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Long-term treatment with nifedipine suppresses coronary hyperconstricting responses and inflammatory changes induced by paclitaxel-eluting stent in pigs *in vivo*: possible involvement of Rho-kinase pathway

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Aims Accumulating evidence indicates that coronary vasoconstricting responses are enhanced at the edges of coronary segment implanted with a drug-eluting stent (DES) compared with a bare-metal stent (BMS) in humans. We have recently demonstrated that Rho-kinase pathway plays an important role in DES-induced coronary hyperconstricting responses associated with inflammatory changes in pigs *in vivo*. This study examined whether long-term treatment with calcium channel blocker suppresses DES-induced coronary hyperconstricting responses in pigs *in vivo*.

Methods and results Paclitaxel-eluting stent (PES) and a BMS were randomly implanted in the left coronary arteries in male domestic pigs with and without long-acting nifedipine (NIF, 4 mg/kg/day) for 4 weeks ($n = 7$ each). Coronary vasomotion was evaluated by quantitative coronary angiography at least 24 h after withdrawal of NIF to avoid its direct effects on coronary vasomotion. In the control group (without NIF), coronary vasoconstricting responses to serotonin (10 and 100 μg/kg, i.c.) were significantly enhanced at the PES site compared with the BMS site ($P = 0.009$), which were abolished by hydroxyfasudil (90 and 300 μg/kg, i.c.), a selective Rho-kinase inhibitor. The PES-induced vasoconstricting responses were significantly inhibited in the NIF group ($P = 0.019$). Histological examination showed that inflammatory cell accumulation and microthrombus formation were enhanced at the PES site compared with the BMS site ($P < 0.05$), both of which were significantly suppressed by NIF associated with reduced Rho-kinase expression and activity ($P < 0.05$).

Conclusion These results indicate that long-term treatment with NIF suppresses PES-induced coronary abnormalities partly through Rho-kinase pathway inhibition *in vivo*.

Keywords Drug-eluting stents • Coronary vasospasm • Calcium channel blockers

Introduction

Drug-eluting stents (DES) have been widely used and have markedly reduced restenosis after percutaneous coronary intervention.[1,2] However, the use of the first generation of DES, such as sirolimus-eluting stent (SES) and paclitaxel-eluting stent (PES), has raised the safety issue concerns, including late stent thrombosis[3] and impairment of coronary vasomotion.[4–6] Indeed, enhanced

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coronary vasoconstriction in response to acetylcholine[4,5] or exercise[6] has been demonstrated in the coronary segments adjacent to DES, but not in those to bare-metal stent (BMS), and even death case was reported among patients with severe coronary vasospasm following DES implantation.[7]

Rho-kinase is a downstream effector of the small guanosine triphosphate-binding protein Rho and consists of two isoforms—ROCK1/Rho-kinase β and ROCK2/Rho-kinase α. We have previously demonstrated that Rho-kinase plays a central role in the molecular mechanism of coronary vasospasm through vascular smooth muscle cell hyperconstriction and down-regulation of endothelial nitric oxide (NO) synthase in endothelial cells.[8–16] Furthermore, we have recently reported that Rho-kinase pathway plays a crucial role in the pathogenesis of DES-induced hyperconstricting responses in pigs *in vivo*.[17]

Long-acting Ca channel blockers (CCBs) are currently the mainstay of the clinical practice of vasospastic angina (VSA).[18] Importantly, the previous studies including ACTION (A Coronary disease Trial Investigating Outcome with Nifedipine gastrointestinal therapeutic system) and ENCORE (Evaluation of Nifedipine on Coronary Endothelial Function) trials demonstrated that nifedipine (NIF) exerts cardiovascular protective effects through inhibition of vascular inflammation and improvement of endothelial function.[19–21] In the present study, we thus examined whether long-acting NIF inhibits PES-induced coronary hyperconstricting responses in pigs *in vivo*, and if so, whether Rho-kinase pathway inhibition is involved.

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).

Nifedipine treatment and experimental animals

As a pilot study, to evaluate plasma levels of NIF, blood samples were collected at 3 weeks during the treatment and at the coronary angiography (CAG) study at 4 weeks ($n = 4$). After centrifugation (1450 *g*, 5 min), the plasma was removed and frozen at -80°C, and was later analysed for concentration of NIF by high-performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) technology in the Toray Research Center, Inc. (Osaka, Japan).

