Original Article

Pitavastatin-Incorporated Nanoparticle-Eluting Stents Attenuate In-Stent Stenosis without Delayed Endothelial Healing Effects in a Porcine Coronary Artery Model

Noriaki Tsukie¹, Kaku Nakano¹, Tetsuya Matoba¹, Seigo Masuda¹, Eiko Iwata¹, Miho Miyagawa¹, Gang Zhao³, Wei Meng³, Junji Kishimoto², Kenji Sunagawa¹ and Kensuke Egashira¹

¹Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

²Digital Medicine Initiative, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

³Department of Cardiovascular Medicine, 6th People's Hospital, Shanghai Jiatong University, Shanghai, China

Aim: The use of currently marketed drug-eluting stents presents safety concerns including increased late thrombosis, which is thought to result mainly from delayed endothelial healing effects (impaired re-endothelialization resulting in abnormal inflammation and fibrin deposition). We recently developed a bioabsorbable polymeric nanoparticle (NP)-eluting stent using a novel cationic electrodeposition technology. Statins are known to inhibit the proliferation of vascular smooth muscle cells (VSMC) and to promote vascular healing. We therefore hypothesized that statin-incorporated NP-eluting stents would attenuate in-stent stenosis without delayed endothelial healing effects.

Methods: Among six marketed statins, pitavastatin (Pitava) was found to have the most potent effects on VSMC proliferation and endothelial regeneration *in vitro*. We thus formulated a Pitava-NP-eluting stent (20 µg Pitava per stent).

Results: In a pig coronary artery model, Pitava-NP-eluting stents attenuated in-stent stenosis as effectively as polymer-coated sirolimus-eluting stents (SES). At SES sites, delayed endothelial healing effects were noted, whereas no such effects were observed in Pitava-NP-eluting stent sites.

Conclusion: Pitava-NP-eluting stents attenuated in-stent stenosis as effectively as SES without the delayed endothelial healing effects of SES in a porcine coronary artery model. This nanotechnology platform could be developed into a safer and more effective device in the future.

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Key words; Statin, Nanotechnology, Drug delivery system, Signal transduction

Introduction

Increased risk of late in-stent thrombosis resulting in acute coronary syndrome (unstable angina, acute myocardial infarction and death) after the use of drug-eluting stent (DES) devices has become a major safety concern¹⁻³. These adverse effects are thought to result mainly from the anti-healing effects of the drugs (sirolimus and paclitaxel) on endothelial cells, leading

to impaired re-endothelialization, excessive inflammation, proliferation and fibrin deposition⁴⁻⁶. These “anti-healing” drugs are used in most newer generation DESs, leading to continued safety concerns; therefore, the cellular and/or molecular targeting of both VSMC proliferation and re-endothelialization is an essential requirement for the development of more efficient and safer DESs. The use of an anti-healing approach that accelerates re-endothelialization, protects against thrombosis and decreases restenosis is warranted. Comparator analysis of endothelial cell coverage in four marketed polymeric DESs in rabbits has demonstrated a disparity in arterial healing; notably, all DESs examined showed a lack of endothelial anticoagulant function, independent of endothelial coverage⁷.

Address for correspondence: Kensuke Egashira, Department of Cardiovascular Medicine, Graduate School of Medical Science, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

E-mail: egashira@cardiol.med.kyushu-u.ac.jp

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We hypothesized that HMG-CoA reductase inhibitors, so-called statins, are appropriate candidate drugs for the anti-healing strategy because the vascular endothelium is a major target for the pleiotropic (non-LDL-related) vasculoprotective effects of statins⁸. In cell culture experiments *in vitro*, statins reportedly promoted endothelial regeneration and inhibited VSMC proliferation and tissue factor expression^{8, 9}. Systemic administration of statins has been reported to inhibit balloon injury-induced and in-stent neointima formation in non-hypercholesterolemic animals. Most of these beneficial effects of statins on neointima formation in animals, however, were observed following daily administration of high doses^{8, 10} (rosuvastatin 20 mg/kg per day¹¹, pitavastatin 40 mg/kg per day¹², simvastatin 40 mg/kg per day¹³); this may lead to serious adverse side effects in a clinical setting. Furthermore, it has been reported that the use of polymer-coated stents with atorvastatin or cerivastatin has no consistent effect on neointima formation in a porcine in-stent stenosis model^{14, 15}. Randomized clinical studies in humans have reported no definite effects of statins within the clinical dose range with respect to indices of restenosis after coronary balloon angioplasty¹⁶⁻¹⁹ or coronary stenting^{20, 21}; therefore, preventing in-stent restenosis via statin-mediated “anti-healing” effects requires an efficient local drug delivery system.

To overcome this problem, we recently introduced nanoparticles (NP) formulated from the bioabsorbable polymer poly(DL-lactide-co-glycolide) (PLGA) and succeeded in formulating an NP-eluting stent by a cationic electrodeposition coating technology²². Therefore, we hypothesized that statin-NP-eluting stents could be an innovative therapeutic “anti-healing” strategy for treating in-stent stenosis *in vivo*.

Materials and Methods

Statins (simvastatin, pitavastatin, atorvastatin, rosuvastatin, fluvastatin, and pravastatin) were purchased, extracted from products, and purified.

Human Coronary Artery Smooth Muscle Cell Proliferation

Human coronary artery smooth muscle cells (SMC) were cultured as previously described^{22, 23} and plated into 96-well culture plates at 1×10^4 cells per well in SMGM2. Proliferation was stimulated by adding human PDGF at 10 ng/mL (Sigma, Tokyo, Japan) or 10% FBS to each well. Either vehicle alone or several concentrations of statins, sirolimus, or paclitaxel were added to the well. Cellular proliferation responses

were determined by evaluating the 5'-bromo-2'-deoxyuridine (BrdU) incorporation rate and/or by a cell counting method, as previously described^{22, 23}.

Human Endothelial Cell Scratch Motility Assay

A scratch motility assay using human umbilical vein endothelial cells (HUVEC) was performed to assess the re-endothelialization response following endothelial denudation. HUVECs were seeded in collagen I-coated 24-well plates at a density of 2×10^4 cells per well and grown to confluence with EGM2. The monolayers were wounded with a pipet tip, washed with PBS twice, photographed and incubated at 37°C with EBM containing 0.5% BSA containing VEGF₁₆₅ (10 ng/mL, R & D) in the presence or absence of statins, sirolimus, or paclitaxel. After five hours, cells were photographed, and the number of cells that had migrated into the wounded area was counted.

