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PRE-CLINICAL RESEARCH

Role of Rho-Kinase in the Pathogenesis of Coronary Hyperconstricting Responses Induced by Drug-Eluting Stents in Pigs In Vivo

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Objectives This study examined whether the Rho-kinase pathway is involved in the pathogenesis of coronary hyperconstricting responses induced by drug-eluting stents (DES) in pigs in vivo.

Background Recent studies showed that coronary vasoconstricting responses are enhanced at the edge of coronary segments implanted with DES compared with bare-metal stents (BMS) in humans. We have previously shown that the activated Rho-kinase pathway plays a central role in the molecular mechanism of coronary vasospasm in animals and humans.

Methods Human coronary artery smooth muscle cells (hCASMCs) were coincubated with various concentrations of paclitaxel (10^{-9} to 10^{-6} mol/l, corresponding levels reported in DES-implanted arterial tissue) for 24 h. A paclitaxel-eluting stent (PES), sirolimus-eluting stent (SES), and BMS were randomly implanted in the left coronary arteries in pigs for 4 weeks.

Results In hCASMCs, paclitaxel significantly enhanced Rho-kinase expression and activity. In a porcine model, coronary vasoconstricting responses to serotonin (10 and 100 μg/kg intracoronary administration) were significantly enhanced at the PES site compared with the BMS site (45 $\pm$ 4% vs. 30 $\pm$ 3%; $p < 0.01$; $n = 12$ each), and were abolished by hydroxyfasudil (90 and 300 μg/kg intracoronary administration), a selective Rho-kinase inhibitor. The PES enhanced inflammatory responses and microthrombus formation at the stent edge, where immunoreactivities for Rho-kinase expression and activity were increased. In organ chamber experiments, serotonin-induced contractions were significantly enhanced in rings from the PES edge site compared with the BMS edge site. The SES also caused similar coronary hyperconstricting responses to serotonin in vivo.

Conclusions These results suggest that the Rho-kinase pathway plays an important role in the pathogenesis of DES-induced coronary hyperconstricting responses. (J Am Coll Cardiol 2009;54:2321–9) © 2009 by the American College of Cardiology Foundation

Drug-eluting stents (DES) have dramatically reduced the rate of restenosis after percutaneous coronary intervention, revolutionizing interventional cardiology (1,2). However, DES have also been shown not to improve patient survival compared with bare-metal stents (BMS) (3). Indeed, recent studies suggest that the early benefits of DES are offset by an increased risk of late stent thrombosis, a potentially fatal complication (4). The DES-induced impairment of coronary vasomotion is another concern regarding the long-term safety of DES (5–10). Enhanced vasoconstriction in response to acetylcholine (5–8) or exercise (9) was shown in the coronary segments adjacent to DES, but not in those adjacent to BMS, and even death was reported among patients with severe coronary vasospasm after DES implantation (10). However, the underlying molecular mechanism for the DES-induced coronary hyperconstriction remains to be elucidated.

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Rho-kinase is one of the down-stream effectors of the small GTP-binding protein Rho and consists of 2 isoforms, Rho-kinase beta (ROCK1) and Rho-kinase alpha (ROCK2)

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Abbreviations and Acronyms

BMS = bare-metal stent(s)
CAG = coronary angiography
DES = drug-eluting stent(s)
ERM = ezrin/radixin/moesin
hCASMC = human coronary artery smooth muscle cell
IC = intracoronary administration
MYPT1 = myosin phosphatase target subunit 1
PES = paclitaxel-eluting stent(s)
RNA = ribonucleic acid
ROCK1 = Rho-kinase beta
ROCK2 = Rho-kinase alpha
SES = sirolimus-eluting stent(s)
VSMC = vascular smooth muscle cell

(11,12). We have previously shown that activation of Rho-kinase plays a central role in the molecular mechanism of coronary vasospasm through vascular smooth muscle cell (VSMC) hypercontraction and down-regulation of endothelial nitric oxide synthase in endothelial cells (13–21).

In the present study, we thus examined whether the Rho-kinase pathway is also involved in the pathogenesis of DES-induced coronary hyperconstriction.

Methods

All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University (20MdA-47).

Cell culture. Human coronary artery smooth muscle cells (hCASMCs) (Lonza, Walkersville, Maryland; passages 4 through 10) were seeded in a growth medium (SmGM-2 Bullet Kit, Lonza) for 24 h and then growth-arrested in a Dulbecco modified Eagle medium (Sigma Aldrich, St. Louis, Missouri) supplemented with 0.1% bovine serum albumin, 100 IU/ml penicillin, and 100 μg/ml streptomycin for 24 h, and used for the experiments.

Real-time polymerase chain reaction for ROCK1 and ROCK2 messenger ribonucleic acid (RNA) expression. The hCASMCs (1×10^5) were coincubated with 10^{-9} to 10^{-6} mol/l paclitaxel for 24 h (n = 9) (22). Cells were lysed, and total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA (600 ng) was reverse-transcribed using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time polymerase chain reaction was performed using the Real-Time Detection System (Bio-Rad Laboratories, Hercules, California). Sequences of the primers were (forward, reverse) 5′-CTGCAACTGGAACTCAACCAAGAA-3′, 5′-TTAGCACGCAATTGCTCAATATCAC-3′ for ROCK1, 5′-TGCTTTAAATTTGCTGGCTACCCTA-3′, 5′-CACACAGCTGCATGTCTGAGGA-3′ for ROCK2, and 5′-TGGCACCCAGCACAATGAA-3′, 5′-CTAAGTC-ATAGTCCGCCTAGAAGCA-3′ for beta-actin, all of which were designed by the Perfect Real Time Support System (Takara Bio Inc., Shiga, Japan). The beta-actin was used as an internal control; SYBR Premix Ex Taq I and II (Takara Bio Inc.) were used for the detection of ROCK1 and ROCK2 cDNA, respectively.