Then, in the main study, a total of 14 domestic male pigs (2–3-month old and weighing 20–30 kg) were randomly divided into the following two groups: seven pigs were orally given aspirin (300 mg/day) and clopidogrel (150 mg/day; control group), and the remaining seven pigs were given long-acting NIF (4 mg/kg/day once a day, Bayer Healthcare, Leverkusen, Germany) in addition to the dual antiplatelet therapy (NIF group). The half-life of long-acting NIF used in the present study is ~20 h, and the present dose and duration of NIF treatment were used in our previous study with pigs *in vivo*.[19] The treatment with the dual anti-platelet agents with or without NIF was started 3 days before stent implantation and was continued for 4 weeks until the final experiment on coronary vasomotion, while NIF was discontinued one day before the final experiment in order to avoid its direct inhibitory effects on coronary vasomotion.

Porcine model of paclitaxel-eluting stent and bare-metal stent implantation *in vivo*

We randomly implanted a PES (Taxus Express 2^{TM}, Boston Scientific, Natick, MA, USA) and a BMS (Express 2^{TM}, Boston Scientific) in the left anterior descending (LAD) and the circumflex coronary arteries (LCX) in the same pig after sedation with ketamine hydrochloride (15 mg/kg, i.m.), anaesthesia with inhaled 2–5% sevoflurane, and heparinization (5000 U i.v.).[17] A segment for stent implantation was selected by using quantitative coronary angiography (QCA) with a stent-to-artery ratio of ~1.1.[22] One stent was implanted in each of the left coronary artery; for example, when PES was implanted in the LAD, BMS was implanted in the LCX in the same pig. Stents in each group were equally distributed. Finally, the number of PES-implanted vessels treated with and without NIF and that of BMS-implanted vessels treated with and without NIF was seven each. As reported previously, we defined the control sites as those at 10–20 mm proximal and distal to the stent edges, and calculated overstretch ratio of stent diameter by dividing a control vessel diameter.[17,23]

Evaluation of coronary vasomotion after paclitaxel-eluting stent and bare-metal stent implantation

Four weeks after the stent implantation, we performed CAG to examine coronary vasomotion.[17] After control CAG, we examined coronary responses to serotonin (10 and 100 μg/kg, i.c.) and then to bradykinin (0.1 μg/kg, i.c.). We re-examined the responses to serotonin after hydroxyfasudil (30 and 100 μg/kg/min i.c. infusion for 3 min), a specific Rho-kinase inhibitor,[10,11] then those to bradykinin after intracoronary infusion of N^{G}-monomethyl-L-arginine (L-NMMA, 1 mg/kg for 10 min), an inhibitor of NO syntheses,[24] and finally those to nitroglycerine (10 μg/kg, i.c.). We performed each protocol at a 30 min interval (see Supplementary material online, *Figure S1*). The QCA analysis was performed with the validated densitometric analysis system (CAAS, Pie Medical Imaging, Maastricht, Netherlands) by an independent observer who was blinded to the type of stent, as previously reported.[13,17] For the clarity of the data, the mean value of vasomotor responses of the proximal and the distal stent edge is presented ($n = 7$ each).

Histological analysis

As previously reported, coronary arteries implanted with stents were embedded in acryl (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany) and were cut into 5 μm thickness with a tungsten hard knife (SHK20WB, Meiwafosis, Osaka, Japan) to preserve stent struts in sections.[17,25] Microthrombus formation and inflammatory response caused by the stents was analysed at each stent strut. Briefly, the extent of microthrombus formation was assessed semi-quantitatively by using the following scale: 0, none; 1, minute peri-stent thrombus; 2, thrombus <50% circular area of peri-stent; and 3, thrombus all around stent strut.[17] The extent of inflammatory responses, including peri-stent leucocytes and macrophages infiltration and adventitial inflammatory changes, was graded by hematoxylin-eosin staining using the following scale: 0, none; 1, <5 inflammatory cells; 2, <20 inflammatory cells; and 3, circumferential dense inflammatory cells infiltration.[25] For each stent, the mean histopathological and histomorphometric values of the 3–5 stent sections were entered into the analysis.