Western Blot Analysis

Human aortic endothelial cells (HAEC) were cultured to confluence in 3 cm dishes and rendered quiescent for 24 hours before stimulation with 1 U/mL thrombin (Sigma). Sirolimus, paclitaxel (both Sigma), and pitavastatin were added to the cells one hour before thrombin (1 U/mL) stimulation. Tissue factor protein expression was determined by Western blot analysis. In another set of experiments, sirolimus was added to HAEC and effects of pitavastatin on sirolimus-induced changes in endothelial nitric oxide synthase (eNOS) and protein kinase B (Akt) protein were examined.

Cell extracts (20 µg) were resolved on 10% reducing SDS-PAGE gels and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Antibodies against human tissue factor (Calbiochem), phosphorylated-Akt (ser473), phosphorylated-eNOS (ser1177), Akt (Cell Signaling), eNOS (Affinity BioReagents) were used. Immune complexes were visualized with horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) using the ECL Plus system (Amersham Biosciences) and were detected with the ECL Detection Kit (Amersham). Blots were normalized against GAPDH expression (Sigma).

Preparation of Cationic PLGA Nanoparticles (NP) with Surface Modification with Chitosan

A lactide/glycolide copolymer (PLGA) with an average molecular weight of 20,000 and a lactide to glycolide copolymer ratio of 75:25 (PLGA7520; Wako Pure Chemical Industries, Osaka, Japan) was used as wall material for the NPs because the bioabsorption

half-life of this product is two weeks in rat tissue (manufacturer's instructions). PLGA NPs incorporated with the fluorescent marker fluorescein isothiocyanate (FITC; Dojindo laboratories, Kumamoto, Japan) or with pitavastatin were prepared by a previously reported emulsion solvent diffusion method in purified water²². FITC- and pitavastatin-loaded PLGA NPs contained 5.0% (w/v) FITC and 6.5% (w/v) pitavastatin, respectively, and were preserved as freeze-dried material. The mean particle size was analyzed by the light scattering method (Microtrack UPA150; Nikkiso, Tokyo, Japan). The average diameter of the PLGA NPs was 226 ± 29 nm. The surface charge (zeta potential) was also analyzed by Zetasizer Nano (Sysmex, Hyogo, Japan) and was found to be cationic ($+36$ mV at pH 4.4).

Preparation of NP-Eluting Stents by Cationic Electrodeposition Coating Technology

The 15 mm-long stainless-steel, balloon-expandable stents (Multilink) were ultrasonically cleaned in acetone, ethanol, and demineralized water. The cationic electrodeposited coating was prepared on cathodic stents in NP solution at a concentration of 5 g/L in distilled water with a current maintained between 2.0 and 10.0 mA by a direct current power supply (DC power supply; Nippon Stabilizer Co, Tokyo, Japan) for different periods under sterile conditions.^{28, 31} The coated stents were then rinsed with demineralized water and dried under a vacuum overnight. This electrodeposition coating procedure produced a coating of 367 ± 77 μ g of the PLGA NPs per stent and 20 ± 4 μ g of pitavastatin per stent ($n=12$). The surfaces of some NP-coating stents were examined with scanning electron microscopy (JXM8600; JEOL, Tokyo, Japan), and it was confirmed that the NPs were structurally intact and cohesive²².

Analysis of Endothelial Surface Coverage by en Face Scanning Electron Microscopy

For evaluation of endothelial coverage, stents were excised seven days after implantation. The stented arteries were fixed *in situ* with 10% neutral-buffered formalin after perfusion with lactated Ringer's solution to remove blood. The samples were further fixed by immersion and then bisected longitudinally with one half processed for scanning electron microscopy (SEM).

Composites of serial en face SEM images acquired at low power ($\times 15$ magnification) were digitally assembled to provide a complete view of the entire luminal stent surface. The images were further enlarged ($\times 200$

magnification), allowing direct visualization of endothelial cells. The extent of endothelial surface coverage above and between stent struts was traced and measured by morphometry software. The results are expressed as a percentage of the total surface area above or between struts or the total and percentage area lacking coverage at each repeated crown along the longitudinal axis from the proximal to the distal orientation. Endothelial cells were identified as sheets of spindle- or polygonal-shaped monolayers in close apposition, a distinguishing feature from other cell types in en face preparations²⁴. By contrast, intimal smooth muscle cells showed elongated processes and were generally stacked in disorganized or haphazard layers²⁴. Other adherent cells present on stent surfaces included platelets, characteristically 1 to 2 μ m in size with an irregular discoid appearance, and inflammatory cells, which were round and varied from 7 to 10 μ m in diameter with a ruffled surface. Struts uncovered by endothelium were completely bare or contained thrombi consisting of focal platelet and fibrin aggregates intermixed with red blood cells and inflammatory cells.

Measurements of Pitavastatin Concentration in Serum and Arterial Tissue

Concentrations of pitavastatin in serum and stented arterial tissue were measured at predetermined time points using a column-switching high performance liquid chromatography (HPLC) system as previously reported²⁵. Briefly, the column-switching HPLC system consists of two LC-10AD pumps, a SIL-10A auto-sampler, a CTO-10A column oven, a six-port column-switching valve, and an SPD-10A UV-detector (all from Shimadzu, Kyoto, Japan). The column temperature was maintained at 40°C. Pre-prepared serum or tissue homogenate sample solutions were injected from the auto-sampler into the HPLC system, and statin in the sample solutions was detected at 250 nm with a UV detector. The detected peak area was measured with Lc solution software (Shimadzu, Kyoto, Japan).

Animal Preparation and Stent Implantation

All *in vivo* experiments were reviewed and approved by the Committee on Ethics in Animal Experiments, Kyushu University Faculty of Medicine, according to the Guidelines of the American Physiological Society.