Western blotting for Rho-kinase activity. The hCASMCs (1×10^5) were coincubated with 10^{-8} mol/l paclitaxel, a comparable concentration in the DES-implanted coronary arteries in pigs (n = 6) for 24 h (23). Western blot analysis was performed for Rho-kinase activity, which was expressed as the extent of phosphorylated ezrin/radixin/moesin (ERM) family, substrates of Rho-kinase, compared with that of total ERM, as previously reported (24).

In vivo study. Domestic male pigs (2 to 3 months old and weighing 20 to 30 kg) were pre-treated orally with aspirin (300 mg/day) and clopidogrel (150 mg/day) for 2 days before stent implantation. After sedation with ketamine hydrochloride (15 mg/kg intramuscularly) and anesthesia with inhaled 2% to 5% sevoflurane and heparinization (5,000 U intravenously), we randomly implanted a paclitaxel-eluting stent (PES) (Taxus Express 2, Boston Scientific, Natick, Massachusetts) and a BMS (Express 2, Boston Scientific) in the left anterior descending and circumflex coronary arteries in the same pig (n = 8). In an additional experiment, another set of comparisons between a sirolimus-eluting stent (SES) (Cypher, Johnson & Johnson, New Brunswick, New Jersey) and a BMS (Velocity, Johnson & Johnson) was performed (n = 6). We defined the control sites as those at 10 to 20 mm proximal and distal to the stent edges, and calculated overstretch ratio of stent diameter by dividing a control vessel diameter (25). The antiplatelet therapy with aspirin and clopidogrel was continued after the stent implantation for 4 weeks.

Four weeks after the stent implantation, we performed coronary angiography (CAG) to examine coronary vasomotion (26). Briefly, after the baseline CAG, we examined coronary responses to serotonin (10 and 100 μg/kg intracoronary administration [IC]) and then to bradykinin (0.1 μg/kg IC). We re-examined the responses to serotonin after hydroxyfasudil (30 and 100 μg/kg/min IC infusion for 3 min), a specific Rho-kinase inhibitor (11), then those to bradykinin after intracoronary infusion of N^G-monomethyl-L-arginine (1 mg/kg for 10 min) (27), and finally those to nitroglycerin (10 μg/kg IC). We performed each protocol at a 30-min interval (26). Quantitative CAG (DFP-2000A, Toshiba Medical, Tokyo, Japan) was performed in a blind manner as previously reported (13,15). For clarity of the data, the mean value of the vasomotor responses of the proximal and the distal stent edges is presented. In the PES protocol, 2 animals were excluded because of >50% coronary restenosis (n = 1) and severe infection (n = 1).

Histological analysis. After the CAG study, animals were euthanized with a lethal dose of sodium pentobarbital (40 mg/kg intravenously), and histological analysis was performed as previously reported (28). The extent of microthrombus formation was assessed semiquantitatively by using the following scale: 0 = none; 1 = minute thrombus peristent; 2 = thrombus <50% circular area of peristent; and 3 = thrombus all around stent strut. The extent of inflammatory responses, including peristent leukocyte and macrophage infiltration and adventitial inflammatory changes, was also assessed by using the following scale: 0 = none; 1 = fewer than 5 inflammatory cells; 2 = fewer than 20 inflammatory cells; and 3 = more than 20 inflammatory cells.

