candesartan alone (CAN, 1.5 mg/kg p.o.; n=7) or candesartan plus pioglitazone (CAN + PIO, 1.5+5 mg/kg, respectively, p.o.; n=7), starting 7 days before stenting and continuing until euthanasia. We implanted SES into the left anterior descending and circumflex arteries of the pigs as described by Schwartz et al. Stent balloons were inflated for 30 s to achieve a 1.1:1 to 1.2:1 stent-to-artery ratio. The electrocardiogram and blood pressure were continuously monitored. Blood pressure was measured using an arterial sheath connected to a transducer.

Preparation

All pigs were killed and all stented coronary arteries were dissected. The acute experiment consisted of a scanning electron microscope (SEM) examination of the stented segment in each longitudinally cut artery to evaluate acute inflammatory response. The early inflammatory response was evaluated in 2 pigs from each group after death (4 coronary segments per group; acute experiment), and endothelial function was examined in 5 pigs (10 coronary segments per group; chronic experiment). We randomly assigned pigs from each group to the acute or the chronic experiment (Figure 1).

Stent Implantation

The pigs were sedated with an i.m. injection of ketamine, xylazine and atropine (30, 5 and 0.05 mg/kg, respectively) on the day of stent implantation. General anesthesia was induced after intubation and the animals were ventilated with isoflurane. A catheter was inserted into the right carotid artery and heparin (5,000U) was administered i.v. The coronary arteriograms were acquired in the front view using a Toshiba cineangiography system. We implanted SES into the left anterior descending and circumflex arteries of the pigs as described by Schwartz et al. Stent balloons were inflated for 30 s to achieve a 1.1:1 to 1.2:1 stent-to-artery ratio. The electrocardiogram and blood pressure were continuously monitored. Blood pressure was measured using an arterial sheath connected to a transducer.

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responses after SES implantation. The adjacent reference segment distal to the SES was immunostained in the acute experiment. The chronic experiment consisted of evaluating endothelial-dependent vasomotor function in adjacent segments (each 3 mm long) located distally to the SES in each artery (stent distal segment). We also examined endothelial function in 3-mm sections located 15 mm beyond the distal adjacent segment to the stents that were defined as intact (non-stented) reference segments (Figure 2).

SEM
Inflammatory cell infiltration into the SES was evaluated on SEM at 3 days after implantation. The histological endpoint was the appearance of inflammatory cell adhesion after stent implantation. Two pigs were killed and 4 stented coronary arteries were dissected in each group. Stented segments from each artery were cut longitudinally, opened along the cut, 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 sputtered with gold and palladium for SEM. The numbers of inflammatory cells were counted in 10 randomly selected fields of view near the stent struts per artery by a laboratory assistant who was unaware of the presence or absence of drug administration, and grades of inflammation were evaluated by scoring each view. Scores were assigned according to the numbers of inflammatory cells observed under high power as: 1+, 2+, 3+ and 4+, for 0–5, 6–10, 11–15 and >15 cells, respectively.

Immunohistochemistry
Immunohistochemistry was evaluated at 3 days after stenting in the acute experiment. To detect tumor necrosis factor-α (TNF-α) and endothelial nitric oxide synthase (eNOS) expression, rings at adjacent segments distal to the SES were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were visualized using an automatic immunostainer (NeXES IHC; Ventana Medical Systems, Tucson, AZ, USA) and a standard 3,3’-diaminobenzidine (DAB) detection kit (Ventana Medical Systems). We applied a mouse monoclonal antibody to TNF-α (R&D Systems, Minneapolis, MN, USA; 7.5 μg/ml) as primary antibodies and a goat monoclonal antibody to eNOS (BD Transduction Laboratories, Lexington, KY, USA; 10 μg/ml). The sections were then washed in distilled water, counterstained with hematoxylin for 3 min and washed in tap water. Finally, the sections were dehydrated in absolute alcohol and placed in xylene followed by mounting medium. In brief, TNF-α and eNOS were detected as brown reaction products generated by DAB in TNF-α- or eNOS-positive tissues. Ten fields of view from each section were selected at random. The proportions of total eNOS- and TNF-α-positive areas per field were determined under high-power magnification (×400) using a KS-400 image analyzing system (Carl Zeiss Imaging Solutions, Hallbergmoos, Germany).