Immunohistological analysis

Immunohistochemistry was performed using mouse anti-human ROCK1 antibody (1:50; BD Biosciences, San Jose, CA, USA), mouse anti-human ROCK2 antibody (1:50; BD Biosciences), and rabbit anti-human phosphorylated myosin phosphatase target subunit 1 (phospho-MYPT1, Thr696; 1:50; Upstate, Billerica, MA, USA), a substrate of Rho-kinase.[17] Non-immune mouse or rabbit IgG was used as a negative control. We semi-quantitatively assessed the extent of ROCK1, ROCK2, and phosphorylated MYPT1, using the following scale: 0, none; 1, slight; 2, moderate; and 3, high.[14]

Statistical analysis

All results are expressed as mean ± SEM. Comparison of the CAG data between the BMS and the PES in the two treatment groups was performed by unpaired Student's *t*-test and Mann–Whitney U-test, in which two-sided tests were used. The histological and immunohistological data were analysed by Mann–Whitney U-test. Statistical analyses were performed with SigmaStat for Windows version 3.00.0 (SPSS Inc, Chicago, IL, USA). A value of $P < 0.05$ was considered to be statistically significant.

Results

Plasma levels of nifedipine

At 3 weeks during the treatment, plasma levels of NIF were increased to 658 ± 133 ng/mL ($n = 4$), which is within the therapeutic range of the CCB.[19] When re-examining 24 h after discontinuation at the CAG study, the plasma levels of NIF were negligible (1.1 ± 0.07 ng/mL; $n = 4$).

Nifedipine suppresses paclitaxel-eluting stent-induced coronary hyperconstricting responses

No difference was noted in the stent implantation procedure between BMS and DES in both groups, including reference vessel diameter, stent diameter, and length or overstretch ratio (*Table 1*). Four weeks after the stent implantation, intracoronary serotonin caused coronary hyperconstriction at the proximal and distal edge segments of the PES site when compared with the BMS site, which was abolished by the intracoronary pre-treatment with hydroxyfasudil in the control group (*Figure 1A–C*). In contrast, in the NIF group, coronary vasoconstricting responses to serotonin were mild and comparable between the PES and the BMS sites (*Figure 1D–F*). Quantitative coronary angiography demonstrated that the coronary hyperconstricting responses to serotonin at the PES site, which were abolished by hydroxyfasudil, were significantly attenuated by the NIF treatment (group data in *Figure 2*, individual data in Supplementary material online, *Figure S2*, and diameter data in Supplementary material online, *Table S1*).

Coronary vasodilating responses to bradykinin did not differ significantly between the control group (PES 1.6 ± 1.1 vs. BMS 1.6 ± 0.6% from the baseline) and the NIF group (PES 0.7 ± 3.0 vs. BMS 1.1 ± 1.4% from the baseline). Similarly, the responses to bradykinin with and without pre-treatment of L-NMMA did not differ significantly between the two groups (data not shown). Coronary vasodilating responses to nitroglycerine were also comparable between the control group (PES 3.5 ± 1.5 vs. BMS 3.8 ± 1.3%) and the NIF group (PES 7.3 ± 2.7 vs. BMS 9.1 ± 2.5%).

Nifedipine suppresses paclitaxel-eluting stent-induced inflammatory responses and microthrombus formation

Histological analysis demonstrated that neointimal formation of the stented coronary segments was significantly suppressed in the PES site compared with the BMS site but was comparable between the control and the NIF groups (*Figures 3A–D*, and *4A*). However, the inflammatory responses at the PES site were significantly suppressed in the NIF group compared with the control group (*Figures 3E–H* and *4B*). Although the microthrombus formation was more enhanced at the PES site compared with the BMS site in both groups, the NIF treatment reduced it in the PES site (*Figures 3F*, *H* and *4C*).

Table 1 Procedural and angiographic findings

	BMS control, $n = 7$	PES control, $n = 7$	*P*-value	BMS NIF, $n = 7$	PES NIF, $n = 7$	*P*-value
Reference vessel diameter (mm)	2.57 ± 0.07	2.45 ± 0.06	0.25	2.32 ± 0.05	2.34 ± 0.09	0.80
Stent diameter (mm)	2.66 ± 0.08	2.54 ± 0.08	0.34	2.39 ± 0.05	2.43 ± 0.04	0.21
Stent length (mm)	15.4 ± 0.6	15.4 ± 0.6	1.0	14.3 ± 0.8	14.3 ± 0.8	1.0
Overstretch ratio	1.04 ± 0.04	1.05 ± 0.03	0.90	1.03 ± 0.02	1.06 ± 0.02	0.34
Proximal	0.96 ± 0.05	0.91 ± 0.03	0.17	0.98 ± 0.04	0.97 ± 0.03	0.90
Distal	1.18 ± 0.05	1.26 ± 0.05	0.29	1.15 ± 0.01	1.20 ± 0.02	0.06
Maximum inflation pressure (a.t.m.)	10.1 ± 1.03	9.0 ± 0.42	0.34	10.1 ± 0.51	11.1 ± 1.22	0.81