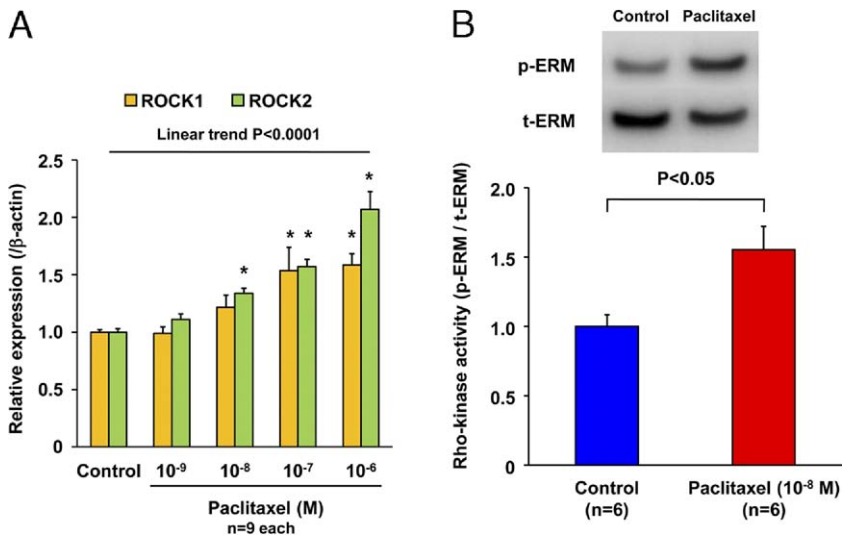

Figure 1 Paclitaxel Increases Rho-Kinase Expression and Activity In Vitro

(A) Paclitaxel significantly enhanced messenger ribonucleic acid expression of Rho-kinase beta (ROCK1) and Rho-kinase alpha (ROCK2) in a concentration-dependent manner. *$p < 0.05$ versus control. **(B)** Rho-kinase activity, as evaluated by the extent of ezrin/radixin/moesin phosphorylation, was significantly increased after 24-h coincubation with paclitaxel (10^{-8} mol/l). p-ERM = phosphorylated ezrin/radixin/moesin; t-ERM = total ezrin/radixin/moesin.

Immunohistological analysis. Immunohistochemical staining was performed using mouse antihuman ROCK1 antibody (1:50, BD Biosciences, San Jose, California), mouse antihuman ROCK2 antibody (1:50, BD Biosciences), and rabbit antihuman phosphorylated myosin phosphatase target subunit 1 (phospho-MYPT1, Thr696) (1:50, Upstate, Billerica, Massachusetts), substrates of Rho-kinase (29). Nonimmune mouse or rabbit immunoglobulin G was used as negative control. We semiquantitatively assessed the extent of ROCK1, ROCK2, and phosphorylated MYPT1 using the following scale: 0 = none; 1 = slight; 2 = moderate; and 3 = high (30).

Organ chamber experiments. Organ chamber experiments were performed (n = 8) at 4 weeks after the stent implantation (15,26). Briefly, the coronary segments just adjacent to the proximal and distal edges of the stent (4-mm-long rings) were removed by gentle rubbing of the luminal surface with a cotton swab. The coronary segment at 20 mm distal to the stent edge was used as a control. The contractions to serotonin (10^{-9} to 3×10^{-6} mol/l) were examined and were expressed as a percentage to the average value of the 3-time pre-contractions to 62 mmol/l KCl (15,26).

Statistical analysis. All results are expressed as mean ± SEM. The results of reverse-transcriptase polymerase chain reaction were analyzed by 1-way analysis of variance followed by the Dunnett test, and the dose-dependent linear trend was also assessed. The results of Western blotting and angiographical data were analyzed by unpaired Student *t* test. The results of organ chamber experiments were analyzed by 2-way analysis of variance followed by a Bonferroni test. The results of histological studies and immunohistological studies were analyzed by Mann-Whitney *U* test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Paclitaxel increases Rho-kinase expression and activity in vitro. In cultured hCASMCs, paclitaxel (10^{-9} to 10^{-6} mol/l for 24 h) increased messenger RNA expression of both ROCK1 and ROCK2 in a concentration-dependent manner (both $p < 0.0001$ for linear trend) (Fig. 1A). Paclitaxel (10^{-8} mol/l for 24 h) also significantly increased the extent of ERM phosphorylation, a marker of Rho-kinase activity (Fig. 1B).

PES induces hydroxyfasudil-sensitive coronary hyperconstricting responses in vivo. In the stent implantation procedure, there was no significant difference in the procedures between the BMS and the PES sites (Table 1). Four weeks after the stent implantation, intracoronary serotonin

Table 1 Procedural and Angiographic Findings, Comparison Between BMS and PES

	BMS	PES	p Value
Control vessel diameter (mm)	2.55 ± 0.08	2.48 ± 0.07	0.54
Stent diameter (mm)	2.72 ± 0.09	2.58 ± 0.08	0.26
Stent length (mm)	16.0 ± 0.0	16.0 ± 0.0	N/A
Overstretch ratio	1.06 ± 0.04	1.04 ± 0.4	0.73
Maximum inflation pressure (atm)	10.7 ± 1.28	8.7 ± 0.42	0.19

Values are expressed as mean ± SEM (n = 6 each). Stent diameter was calculated by averaging the diameters at the proximal edge, mid portion, and distal edge of the stented coronary artery. Overstretch ratio is the stent diameter divided by the control vessel diameter. Nominal pressure was 9 atm for both BMS (Express 2, Boston Scientific, Natick, Massachusetts) and PES.
BMS = bare-metal stent(s); PES = paclitaxel-eluting stent(s); N/A = not available.

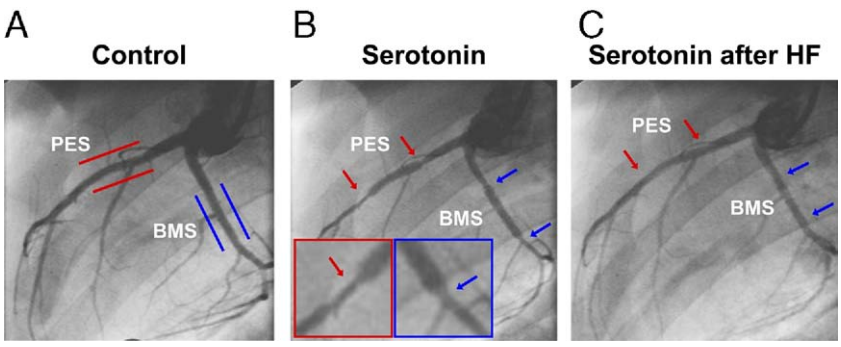

Figure 2 PES Enhances Coronary Vasoconstricting Responses in Pigs In Vivo

Representative left coronary angiograms under control condition **(A)**, after intracoronary serotonin (100 μg/kg intracoronary administration) without **(B)** and with **(C)** hydroxyfasudil (HF) (300 μg/kg intracoronary administration). The **red lines** indicate the site of paclitaxel-eluting stent (PES) implantation, and **blue lines** indicate the site of bare-metal stent (BMS) implantation. **Red arrows** indicate the proximal and distal edges of PES, and **blue arrows** indicate those of BMS. Magnified images of the distal edge of PES and BMS are shown in the **boxes (B)**.