Measurement of Changes in Force
We examined the endothelium-dependent relaxation of porcine coronary rings in response to bradykinin. Porcine coronary artery segments were immediately transferred to cold Krebs-HEPES (25 mmol/L) buffer (pH 7.4) after dissection, and all samples were investigated on the same day. Surrounding connective tissue and fat were removed and then vessel segments were cut into rings (approximately 3 mm long), while avoiding contact with 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. Briefly, the arterial rings were mounted on wire hooks attached to force displacement transducers (Nihon Kohden, Tokyo, Japan) and then changes in isometric force were recorded on a polygraph (Rikadenki, Tokyo, Japan). The rings were incubated in individual 10-ml baths at 37°C for 120 min at an optimal passive tension of 5 g in Krebs bicarbonate buffer containing the following (in mmol/L): 118 NaCl, 4.7 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄, 1.2 KH₂PO₄, and 5.6 glucose, and bubbled with a 21% O₂–5% CO₂ (balanced N₂) mixture. After contraction with Krebs bicarbonate buffer containing KCl instead of NaCl (to generate maximal force and to enhance the reproducibility of subsequent contractions), the vessels were washed with fresh Krebs bicarbonate buffer, re-equilibrated for 30 min and initially contracted with a stable prostaglandin F₂α analog (U-46619; 30 μmol/L). Cumulative amounts of bradykinin (0.1–1 μmol/L) were added when contraction reached a steady state, and then endothelium-dependent 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 except for changes in force, which are shown as mean±SE. Groups were compared using a Student’s t-test or a 2-way analysis of variance (ANOVA). The significance of differences among the groups in endothelial function was determined by 2-tailed multiple t-test with Bonferroni correction after ANOVA. Categorical variables were analyzed using the chi-squared test and Fisher’s exact test. P<0.05 was considered statistically significant. All data were statistically analyzed using SPSS version 18.0 (SPSS, Chicago, IL, USA) and JMP version 7.0 (SAS Institute, Cary, NC, USA).
**Table. Changes in Body Weight and Blood Pressure**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=7)</th>
<th>CAN (n=7)</th>
<th>CAN+PIO (n=7)</th>
<th>P value</th>
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<tr>
<td><strong>Day 0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>31.5±2.1</td>
<td>32.4±2.6</td>
<td>30.1±2.0</td>
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<tr>
<td>SBP (mmHg)</td>
<td>125.4±13.5</td>
<td>125.6±10.4</td>
<td>128.2±18.0</td>
<td>0.943</td>
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<tr>
<td>DBP (mmHg)</td>
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<td>88.2±8.6</td>
<td>89.6±13.1</td>
<td>0.962</td>
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<tr>
<td><strong>Day of death</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
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<td>33.5±1.4</td>
<td>33.4±1.2</td>
<td>0.217</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
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<td>133.8±16.2</td>
<td>131.0±10.8</td>
<td>0.881</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>91.2±8.8</td>
<td>94.2±15.1</td>
<td>92.4±11.9</td>
<td>0.895</td>
</tr>
</tbody>
</table>

CAN, candesartan; CAN+PIO, candesartan plus pioglitazone; SBP, systolic blood pressure; DBP, diastolic blood pressure.

**Figure 3.** Scanning electron microscopy of vessels implanted with sirolimus-eluting stents (SES) at 3 days after stenting. (A) Control; (B) candesartan (CAN); (C) candesartan plus pioglitazone (CAN+PIO: all×2,000). Globular cells are monocytes, many of which are evident in the control group; CAN and CAN+PIO groups have far fewer inflammatory cells. (D) Proportion (%) of inflammatory grades in total 40 fields per group. Inflammation scores in CAN+PIO, CAN and control groups: 1+, 35.0%, 22.5% and 10.0%, respectively; 2+, 30.0%, 27.5% and 17.5%, respectively; 3+, 27.5%, 30.0% and 37.5%, respectively; 4+, 7.5%, 20.0% and 35.0%, respectively. Rates of high inflammation fields in stents are reduced in the CAN group and further decreased in the CAN+PIO group compared with controls (P=0.039 and P<0.001, respectively). Scale bar, 10 μm.

**Results**

The animals in all groups were matched for age and weight at the start of the experiment. Forty-two SES were implanted without complications into 21 animals, all of which survived without any adverse events. Blood pressure and body weight did not significantly differ among the groups at day 0 and at the time of death (Table).