Results are expressed as mean ± SEM. Stent diameter was calculated by averaging the diameters at the proximal edge, mid-portion, and distal edge of the stented coronary artery. Overstretch ratio, stent diameter divided by reference vessel diameter; proximal overstretch ratio, proximal stent diameter divided by proximal reference vessel diameter; distal overstretch ratio, distal stent diameter divided by distal reference vessel diameter. Nominal pressure was 9 atm for both BMS and PES.
BMS, bare-metal stent; PES, paclitaxel-eluting stent; NIF, nifedipine.

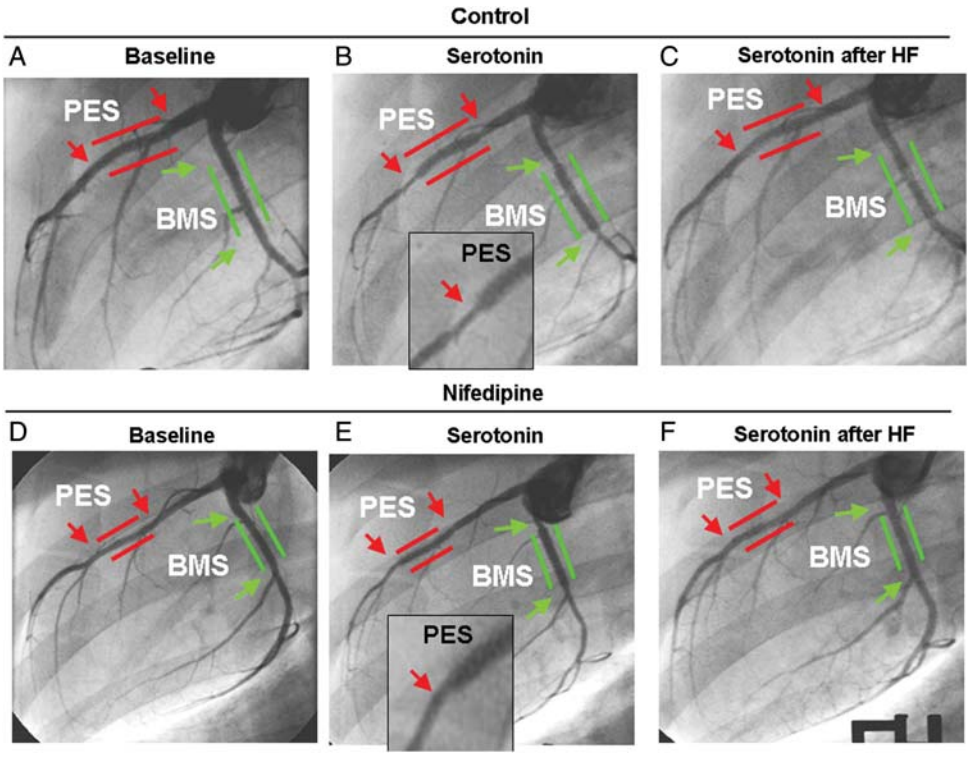

Figure 1 Nifedipine attenuates paclitaxel-eluting stent-induced coronary hyperconstricting responses. Representative left coronary angiograms of the control group (*A*–*C*) and the nifedipine (NIF) group (*D*–*F*), under baseline condition (*A* and *D*), after intracoronary serotonin (100 μg/kg, i.c.) without (*B* and *E*) and with hydroxyfasudil (HF, 300 μg/kg, i.c.) (*C* and *F*). Red lines indicate the site of paclitaxel-eluting stent implantation and green lines the site of bare-metal stent implantation. Red arrows indicates the proximal and distal edges of paclitaxel-eluting stent and green arrows those of bare-metal stent. Magnified images of the distal edge of paclitaxel-eluting stent and bare-metal stent are shown in the boxes of (*B*) and (*D*).