caused hyperconstriction at the proximal and distal edge segments of the PES site as compared with the BMS site, which was abolished by intracoronary pre-treatment with hydroxyfasudil, a selective Rho-kinase inhibitor (Fig. 2). Quantitative analysis showed that the responses of the stent edges were significantly enhanced at the PES site compared with the BMS site and were abolished by hydroxyfasudil (Fig. 3A). In contrast, the vasoconstricting responses to serotonin were comparable in the control (nonstented) sites between the BMS and the PES sites (Fig. 3B).

Coronary vasodilating responses to bradykinin did not differ significantly between the PES and the BMS sites (PES 1.6 ± 1.1%, BMS 1.4 ± 0.6% from baseline) and were equally impaired as compared with the control sites (PES 5.8 ± 1.3%, BMS 6.0 ± 0.9%, both $p < 0.01$). Moreover, responses to bradykinin with and without pre-treatment of N^G-monomethyl-L-arginine did not differ significantly between the BMS and the PES sites. Coronary vasodilating responses to nitroglycerin were comparable between the 2 stent sites (PES 3.5 ± 1.4%, BMS 3.9 ±

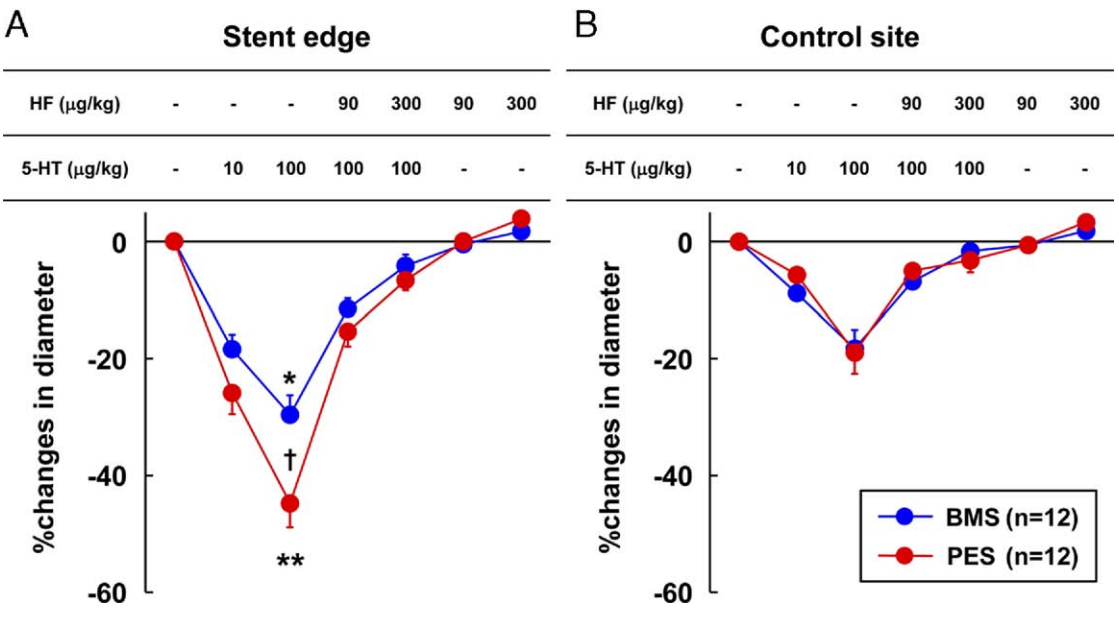

Figure 3 PES Enhances Coronary Vasoconstricting Responses in Pigs In Vivo

Coronary vasoconstricting responses to intracoronary serotonin (5-HT) before and after the pre-treatment with HF in the stent edges **(A)** and the control sites **(B)**. The vasoconstricting responses are expressed as percent changes in diameter from the level with nitroglycerin (10 μg/kg intracoronary administration). Results are expressed as mean ± SEM. *$p < 0.05$ versus control site. †$p < 0.01$ versus BMS. **$p < 0.01$ versus control site. Abbreviations as in Figure 2.

1.5%) with no significant difference with the control sites (PES 9.0 ± 2.0%, BMS 6.9 ± 1.2%).

PES enhances coronary microthrombus formation and inflammatory responses in vivo. Histological analysis showed that neointimal formation of the coronary artery was significantly suppressed in the PES site compared with the BMS site (Figs. 4A to 4C). However, the extent of peristent microthrombus formation (Figs. 4D to 4F) and that of inflammatory responses (Figs. 4G to 4I) were significantly enhanced at the PES site compared with the BMS site.