Domestic male pigs (Kyudo, Tosu, Japan; aged 2 to 3 months and weighing 25 to 30 kg) received orally aspirin (330 mg/day) and ticlopidine (200 mg/day) until euthanasia from 3 days before stent implantation

Table 1. Inhibitory effects of 6 commercially available statins on proliferation of human coronary artery smooth muscle cells

	IC ₅₀ values (nmol/L)	efficacy ratio	95% Wald confidence intervals	<i>p</i> value
pitavastatin	193	1	—	—
fluvastatin	836	0.230	0.119, 0.446	<0.001
atorvastatin	2512	0.077	0.039, 0.150	<0.001
simvastatin	3951	0.049	0.023, 0.104	<0.001
rosuvastatin	Not calculated	Not calculated	Not calculated	Not calculated
pravastatin	Not calculated	Not calculated	Not calculated	Not calculated

n = 6 each. *p* values versus pitavastatin by Wald tests in 4-parameter logistic regression model.

procedure. Animals were anesthetized with ketamine hydrochloride (15 mg/kg, IM) and pentobarbital (20 mg/kg, IV). They were then intubated and mechanically ventilated with room air. A preshaped Judkins catheter was inserted into the carotid artery and advanced into the orifice of the left coronary artery. After systemic heparinization (100 IU/kg) and intracoronary administration of nitroglycerin, coronary angiography of the left coronary artery was performed with the use of contrast media (iopamidol 370) in a left oblique view with the angiography system (Toshiba Medical, Tokyo, Japan).

Animals were divided into groups, which underwent deployment of either non-coated bare metal stents (1 week: *n* = 3, 4 weeks: *n* = 12), FITC-incorporated NP-eluting stents (4 weeks: *n* = 12), pitavastatin-incorporated NP-eluting stents (1 week: *n* = 3, 4 weeks: *n* = 12), or sirolimus-eluting stents (Cypher; 3 mm × 15 mm) (1 week: *n* = 3, 4 weeks: *n* = 12) in the left anterior descending (LAD) or the left circumflex (LCx) coronary arteries. After arterial blood samples were taken, animals were given a lethal dose of anesthesia after 1 or 4 weeks, and the stented arterial sites and contralateral non-stented sites were excised for biochemical, immunohistochemical, and morphometric analyses.

As another set of experiments, animals were treated with intracoronary administration of pitava-NP at 300 μg (a similar dose to that coated on pitavastatin-NP eluting stent, 4 weeks: *n* = 6) and 3000 μg (10x dose coated on pitavastatin-NP eluting stent, 4 weeks: *n* = 6) containing 20 and 200 μg pitavastatin, respectively, immediately after deployment of bare metal stents. Pitava-NPs were diluted with 10 mL saline. Intracoronary administration of 10 mL saline was used as a control experiment (4 weeks: *n* = 5).

A segment with a mean coronary diameter of 2.5 mm was selected by using quantitative coronary angiography with a stent-to-artery ratio of approximately

1.1 : 1.2. A balloon catheter mounted with a stent was then advanced to the pre-selected coronary segments for deployment over a standard guide-wire. The balloon catheter was inflated at 15 atm for 60 seconds once and was then slowly withdrawn, leaving the stent in place.

Quantitative coronary angiography (Toshiba Medical, Tokyo, Japan) was performed before, immediately after, and 4 weeks after stent implantation to examine the coronary arterial diameter at stented and non-stented sites. The image of a Judkins catheter was used as reference diameter. Arterial pressure, heart rate, and ECG were continuously monitored and recorded on a recorder.

Histopathological Study

Four weeks after the coronary angiographic study, animals were euthanized with a lethal dose of sodium pentobarbital (40 mg/kg intravenously), and histological analysis was performed. The left coronary artery was perfused with 10% buffered formalin at 120 mmHg and fixed for 24 hours. The stented artery segments were isolated and processed as described previously²⁶. The segment was divided into two parts at the center of the stent and then embedded in methyl methacrylate mixed with n-butyl methacrylate to allow for sectioning through the metal stent struts. Serial sections were stained with elastica van Gieson and hematoxylin-eosin (HE). The neointimal area, the area within the internal elastic lamina (IEL), and the lumen area were measured by computerized morphometry, which was carried out by a single observer who was blinded to the experimental protocol. All images were captured by an Olympus microscope equipped with a digital camera (HC-2500) and were analyzed using Adobe Photoshop 6.0 and Scion Image 1.62 software. The injury, inflammation, fibrin, hemorrhage and re-endothelialization scores were determined at each strut site, and mean values were calculated for each stented segment.

Statistical Analysis

Data are expressed as the means $\pm$ SE. Statistical analysis of differences between two groups was performed with the unpaired *t*-test, and differences among groups were analyzed using ANOVA and multiple comparison tests.

Efficacy ratios (IC₅₀ values) of the statins were tested using Wald tests in a four-parameter logistic regression model. Point estimates and Wald 95% confidence intervals for efficacy ratios were calculated. Statistical calculations were performed with SAS pre-clinical package software version 9.1.3 (SAS Institute Inc., Japan, Tokyo). *P* values < 0.05 were considered significant.

Results

Effects of Statins on Human Coronary Artery Smooth Muscle Cell Proliferation and Endothelial Cell Scratch Motility Assay

To incorporate statins into the NP-eluting stent design, the effects of statins were compared. In the human CASC proliferation assay (% inhibition of BrdU index), hydrophilic statins (rosuvastatin and pravastatin) had no effects on PDGF-induced proliferation; thus, calculation of the IC₅₀ value was impossible for those statins (Table 1). By contrast, the other four statins showed concentration-dependent inhibition. The IC₅₀ values of the four statins are shown in Table 1, which indicates that the value for pitavastatin is lowest.

In the human endothelial cell scratch motility assay (re-endothelialization *in vitro*), only pitavastatin increased the re-endothelialization response following scratch injury; the other five statins showed no such effects (Fig. 1). In addition, there was no difference in the re-endothelialization response among the five statins.

Effects of Pitavastatin, Sirolimus, and Paclitaxel on Human Coronary Artery Smooth Muscle Cell Proliferation, Endothelial Cell Scratch Motility Assay, and Tissue Factor Expression in Human Endothelial Cells

To incorporate pitavastatin into the NP-eluting stent design, the effects of pitavastatin were then compared with those of sirolimus and paclitaxel. All three drugs inhibited human CASC proliferation (Fig. 2). In the human endothelial cell scratch motility assay, sirolimus did not affect the re-endothelialization response, whereas the response was significantly delayed in paclitaxel-treated endothelial cells (Fig. 1).