**Scanning Electron Microscopy**

Scanning electron microscopy showed leukocytes attached to the luminal surface near the stent struts as in the acute phase.
Figure 4. Immunostaining for tumor necrosis factor-α (TNF-α) in porcine coronary arteries at 3 days after stenting. Immunostaining of TNF-α in vessels from (A) control; (B) candesartan (CAN); (C) candesartan plus pioglitazone (CAN+PIO); and (D) negative control groups (all×400). (E) Proportions (%) of areas positive for TNF-α; these differed significantly among groups (48.8±18.7%, 28.1±14.2% and 19.9±11.5%; P<0.01). Scale bar, 100 μm.

Figure 5. Immunostaining for endothelial nitric oxide synthase (eNOS) in porcine coronary arteries at 3 days after stenting. Immunostaining of eNOS in vessels from (A) control; (B) candesartan (CAN); (C) candesartan plus pioglitazone (CAN+PIO); and (D) negative control groups (all×400). (E) Proportions (%) of areas positive for eNOS significantly differed among groups (5.8±2.9%, 8.5±4.4% and 9.7±4.4%; P<0.05). Scale bar, 100 μm.
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at 3 days after stenting. The SEM findings of vessels are given in Figure 3A for the control, Figure 3B for the CAN, and Figure 3C for the CAN + PIO groups. Globular cells are inflammatory cells (including monocytes and granular leukocytes). The proportion (%) of each inflammatory grade in 40 fields from each group is shown in Figure 3D. Inflammation was graded as 1+ in 35.0% of the fields in the CAN + PIO group compared to 22.5% and 10.0% in the CAN and control groups, respectively. High-grade inflammation was found in 35.0%, 50.0% and 72.5% of fields in the CAN + PIO, CAN and control groups, respectively (P=0.024). Figure 3 indicates that the rates of high-inflammation fields in the stents were reduced in the CAN group, and further decreased in the CAN + PIO group compared with controls (P=0.039 and P<0.001, respectively). In addition, the rates of high-inflammation fields tended to be lower in the CAN + PIO group than in the CAN group (P=0.067).

Immunostaining of TNF-α and eNOS

Figures 4A–C shows immunostaining of TNF-α in vessels from the control, CAN and CAN + PIO groups, respectively, at 3 days after stenting, and Figure 4E shows TNF-α-positive cells. The % positive areas of TNF-α at adjacent distal segments of the SES (control, 48.8±18.7%) were lower in the CAN and CAN + PIO groups than in the control group (28.1±14.2%, P<0.001; 19.9±11.5%, P<0.001).

Figures 5A–C shows immunostaining for eNOS in vessels from the control, CAN and CAN + PIO groups, respectively,
at 3 days after stenting, and Figure 5E shows eNOS-positive areas. The % positive areas of eNOS in adjacent segments distal to the SES (control, 5.8±3.0%) were higher in the CAN and CAN+PIO groups than in the control group (8.5±4.4%, P=0.021; 9.7±4.4%, P=0.002).

### Endothelial-dependent Vasodilatation

We investigated whether adding bradykinin to all coronary rings with endothelium resulted in their concentration-dependent relaxation. Endothelium-dependent relaxation by bradykinin at adjacent segments distal to the SES was significantly impaired compared to intact segments in the control group (n=10, respectively; Figure 6A).

Bradykinin-induced relaxation was significantly improved in the CAN+PIO and CAN groups compared with the control group (n=10, respectively). In addition, the ratio of the improvement at 10⁻⁶ and 10⁻⁷ mol/L bradykinin was more obvious in the CAN+PIO group than in the CAN alone group (P=0.013 and P=0.035, respectively, Figure 6B).

### Discussion

The present study showed that candesartan improves endothelial dysfunction caused by SES implantation in the porcine coronary model in the short term and that pioglitazone has an additive effect on improving endothelial dysfunction. Combining candesartan with pioglitazone further relieved acute inflammation due to SES injury from the early stage.

We confirmed that SES implantation impairs coronary arterial endothelial function in adjacent segments distal to the SES in a porcine model. The important role of the endothelium in regulating vascular tone, inflammatory responses, coagulation and thrombocyte adhesion is now recognized. In contrast, sirolimus (rapamycin) is a potent immunosuppressive agent with anti-proliferative effects that are exerted during cell-cycle arrest during the late G1 phase. The effects of sirolimus on vasomotion are hypothetically due to a direct effect on the endothelium, on the signaling pathway of the endothelium to medial smooth muscle cells or a direct effect on the media. Evidence in vivo has shown that SES implantation is associated with persistently impaired endothelial vasodilator function in arterial segments for up to 6 months after implantation. In addition, SES implantation also causes endothelial and vasomotor dysfunction of coronary artery segments distal to implanted segments in porcine coronary arteries. Thus, we consider that the porcine model of stent-induced endothelial dysfunction is useful for evaluating effective treatment strategies.