PES enhances coronary Rho-kinase expression and Rho-kinase activity. Immunohistological analysis showed that ROCK1 (Figs. 5A to 5D), ROCK2 (Figs. 5E to 5H), and phospho-MYPT1 (Figs. 5I to 5L) were highly expressed in the PES as compared with the BMS site.

PES enhances contractions to serotonin of isolated coronary arteries. In organ chamber experiments, serotonin caused concentration-dependent contractions of coronary rings without endothelium. The extent of the contractions at the stent edge segments was significantly greater at the PES site compared with the BMS site (Fig. 6A). In contrast, the extent of the contractions at the control sites was comparable between the BMS and the PES sites (Fig. 6B).

SES induces coronary hyperconstricting responses similar to those of PES in vivo. In an additional experiment, we performed a similar in vivo protocol with SES to examine whether other DES also cause coronary hyperconstricting responses. The stent implantation procedures were comparable between the BMS (Velocity) and the SES sites (Table 2). Coronary hyperconstricting responses to serotonin were also noted at the proximal and distal edge segments of the SES site as compared with the BMS site, which was abolished by intracoronary pre-treatment with hydroxyfasudil (Figs. 7 and 8). The histological analysis showed the higher score of microthrombus formation (SES 2.33 ± 0.33, BMS 0.75 ± 0.28) and of inflammatory responses (SES 5.00 ± 1.30, BMS 0.70 ± 0.12) in SES sites than in BMS sites (both $p < 0.01$).

Discussion

The major findings of this study were: 1) paclitaxel increased Rho-kinase expression and activity in hCASMCs

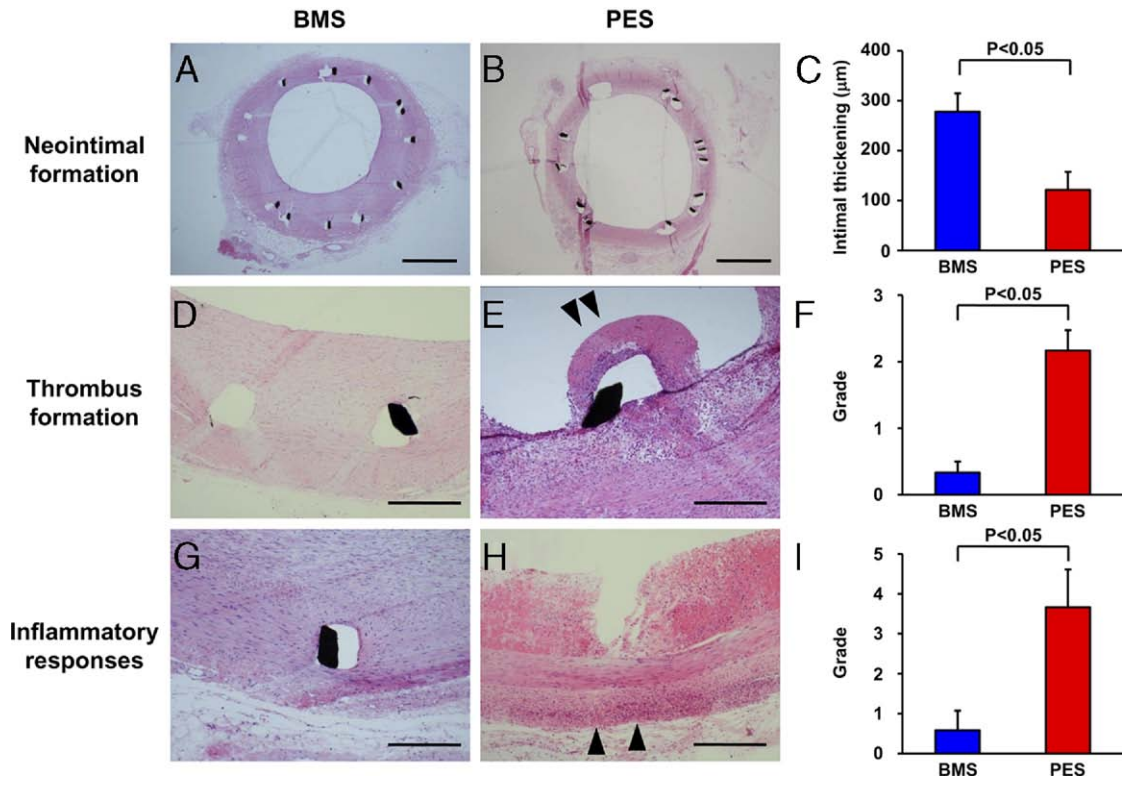

Figure 4 PES Enhances Coronary Microthrombus Formation and Inflammatory Responses in Pigs In Vivo

Representative photomicrographs of BMS-treated arteries **(A, D, G)** and PES-treated arteries **(B, E, H)**. Scale bars represent 1 mm **(A, B)** and 200 μm **(D, E, G, H)**, respectively. Semiquantitative analysis showed that although neointimal formation was significantly suppressed in the PES site **(C)**, peristent microthrombus formation **(arrowheads in E, panel F)** and inflammatory responses **(arrowheads in H, panel I)** were significantly enhanced at the PES site compared with the BMS site (n = 6 each). Abbreviations as in Figure 2.