Because tissue factor has a primary role in stent

Fig. 1. Human umbilical vein endothelial cell scratch motility assay *in vitro*. Vertical axis denotes the number of migrated cells into the scratched area. **p* < 0.05 vs no treatment by one-way ANOVA followed with Dunnett's multiple comparison test (*n* = 6–8 each).

thrombosis, its protein expression was examined in human endothelial cells. As previously reported by others²⁷⁾, both sirolimus and paclitaxel enhanced the thrombin-induced tissue factor expression (Fig. 2). By contrast, pitavastatin inhibited tissue factor expression (Fig. 2).

Effects of Pitavastatin on Sirolimus-Induced Down Regulation of eNOS in Human Endothelial Cells

Sirolimus down regulated phosphorylated-eNOS and decreased phosphorylated Akt (a representative down stream signal of eNOS), while sirolimus had no effects on eNOS and Akt protein expression (Fig. 3).

Fig. 2. Effects of pitavastatin, sirolimus, and paclitaxel on human CASC proliferation and human AEC tissue factor expression.

A, PDGF-induced proliferation (% increase in cell number) of human CASCs. Data are the mean $\pm$ SEM ($n=6$ each). * $p<0.01$ versus control by one-way ANOVA followed with Dunnett's multiple comparison test. B, Thrombin-induced tissue factor protein expression in human AECs. Data are the mean $\pm$ SEM ($n=6$ each). * $p<0.01$ versus thrombin alone by one-way ANOVA followed with Dunnett's multiple comparison test.

Pitavastatin nearly normalized the sirolimus-induced decrease in p-eNOS and p-Akt protein expression.

Effects of Pitavastatin and Pitavastatin-NP on Human CASC Proliferation

Both pitavastatin and pitavastatin-NP inhibited the FBS-induced proliferation of human CASC. The inhibitory activities of pitavastatin-NP were greater than those of 0.01, 0.1, and 1 μ M pitavastatin only (Fig. 4).

Effects of Pitavastatin-NP-Eluting Stents and Sirolimus-Eluting Stents on Neointima Formation Four Weeks after Stent Implantation

Two animals in the control bare metal stent

group died suddenly between weeks three and four; therefore, these animals were excluded from angiographic and histopathological analyses, which were performed in 34 pigs (10 in the control bare metal stent group, 12 in the FITC-NP-eluting stent group, 12 in the pitavastatin-NP eluting stent group, and 12 in the sirolimus-eluting stent group).

Quantitative coronary arteriography revealed that (1) there was no significant difference in the coronary diameter before and immediately after stent implantation or in the stent-to-artery ratio among the four groups and (2) the coronary diameter was less in the control bare metal and the FITC-NP-eluting stent sites than in the pitavastatin-NP-eluting stent and sirolimus-eluting stent sites four weeks after stenting.

Fig. 3. Effects of pitavastatin on sirolimus-induced down regulation of eNOS and Akt (a representative down stream signal of eNOS) in human aortic endothelial cells. Pitavastatin normalized sirolimus-induced decrease in phosphorylated (activated) eNOS and Akt. Data are the mean $\pm$ SEM ($n=6$ each), $p<0.05$ or 0.01 versus no treatment by one-way ANOVA followed with Dunnett's multiple comparison test.

(Table 2). Thus, the angiographically examined in-stent stenosis was less in the pitavastatin-NP group than in the control and FITC-NP groups. The sirolimus-eluting stent showed similar inhibitory effects on markers of in-stent stenosis.

Histological analysis demonstrated that a significant in-stent neointima formed similarly at the bare metal stent and FITC-NP-eluting stent sites (Fig. 5). Quantitative analysis demonstrated that pitavastatin-NP-eluting stents attenuated in-stent neointima formation as effectively as sirolimus-eluting stents.

Effects of Pitavastatin-NP-Eluting Stents Versus Sirolimus-Eluting Stents on Inflammation and Fibrin Deposition

Micrographs of coronary artery cross-sections stained with hematoxylin-eosin from FITC-NP-eluting, pitavastatin-NP-eluting, and sirolimus-eluting stent groups are shown in Fig. 6. A semi-quantitative histological scoring system demonstrated that there was no significant difference in the injury score among the four groups four weeks after stenting (Table 3).

Inflammatory cells consisting mainly of macrophages were recruited around the stent wire and into the neointimal layer in all four sites; however, the inflammation score was significantly less at the pitavastatin-NP-eluting stent site than in the bare metal stent site (Table 3). At the sirolimus-eluting stent site, the inflammation score was greater than at the bare metal stent and pitavastatin-NP-eluting stent

FBS-induced proliferation of human coronary artery SMCs (cell number/well)

Fig. 4. Effects of pitavastatin and pitavastatin-NP on the FBS-induced proliferation of human CSMCs. Data are the mean $\pm$ SEM ($n=6$ each). * $p<0.01$ versus no treatment by one-way ANOVA followed with Dunnett's multiple comparison test.

sites (Table 3).

Minor fibrin deposition was observed at bare metal, FITC-NP-eluting, and pitavastatin-NP-eluting stent sites (Table 3). At the sirolimus-eluting stent site, the fibrin score was significantly higher than at the bare metal stent site, and this difference was even more significant when compared with the pitavastatin-NP-eluting stent site.