We demonstrated that candesartan could improve endothelial function and inflammatory reactions in the porcine model of coronary SES implantation. Various mechanisms must be involved in the improvement in endothelial function induced by ARB. They reduce AT1 receptor-dependent vascular signaling, in particular AT1 receptor-dependent vascular production of reactive oxygen species (ROS). AT1 receptor signaling also augments the apoptosis of endothelial cells during oxidative stress-induced vascular injury. In addition, sirolimus causes obvious vascular dysfunction and nitrate resistance, and such impaired vasorelaxation is induced by upregulated ROS as well as by the upregulated NADPH oxidase-driven superoxide production. Moreover, functional cross-regulation has been established between the renin–angiotensin system and pro-inflammatory cytokine TNF-α. Pleiotropic TNF-α might regulate cell proliferation, differentiation and apoptosis through the induction of immunomodulatory molecules. This cytokine also exerts detrimental effects on endothelial function, reduces the expression of constitutive eNOS and activates the expression of inducible nitric oxide synthase, which increases ROS production. Candesartan might correct such dysfunction by decreasing vascular ROS production and the expression of prototypical pro-inflammatory and fibrogenic genes. Consequently, we consider that the present findings demonstrate that candesartan inhibits endothelial dysfunction by reducing vascular oxidative stress and inflammatory gene production independently of a reduction in blood pressure.

We also examined the effect of adding pioglitazone to ARB on coronary vascular function. We previously showed that PPAR-γ stimulation with pioglitazone exerts beneficial vascular effects independently of glycemic control. Other studies have demonstrated that PPAR-γ is expressed in vitro in human endothelial cells, where it might enhance nitric oxide release, and in vascular smooth muscle cells, where it might downregulate AT1 receptor expression, inhibit cell migration and reduce the release of matrix-degrading enzymes.

Because PPAR-γ activation inhibits the expression of inflammatory genes, the effect of thiazolidinediones on endothelial function could potentially be mediated by an improvement in inflammation. Furthermore, pioglitazone induces PPAR-γ-independent relaxation caused by endothelium-derived NO and the opening of smooth muscle K channels in isolated blood vessels. Moreover, the inhibitory effects of sirolimus on circulating endothelial progenitor cells would strongly affect re-endothelialization after SES implantation. The benefits of PPAR-γ agonists on endothelial progenitor cell endothelialization capacity provide a potential explanation for their anti-restenotic effects and improved endothelial dysfunction when administered after coronary intervention. Therefore, we considered that adding pioglitazone would be more effective than candesartan alone against impaired coronary endothelial function with vascular inflammation.

Candesartan combined with pioglitazone exerts significant benefits upon vascular endothelium-independent relaxation compared to either drug alone. The present results also demonstrate that the combination of these agents further improves the impaired endothelium-dependent relaxation response to bradykinin as well as vascular inflammation compared with candesartan alone. The additional benefits of the combination might be the result of several interacting mechanisms. Recent experimental studies have demonstrated cross-talk between PPAR-γ and angiotensin II. Such cross-talk is regulated by ARB by inhibiting nuclear factor-κB that is activated by oxygen free radicals; and PPAR-γ ligands suppress the expression of angiotensin II type 1 receptor mRNA and protein. Therefore, we believe that the combined therapy will benefit patients with coronary artery diseases that require SES implantation.

Several limitations were associated with the present study. First, the sample size was relatively small, particularly in the acute experiment. Second, coronary stents were implanted into the non-atherosclerotic arteries of pigs. Biological findings from normal porcine coronary arteries after SES implantation might not be representative of those in human atherosclerotic coronary arteries. Findings of similar studies, however, have served extensively as the basis for clinical research and the development of drug-eluting stents. Third, we did not investigate the long-term effect of drugs in the porcine coronary SES model, but we believe that the protection afforded from the early stage soon after SES implantation contributes to long-term benefits against endothelial dysfunction and vascular inflammation. Further studies are warranted to evaluate these types of disorders over the long term.
Conclusions
The present study has demonstrated that SES implantation causes endothelial dysfunction and inflammation in adjacent segments distal to stents in the porcine coronary artery. Candesartan improved coronary endothelial dysfunction and early inflammatory responses in the short term after SES implantation. Furthermore, pioglitazone, when administered together with candesartan, increased coronary arterial protection.

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Disclosure
Conflict of interest: none declared.

References

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