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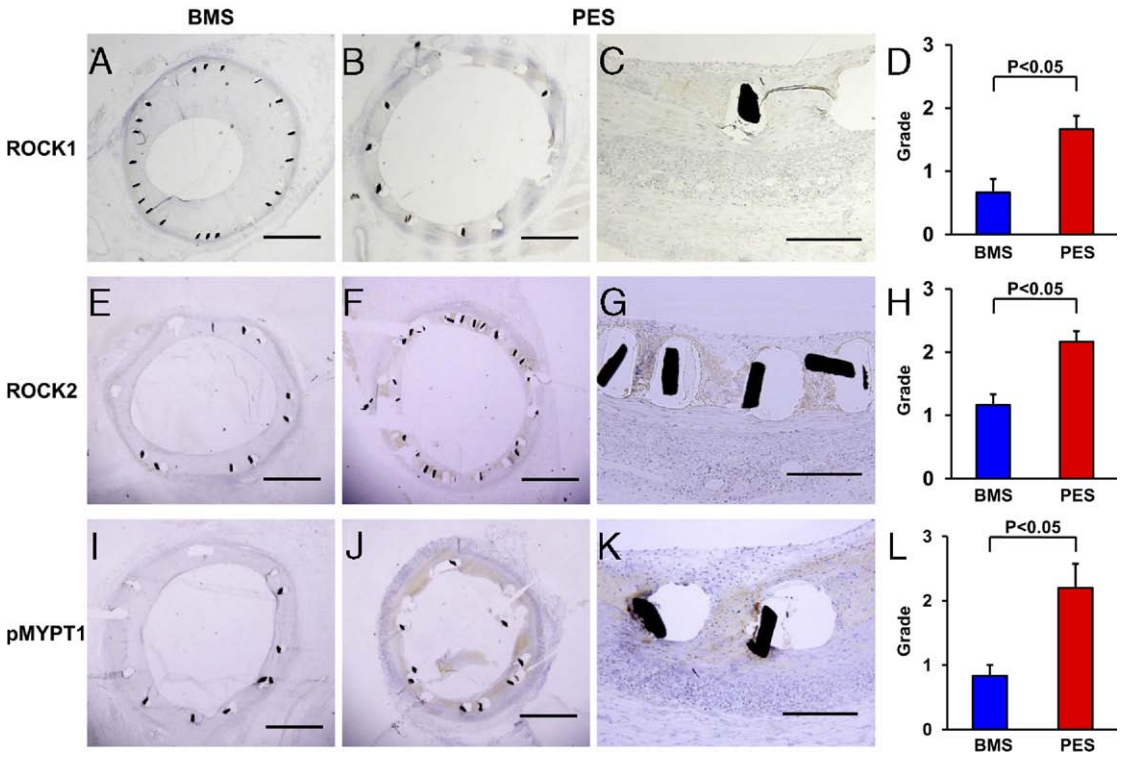

Figure 5 PES Enhances Coronary Rho-Kinase Expression and Activity in Pigs In Vivo

Representative immunostainings of BMS-treated arteries **(A, E, I)**, PES-treated arteries **(B, F, J)**, and magnified images of the PES-treated arteries **(C, G, K)**. Scale bars represent 1 mm **(A, B, E, F, I, J)** and 200 μm **(C, G, K)**, respectively. Semiquantitative analysis showed that ROCK1 **(D)**, ROCK2 **(H)**, and phosphorylated myosin phosphatase target subunit 1 (pMYPT1) **(L)** were expressed to a greater extent in the PES site compared with the BMS site (n = 6 each). Abbreviations as in Figures 1 and 2.

in vitro; 2) PES enhanced coronary vasoconstricting responses to serotonin as compared with BMS in pigs both in vivo and in vitro; 3) the hyperconstrictive responses were abolished by hydroxyfasudil, a selective Rho-kinase inhibitor; 4) those functional alterations of the coronary arteries were associated with enhanced microthrombus formation and inflammatory cell infiltration, where immunoreactivities for ROCK1, ROCK2,

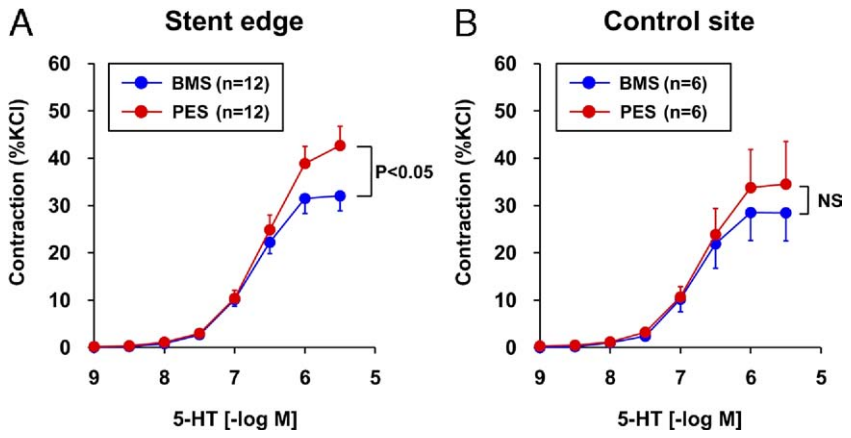

Figure 6 PES Causes Hypercontractions of Porcine Coronary Arteries In Vitro

Serotonin-induced contractions of isolated coronary rings without endothelium, when expressed as percent contraction to 62 mmol/l KCl, were significantly enhanced at the edge of the PES site compared with the BMS site **(A)**. In contrast, no difference was noted at the control site **(B)**. Results are expressed as mean ± SEM. NS = not significant; other abbreviations as in Figure 2.