To determine whether the therapeutic effects of

Table 2. Coronary angiographic parameters (coronary artery diameter, in-stent stenosis) before, immediately after, and 4 weeks after stent implantation in porcine coronary artery

	BMS (<i>n</i> = 10)	FITC-NP stent (<i>n</i> = 12)	Pitavastatin -NP stent (<i>n</i> = 12)	SES (<i>n</i> = 12)	<i>p</i> value
Coronary diameter before stent implantation	2.19 ± 0.04	2.25 ± 0.05	2.41 ± 0.06	2.42 ± 0.10	NS
Coronary diameter immediately after stent implantation	2.70 ± 0.06	2.75 ± 0.05	2.79 ± 0.03	2.89 ± 0.04	NS
Stent-to-artery ratio immediately after stent implantation	1.20 ± 0.02	1.22 ± 0.03	1.13 ± 0.02	1.19 ± 0.04	NS
Coronary diameter 4 weeks after stent implantation	1.45 ± 0.13	1.51 ± 0.10	2.02 ± 0.12*	2.02 ± 0.15*	0.0025
Angiographically-examined in-stent restenosis 4 weeks after stent implantation (% diameter stenosis)	42.9 ± 4.9	46.0 ± 3.4	25.8 ± 3.5*	27.2 ± 3.7*	0.0003

Data are the mean ± SEM. NS = not significant

**p* < 0.05 versus control bare metal stent by Bonferroni's multiple comparison tests

the pitavastatin-NP-eluting stent are mediated by local or systemic mechanisms, effects of intracoronary administration of pitavastatin-NP on histopathological features after deployment of bare metal stents were examined (Table 4). Pitavastatin-NP at 300 µg showed no therapeutic effects on indices of in-stent stenosis and adverse effects on histopathological scoring. Pitavastatin-NP at 3000 µg also had no effects on indices of in-stent stenosis, but modestly but significantly decreased fibrin deposition.

Effects of Pitavastatin-NP-Eluting Stents Versus Sirolimus-Eluting Stents on Endothelial Surface Coverage

As previously reported, re-endothelialization was not impaired at sirolimus-eluting stent sites four weeks post-stenting. There was no significant difference in the re-endothelialization score among the four groups four weeks after stenting (Table 3); therefore, the endothelial surface coverage was examined at earlier time points (seven days post-stenting) by scanning electron microscopy. At bare metal stent sites, almost complete endothelial coverage was noted above and between stent struts at seven days (Fig. 7). Similar magnitudes of endothelial coverage were observed at FITC-NP-eluting stent and pitavastatin-NP-eluting stent sites. In contrast, endothelial coverage was impaired at sirolimus-eluting stent sites; regions lacking endothelial coverage were generally associated with platelet aggregation and adherent leukocytes.

Serum and Tissue Concentrations of Pitavastatin

Tissue concentrations of pitavastatin were below the limit of detection (2.5 ng/g protein) in coronary artery segments three hours after the deployment of pitavastatin-NP-eluting stents. In addition, serum levels of pitavastatin were below the limit of detection (1 ng/mL) one and three hours after the deployment of pitavastatin-NP-eluting stents.

Discussion

We here report the first successful development of pitavastatin-NP-eluting stents with a newly invented cationic electrodeposition coating technology. We previously showed that (1) PLGA-NP was taken up actively by VSMC and endothelial cells mainly via endocytosis and was retained stably in the intracellular space and that (2) NPs may slowly release encapsulated drugs as PLGA is hydrolyzed^{22, 23}). This bioabsorbable polymeric NP-eluting stent system has unique aspects with respect to vascular compatibility and efficient drug delivery (stable delivery of NPs into the neointima and medial layers until day 28 after deployment of the NP-eluting stent), as compared to dip-coated polymer-eluting stents²²). Importantly, this NP drug delivery system can carry hydrophilic agents such as statins, which offer advantages over the current stent-coating technology. In addition, this NP-eluting stent system provided an effective means of delivering NP-incorporated drugs or genes that target intracellular proteins involved in the pathogenesis of

Fig. 5. Histopathological analysis of in-stent neointima formation four weeks after stent implantation.

A, Coronary artery cross-sections stained with hematoxylin-eosin from bare metal stent (BMS), FITC-NP-eluting, pitavastatin-NP-eluting, and sirolimus-eluting stent (SES) groups. Bar = 0.5 mm. B, Neointima area (mm²) and % stenosis [$100 \times (\text{area of internal elastic lamia} - \text{neointima area}) / \text{area of internal elastic lamia}$] in bare metal stent ($n = 10$), FITC-NP-eluting stent ($n = 12$), pitavastatin-NP-eluting stent ($n = 12$) stent, and sirolimus-eluting stent ($n = 12$) groups.

in-stent neointima formation; therefore, this NP-eluting stent system may work as an excellent platform for delivering therapeutic agents.

The pleiotropic (non-LDL-related) vasculoprotective effects of statins are mediated through reduced levels of cholesterol biosynthesis intermediates that serve as lipid attachments for posttranslational modifi-

cations (isoprenylation) of proteins, including Rho and Rac⁸). In the present study, pitavastatin was selected as the nanoparticulation compound because this compound elicited (1) the most potent inhibitory effects on human VSMC proliferation (**Table 1**), and (2) the greatest effects on the re-endothelialization response of human endothelial cells and on the inhibition of tis-

Fig. 6. Representative low- (left panels) and high-magnification (right panels) micrographs of coronary artery cross-sections stained with hematoxylin-eosin after four weeks from FITC-NP-eluting, pitavastatin-NP-eluting, and sirolimus-eluting stent groups.

sue factor expression (**Fig. 1** and **2**). A possible explanation for the discrepancy in the effect of VSMC proliferation between hydrophilic and lipophilic statins is that, under *in vitro* conditions, hydrophilic statins are hardly incorporated into vascular tissues or vascular smooth muscle cells while lipophilic statins are more widely taken up by vascular tissues and cells via passive diffusion. Pitavastatin normalized sirolimus-induced inhibition of eNOS activity in human endothelial cells (**Fig. 3**). In addition, pitavastatin-NP was as effective as non-nanoparticulated pitavastatin at an approximately 100-fold lower dose for inhibition of VSMC proliferation (**Fig. 4**). Collectively, these *in vitro* data suggest that the NP-mediated delivery of

pitavastatin may be more effective than pitavastatin alone in inhibiting VSMC proliferation and tissue factor expression and in promoting re-endothelialization.

We previously reported the central role of monocyte-mediated inflammation in the pathogenesis of in-stent neointima formation²⁸⁻³¹ and the formulation of polymeric gene-eluting stents or nuclear factor kappa-B decoy, inhibiting in-stent stenosis^{28, 32}; however, although advanced polymer technology was used, we were not able to formulate appropriate statin coating on metallic stents (authors' unpublished observation). An important finding of this study is that pitavastatin-NP-eluting stents attenuated in-stent stenosis (neointima formation) as effectively as sirolimus-eluting

Table 3. Re-endothelialization, injury score, inflammation score, and fibrin score 4 weeks after stenting

	BMS (n = 10)	FITC-NP stent (n = 12)	Pitavastatin -NP stent (n = 12)	SES (n = 12)	p value
Re-endothelialization score	2.70 ± 0.15	2.83 ± 0.11	2.83 ± 0.11	2.08 ± 0.43	NS
Injury score	1.31 ± 0.03	1.39 ± 0.07	1.36 ± 0.05	1.42 ± 0.11	NS
Inflammation score	1.65 ± 0.14	1.64 ± 0.07	1.08 ± 0.14*	2.38 ± 0.12**	p < 0.0001
Fibrin score	0.30 ± 0.15	0.58 ± 0.19	0.65 ± 0.18	2.00 ± 0.21**	p < 0.0001

Data are the mean ± SEM. * $p < 0.05$, ** $p < 0.01$ versus control bare metal stent by Kruskal-Wallis test followed with non-parametric Dunn's multiple comparison tests. NS: Not Significant.

The re-endothelialization score was defined as the extent of the circumference of the arterial lumen covered by endothelial cells and was scored from 1 to 3 (1 = 25%; 2 = 25% to 75%; 3 = >75%).

The injury score was determined at each strut site, and mean values were calculated for each stented segment. In brief, a numeric value from 0 (no injury) to 3 (most injury) was assigned: 0 = endothelial denudate, internal elastica lamina (IEL) intact; 1 = IEL lacerated, media compressed, not lacerated; 2 = IEL lacerated, media lacerated, external elastica lamina (EEL) compressed, not lacerated; and 3 = media severely lacerated, EEL lacerated, adventitial may contain stent strut. The average injury score for each segment was calculated by dividing the sum of injury scores by the total number of struts in the examined section.

The inflammation score took into consideration the extent and density of the inflammatory infiltrate in each individual strut. With regard to the inflammatory score for each individual strut, the grading is: 0 = no inflammatory cells surrounding the strut; 1 = light, non-circumferential inflammatory cell infiltrate surrounding the strut; 2 = localized, moderate to dense cellular aggregate surrounding the strut non-circumferentially; and 3 = circumferential dense inflammatory cell infiltration of the strut. The inflammatory score for each cross section was calculated in the same manner as for the injury score (sum of the individual inflammatory scores, divided by the number of struts in the examined section).

The intimal fibrin content was graded as 0, no residual fibrin; 1, focal regions of residual fibrin involving any portion of the artery or moderate fibrin deposition adjacent to the strut involving <25% of the circumference of the artery; 2, moderate fibrin involving >25% of the circumference of the artery or heavy deposition involving <25% of the circumference of the artery; or 3, heavy fibrin deposition involving >25% of the circumference of the artery.

Table 4. Effects of intracoronary saline or Pitava-NP on histopathological morphometry and scoring 4 weeks after stenting

	saline (n = 5)	Pitavastatin-NP 300 μ g (n = 6)	Pitavastatin-NP 3000 μ g (n = 6)	p value
Morphometry at stent sites				
Lumen area	2.88 ± 0.25	2.29 ± 1.01	2.72 ± 0.88	NS
Media area	0.95 ± 0.12	1.25 ± 0.32	0.92 ± 0.13	NS
Neointimal area	1.80 ± 0.14	2.30 ± 0.93	1.92 ± 0.88	NS
Scores at stent sites				
Injury score	1.00 ± 0.0	1.22 ± 0.13	1.04 ± 0.05	NS
Inflammation score	1.33 ± 0.0	1.47 ± 0.46	1.23 ± 0.08	NS
Fibrin score	0.75 ± 0.09	0.74 ± 0.27	0.39 ± 0.16*	p < 0.05
Endothelization score	2.00 ± 0.0	1.80 ± 0.27	1.98 ± 0.03	NS

Data are the mean ± SEM. * $p < 0.05$ versus control bare metal stent by Kruskal-Wallis test followed with non-parametric Dunn's multiple comparison tests. NS: not significant.

stents in a porcine coronary artery model. It is likely that local mechanisms are involved in the mechanism of the therapeutic effects of pitavastatin-NP eluting stents, because intracoronary administration of pitavastatin-NP at 300 μ g (an equivalent dose to that coated on pitavastatin-NP-eluting stent) showed no effects. A prior study reported that oral administration of pitavastatin at 40 mg/body per day for five weeks inhibited in-stent stenosis in a porcine coronary artery model¹². The estimated dose of pitavastatin loaded on our NP-eluting stent was 20 ± 4 μ g/stent, which is

much lower than the cumulative systemic dose used in a prior study (40 × 35 days = 1350 mg/body)¹². We measured the local tissue concentrations of pitavastatin with HPLC and found them to be under the limit of detection immediately after the deployment of the pitavastatin-NP-eluting stent; therefore, the local concentration of pitavastatin after deployment of the pitavastatin-NP-eluting stent is unclear, but is less than the limit of detection (2.5 ng/g protein or 1 ng/mL).

Delayed endothelial healing effects characterized

Fig. 7. Endothelial surface coverage analysis by scanning electron microscopy.

A, Representative micrographs of the endothelial surface by scanning electron microscopy one week after stent implantation. B, Endothelial coverage score one week after stent implantation in bare metal stent (BMS), FITC-NP-eluting stent (FITC-NP), pitavastatin-NP-eluting stent (Pitava-NP) stent, and sirolimus-eluting stent (SES) groups ($n = 3$ each).

by impaired re-endothelialization and persistence of fibrin and inflammation are an important histopathological feature in arteries exposed to currently marketed DESs in experimental animals^{33, 34)} and in humans⁴⁻⁶⁾. These delayed endothelial healing effects are presumed to result from the effects of drugs coated on DESs (see Introduction). Using human pathological data from autopsy patients in whom death occurred >30 days after drug-eluting stent placement, Finn *et al.*⁶⁾ dem-

onstrated that endothelialization of the stent was the best predictor of late in-stent thrombosis. Heterogeneity of healing is a common finding in cases of late drug-eluting stent thrombosis. In this study, we confirmed the delayed endothelial healing effects of sirolimus-eluting stents in porcine coronary arteries (**Fig. 5, 6, and 7**) reported by Virmani's group⁵⁾; however, we found that pitavastatin-NP-eluting stents had no such delayed endothelial healing effects in porcine coronary

arteries *in vivo*. The opposing effects of pitavastatin-NP-eluting stents and sirolimus-eluting stents might be explained by our *in vitro* study (Fig. 1 and 2). Collectively, these data on anti-endothelial healing properties suggest that pitavastatin-NP-eluting stents may have an advantage over currently marketed polymeric DES devices. Future studies are needed to prove this point.