Table 2 Procedural and Angiographic Findings, Comparison Between BMS and SES

	BMS	SES	p Value
Control vessel diameter (mm)	2.69 ± 0.12	2.58 ± 0.24	0.24
Stent diameter (mm)	2.62 ± 0.21	2.70 ± 0.18	0.49
Stent length (mm)	19.7 ± 1.1	19.2 ± 1.3	0.77
Overstretch ratio	0.97 ± 0.08	1.03 ± 0.07	0.21
Maximum inflation pressure (atm)	10.8 ± 1.1	12.0 ± 0.0	0.15

Values are expressed as mean ± SEM (n = 6 each). Stent diameter was calculated by averaging the diameters at the proximal edge, mid portion, and distal edge of the stented coronary artery. Overstretch ratio is the stent diameter divided by the control vessel diameter. Nominal pressure was 8 atm for bare-metal stents (BMS) (Velocity, Johnson & Johnson, New Brunswick, New Jersey) and 12 atm for sirolimus-eluting stents (SES), respectively.

and Rho-kinase activity (phospho-MYPT1) were increased; and 5) SES also caused similar coronary hyperconstricting responses in vivo as did PES.

DES and Rho-kinase. Paclitaxel, a tubulin polymerizing agent (31), is now widely used for the pharmacologic component of DES because it inhibits VSMC proliferation and migration in vitro (22) and suppresses neointimal thickening in animal models in vivo (32). The present result with hCASMCs provides new findings that paclitaxel significantly enhances ROCK1 and ROCK2 messenger RNA expression and Rho-kinase activity at its clinically relevant concentration. The previous studies showed that Rho guanine triphosphatases control organization of the actin cytoskeleton (33) and that Rho could be activated by paclitaxel, possibly through interfering with microtubules or actin polymerization (34), suggesting that paclitaxel may activate Rho-kinase in part by cytoskeletal reorganization.

Enhanced Rho-kinase activity plays a central role in the pathogenesis of coronary vasospasm (11). Intracoronary administration of fasudil or hydroxyfasudil (11), selective Rho-kinase inhibitors, markedly inhibits coronary vasospasm in porcine models with various inflammatory stimuli in vivo (13–18) and in humans (19,20). In the present porcine model, serotonin-induced coronary hyperconstriction was significantly enhanced at the PES as well as the SES site as compared with the BMS site. This finding was duplicated in organ chamber experiments using coronary rings without endothelium. Endothelial function was equally but modestly reduced at the PES and BMS sites in vivo. The previous study reported that paclitaxel did not impair endothelium nitric oxide synthase activity or nitric oxide release from coronary artery endothelial cells (35). Thus, in the present porcine model, the coronary hyperconstricting responses are mainly caused by VSMC hypercontraction through a Rho-kinase–mediated mechanism rather than endothelial dysfunction, a finding consistent with the previous studies (27,36).

Mechanisms of DES-induced Rho-kinase activation. A DES consists of 3 distinct components, including platform, drug, and polymer. In the present study, a possible adverse effect of platform can be excluded because we used the same platform and the procedural data were well comparable between the 2 stent sites. In the present study, neointimal formation was more suppressed and peristent microthrombus formation was more enhanced at the DES site. These histological findings reflect antiproliferative effects of paclitaxel on both VSMC and endothelial cells, leading to delayed re-endothelialization and resultant thrombus formation (37). Activated platelets may be involved in the thrombus formation through Rho/Rho-kinase pathways by releasing serotonin and platelet-derived growth factors (11) and interactions with thrombin (38).

The present study also showed that inflammatory responses were accelerated at the DES site. These changes could be caused by a local hypersensitivity reaction to the nonbioabsorbing polymer used in DES (37). Indeed, we have previously shown that the expression of Rho-kinase

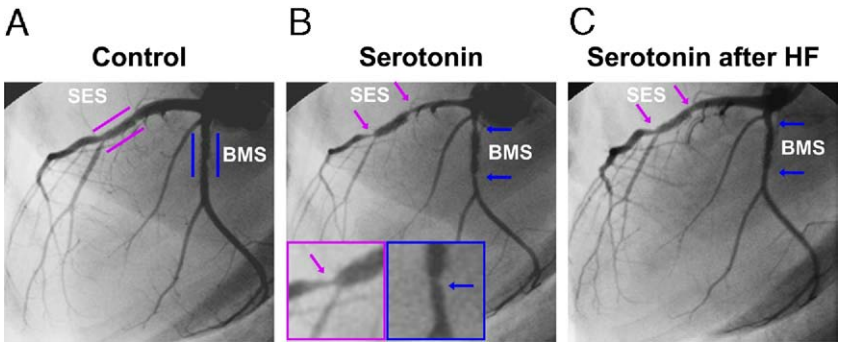

Figure 7 SES Enhances Coronary Vasoconstricting Responses in Pigs In Vivo

Representative left coronary angiograms under control condition **(A)**, after intracoronary serotonin (100 μg/kg intracoronary administration) **(B)**, and after intracoronary serotonin with HF (300 μg/kg intracoronary administration) **(C)**. **Pink lines** indicate the site of sirolimus-eluting stent (SES) implantation, and **blue lines** indicate the site of BMS implantation. **Pink arrows** indicate the proximal and distal edges of SES, and **blue arrows** indicate those of BMS. Magnified images of the distal edge of SES and BMS are shown in the **boxes (B)**. Abbreviations as in Figure 2.