A major limitation of this study is that this study was performed in normal pigs without pre-existing atherosclerotic coronary lesions, although this porcine coronary artery model is regarded as an appropriate and standard preclinical study model³⁵. A long-term efficacy study is also needed.

In conclusion, pitavastatin-NP-eluting stents not only attenuated in-stent stenosis as effectively as polymer-coated sirolimus-eluting stents but also elicited endothelial healing effects in a porcine coronary artery model. Delayed endothelial healing is a common feature at atherosclerotic arterial sites deployed with currently marketed DESs, which may lead to an increased risk of late stent thrombosis. Our present technology can be one approach to overcome the adverse effects of current DESs and may provide the optimal anti-healing combination: the inhibition of VSMC proliferation to decrease restenosis and the promotion of re-endothelialization to protect from late stent thrombosis. This NP-eluting stent system may be developed as an innovative platform for the future treatment of atherosclerotic vascular disease.

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Conflict of Interest

Dr. Egashira holds a patent on the results reported in the present study. The remaining authors report no conflicts.

References

- 1) Laskey WK, Yancy CW, Maisel WH: Thrombosis in coronary drug-eluting stents: Report from the meeting of the circulatory system medical devices advisory panel of the food and drug administration center for devices and radiologic health, december 7-8, 2006. *Circulation*, 2007; 115: 2352-2357
- 2) Serruys PW, Kutryk MJ, Ong AT: Coronary-artery stents. *N Engl J Med*, 2006; 354: 483-495
- 3) Shuchman M: Trading restenosis for thrombosis? New questions about drug-eluting stents. *N Engl J Med*, 2006; 355: 1949-1952
- 4) Luscher TF, Steffel J, Eberli FR, Joner M, Nakazawa G, Tanner FC, Virmani R: Drug-eluting stent and coronary thrombosis: Biological mechanisms and clinical implications. *Circulation*, 2007; 115: 1051-1058
- 5) Finn AV, Nakazawa G, Joner M, Kolodgie FD, Mont EK, Gold HK, Virmani R: Vascular responses to drug eluting stents. Importance of delayed healing. *Arterioscler Thromb Vasc Biol*, 2007; 27: 1500-1510
- 6) Finn AV, Joner M, Nakazawa G, Kolodgie F, Newell J, John MC, Gold HK, Virmani R: Pathological correlates of late drug-eluting stent thrombosis: Strut coverage as a marker of endothelialization. *Circulation*, 2007; 115: 2435-2441
- 7) Joner M, Nakazawa G, Finn AV, Quee SC, Coleman L, Acampado E, Wilson PS, Skorija K, Cheng Q, Xu X, Gold HK, Kolodgie FD, Virmani R: Endothelial cell recovery between comparator polymer-based drug-eluting stents. *J Am Coll Cardiol*, 2008; 52: 333-342
- 8) Takemoto M, Liao JK: Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol*, 2001; 21: 1712-1719
- 9) Eto M, Kozai T, Cosentino F, Joch H, Luscher TF: Statin prevents tissue factor expression in human endothelial cells: Role of rho/rho-kinase and akt pathways. *Circulation*, 2002; 105: 1756-1759
- 10) Thyberg J: Re-endothelialization via bone marrow-derived progenitor cells: Still another target of statins in vascular disease. *Arterioscler Thromb Vasc Biol*, 2002; 22: 1509-1511
- 11) Werner N, Priller J, Laufs U, Endres M, Bohm M, Dirnagl U, Nickenig G: Bone marrow-derived progenitor cells modulate vascular reendothelialization and neointimal formation: Effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition. *Arterioscler Thromb Vasc Biol*, 2002; 22: 1567-1572
- 12) Yokoyama T, Miyauchi K, Kurata T, Satoh H, Daida H: Inhibitory efficacy of pitavastatin on the early inflammatory response and neointimal thickening in a porcine coronary after stenting. *Atherosclerosis*, 2004; 174: 253-259
- 13) Indolfi C, Cioppa A, Stabile E, Di Lorenzo E, Esposito G, Pisani A, Leccia A, Cavuto L, Stingone AM, Chieffo A, Capozzolo C, Chiariello M: Effects of hydroxymethylglutaryl coenzyme a reductase inhibitor simvastatin on smooth muscle cell proliferation in vitro and neointimal formation in vivo after vascular injury. *J Am Coll Cardiol*, 2000; 35: 214-221
- 14) Scheller B, Schmitt A, Bohm M, Nickenig G: Atorvastatin stent coating does not reduce neointimal proliferation after coronary stenting. *Zeitschrift fur Kardiologie*, 2003; 92: 1025-1028
- 15) Miyauchi K, Kasai T, Yokoyama T, Aihara K, Kurata T, Kajimoto K, Okazaki S, Ishiyama H, Daida H: Effectiveness of statin-eluting stent on early inflammatory response

- and neointimal thickness in a porcine coronary model. *Circ J*, 2008; 72: 832-838
- 16) Serruys PW, Foley DP, Jackson G, Bonnier H, Macaya C, Vrolix M, Branzi A, Shepherd J, Suryapranata H, de Feyter PJ, Melkert R, van Es GA, Pfister PJ: A randomized placebo-controlled trial of fluvastatin for prevention of restenosis after successful coronary balloon angioplasty; final results of the fluvastatin angiographic restenosis (flare) trial. *Eur Heart J*, 1999; 20: 58-69
 - 17) Bertrand ME, McFadden EP, Fruchart JC, Van Belle E, Commeau P, Grollier G, Bassand JP, Machecourt J, Casagnes J, Mossard JM, Vacheron A, Castaigne A, Danchin N, Lablanche JM: Effect of pravastatin on angiographic restenosis after coronary balloon angioplasty. The predict trial investigators. Prevention of restenosis by elisor after transluminal coronary angioplasty. *J Am Coll Cardiol*, 1997; 30: 863-869
 - 18) Weintraub WS, Bocuzzi SJ, Klein JL, Kosinski AS, King SB, 3rd, Ivanhoe R, Cedarholm JC, Stillabower ME, Talley JD, DeMaio SJ, et al: Lack of effect of lovastatin on restenosis after coronary angioplasty. Lovastatin restenosis trial study group. *N Engl J Med*, 1994; 331: 1331-1337
 - 19) Onaka H, Hirota Y, Kita Y, Tsuji R, Ishii K, Ishimura T, Kawamura K: The effect of pravastatin on prevention of restenosis after successful percutaneous transluminal coronary angioplasty. *Jpn Circ J*, 1994; 58: 100-106
 - 20) Bae JH, Bassenge E, Kim KY, Synn YC, Park KR, Schwemmer M: Effects of low-dose atorvastatin on vascular responses in patients undergoing percutaneous coronary intervention with stenting. *J Cardiovasc Pharmacol Ther*, 2004; 9: 185-192
 - 21) Petronio AS, Amoroso G, Limbruno U, Papini B, De Carlo M, Micheli A, Ciabatti N, Mariani M: Simvastatin does not inhibit intimal hyperplasia and restenosis but promotes plaque regression in normocholesterolemic patients undergoing coronary stenting: A randomized study with intravascular ultrasound. *Am Heart J*, 2005; 149: 520-526
 - 22) Nakano K, Egashira K, Masuda S, Funakoshi K, Zhao G, Kimura S, Matoba T, Sueishi K, Endo Y, Kawashima Y, Hara K, Tsujimoto H, Tominaga R, Sunagawa K: Formulation of nanoparticle-eluting stents by a cationic electro-deposition coating technology efficient nano-drug delivery via bioabsorbable polymeric nanoparticle-eluting stents in porcine coronary arteries. *JACC Cardiovasc Interv*, 2009; 2: 277-283
 - 23) Kimura S, Egashira K, Nakano K, Iwata E, Miyagawa M, Tsujimoto H, Hara K, Kawashima Y, Tominaga R, Sunagawa K: Local delivery of imatinib mesylate (sti571)-incorporated nanoparticle ex vivo suppresses vein graft neointima formation. *Circulation*, 2008; 118: S65-70
 - 24) Masuda S, Nakano K, Funakoshi K, Zhao G, Meng W, Kimura S, Matoba T, Miyagawa M, Iwata E, Sunagawa K, Egashira K: Imatinib mesylate-incorporated nanoparticle-eluting stent attenuates in-stent neointimal formation in porcine coronary arteries. *J Atheroscler Thromb*, 2011; 18: 1043-1053
 - 25) Aoki T, Nishimura H, Nakagawa S, Kojima J, Suzuki H, Tamaki T, Wada Y, Yokoo N, Sato F, Kimata H, Kitahara M, Toyoda K, Sakashita M, Saito Y: Pharmacological profile of a novel synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme a reductase. *Arzneimittel-Forschung*, 1997; 47: 904-909
 - 26) Ohtani K, Usui M, Nakano K, Kohjimoto Y, Kitajima S, Hirouchi Y, Li XH, Kitamoto S, Takeshita A, Egashira K: Antimonocyte chemoattractant protein-1 gene therapy reduces experimental in-stent restenosis in hypercholesterolemic rabbits and monkeys. *Gene Ther*, 2004; 11: 1273-1282
 - 27) Camici GG, Steffel J, Akhmedov A, Schafer N, Baldinger J, Schulz U, Shojaati K, Matter CM, Yang Z, Luscher TF, Tanner FC: Dimethyl sulfoxide inhibits tissue factor expression, thrombus formation, and vascular smooth muscle cell activation: A potential treatment strategy for drug-eluting stents. *Circulation*, 2006; 114: 1512-1521
 - 28) Egashira K, Nakano K, Ohtani K, Funakoshi K, Zhao G, Ihara Y, Koga J, Kimura S, Tominaga R, Sunagawa K: Local delivery of anti-monocyte chemoattractant protein-1 by gene-eluting stents attenuates in-stent stenosis in rabbits and monkeys. *Arterioscler Thromb Vasc Biol*, 2007; 27: 2563-2568
 - 29) Usui M, Egashira K, Ohtani K, Kataoka C, Ishibashi M, Hiasa K, Katoh M, Zhao Q, Kitamoto S, Takeshita A: Anti-monocyte chemoattractant protein-1 gene therapy inhibits restenotic changes (neointimal hyperplasia) after balloon injury in rats and monkeys. *FASEB J*, 2002; 16: 1838-1840
 - 30) Egashira K, Zhao Q, Kataoka C, Ohtani K, Usui M, Charo IF, Nishida K, Inoue S, Katoh M, Ichiki T, Takeshita A: Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circ Res*, 2002; 90: 1167-1172
 - 31) Egashira K: Molecular mechanisms mediating inflammation in vascular disease: Special reference to monocyte chemoattractant protein-1. *Hypertension*, 2003; 41: 834-841
 - 32) Ohtani K, Egashira K, Nakano K, Zhao G, Funakoshi K, Ihara Y, Kimura S, Tominaga R, Morishita R, Sunagawa K: Stent-based local delivery of nuclear factor-kappaB decoy attenuates in-stent restenosis in hypercholesterolemic rabbits. *Circulation*, 2006; 114: 2773-2779
 - 33) van der Giessen WJ, Lincoff AM, Schwartz RS, van Beusekom HM, Serruys PW, Holmes DR Jr, Ellis SG, Topol EJ: Marked inflammatory sequelae to implantation of biodegradable and nonbiodegradable polymers in porcine coronary arteries. *Circulation*, 1996; 94: 1690-1697
 - 34) Lincoff AM, Furst JG, Ellis SG, Tuch RJ, Topol EJ: Sustained local delivery of dexamethasone by a novel intravascular eluting stent to prevent restenosis in the porcine coronary injury model. *J Am Coll Cardiol*, 1997; 29: 808-816
 - 35) Schwartz RS, Edelman ER, Carter A, Chronos N, Rogers C, Robinson KA, Waksman R, Weinberger J, Wilensky RL, Jensen DN, Zuckerman BD, Virmani R: Drug-eluting stents in preclinical studies: Recommended evaluation from a consensus group. *Circulation*, 2002; 106: 1867-1873