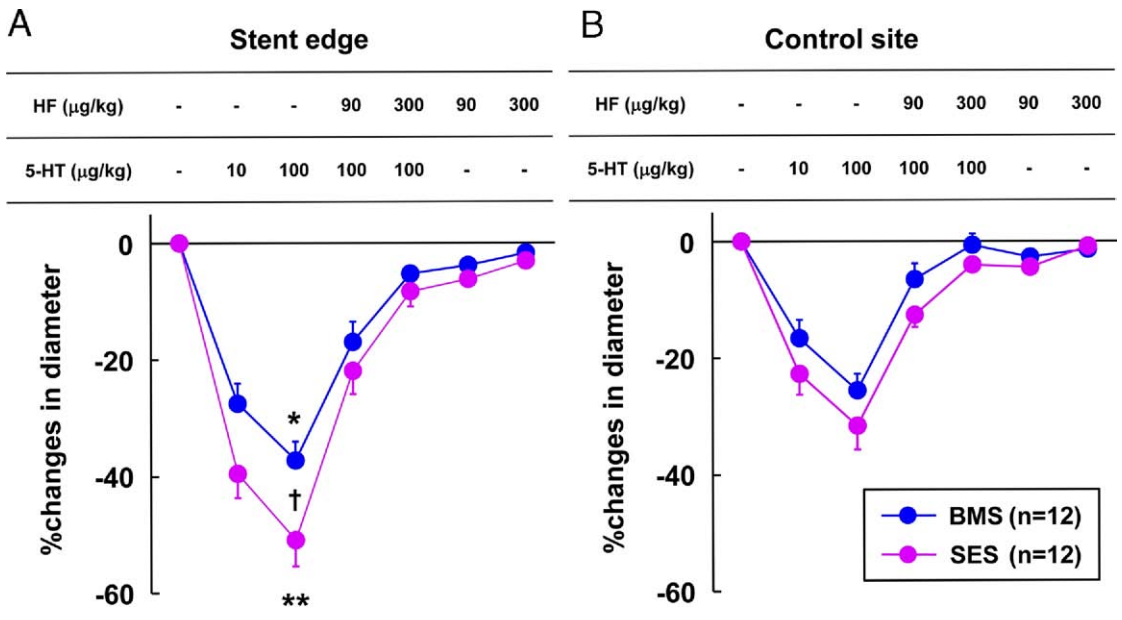

Figure 8 SES Enhances Coronary Vasoconstricting Responses in Pigs In Vivo

Coronary vasoconstricting responses to intracoronary serotonin (5-HT) before and after the pre-treatment with HF, a selective Rho-kinase inhibitor, in the stent edges **(A)** and the control sites **(B)**. The vasoconstricting responses are expressed as percent changes in diameter from the level with nitroglycerin (10 μg/kg intracoronary administration). Results are expressed as mean ± SEM. *$p < 0.05$ versus control site. †$p < 0.05$ versus BMS. **$p < 0.01$ versus control site. SES = sirolimus-eluting stents; other abbreviations as in Figure 2.

itself is accelerated by inflammatory stimuli, such as angiotensin II and interleukin-1 beta, through protein kinase C/nuclear factor kappa beta pathway (39). Thus, it is conceivable that DES-induced inflammatory responses also enhance Rho-kinase activity with a resultant coronary hyperconstricting response and thrombus formation. Indeed, in association with those changes, immunoreactivities of ROCK1, ROCK2, and phospho-MYPT1, a reliable marker of Rho-kinase activity (29), were enhanced.

Study limitations. First, we were unable to dissect the roles of ROCK1 and ROCK2. Recently, it was reported that ROCK isoforms may have different roles in neointimal formation (40). Furthermore, the localization of Rho-kinase activation and the role of other G-proteins (e.g., Rac-1) remain to be examined in future studies. Second, the present study was performed in normal juvenile pigs without preexisting atherosclerotic coronary lesions. This might explain, at least in part, the discrepancy between the present animal study (normal vascular function at the distal segment) and the previous clinical study (coronary hyperconstricting responses even at the distal segment of DES-implanted arteries) (6). Finally, in the present study, we used intracoronary serotonin administration to examine coronary vasomotor responses. In the clinical setting, acetylcholine is now frequently used to provoke coronary spasm. However, it has been reported that serotonin better mimics spontaneous vasospasm in humans than acetylcholine (41).

Conclusions

The present study suggests that the activated Rho-kinase pathway plays an important pathogenetic role in the DES-induced coronary hyperconstricting responses. Use of Rho-kinase inhibitors and other vasculoprotective agents (e.g., calcium-channel blockers and statins), in addition to developing innovative devices, may help to optimize the efficacy and safety of DES.

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