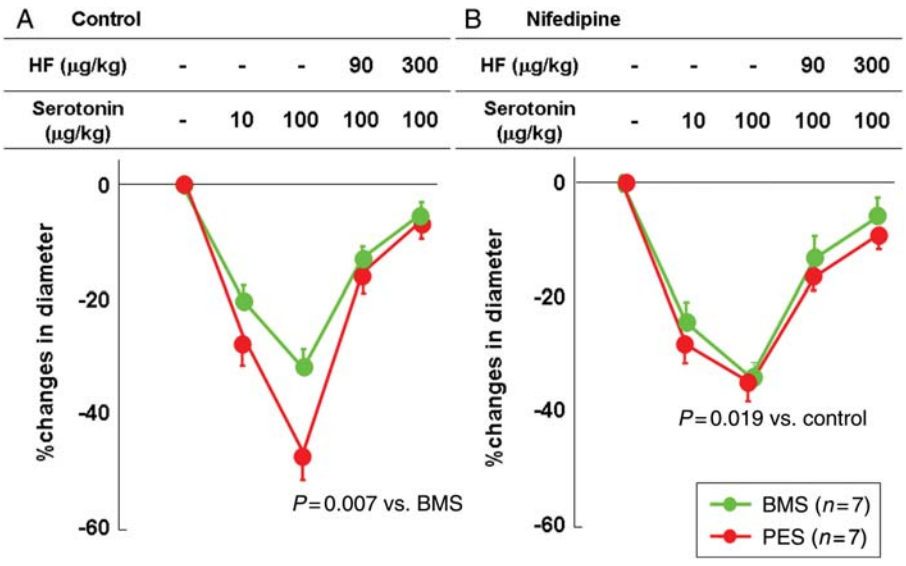

Figure 2 Nifedipine attenuates paclitaxel-eluting stent-induced coronary hyperconstricting responses. Coronary vasoconstricting responses to intracoronary serotonin before and after the pre-treatment with hydroxyfasudil (HF) at the stent edges in the control group (*A*) and the nifedipine (NIF) group (*B*). The mean value of vasomotor responses of the proximal and the distal stent edge is presented. The vasoconstricting responses are expressed as % changes in diameter from the level with nitroglycerine (10 μg/kg, i.c.). Results are expressed as mean ± SEM.

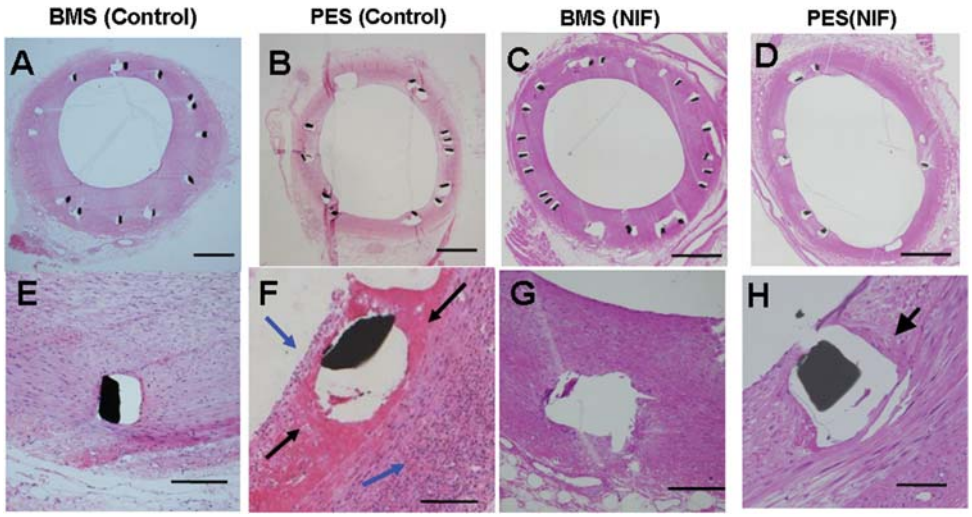

Figure 3 Histology of stented porcine coronary arteries. Representative photomicrographs of bare-metal stent- and paclitaxel-eluting stent-implanted porcine coronary arteries in the control and the nifedipine groups. Scale bars represent 1 mm (*A–D*) and 200 μm (*E–H*). Neointimal formation was suppressed in the paclitaxel-eluting stent-treated arteries compared with the bare-metal stent-treated arteries in both the control and the nifedipine groups (*A–D*). In contrast, enhanced inflammatory cell infiltration and microthrombus formation (*E–H*) at the paclitaxel-eluting stent site were attenuated in the nifedipine group. Blue arrows indicate inflammatory cell infiltration and black arrows microthrombus formation.

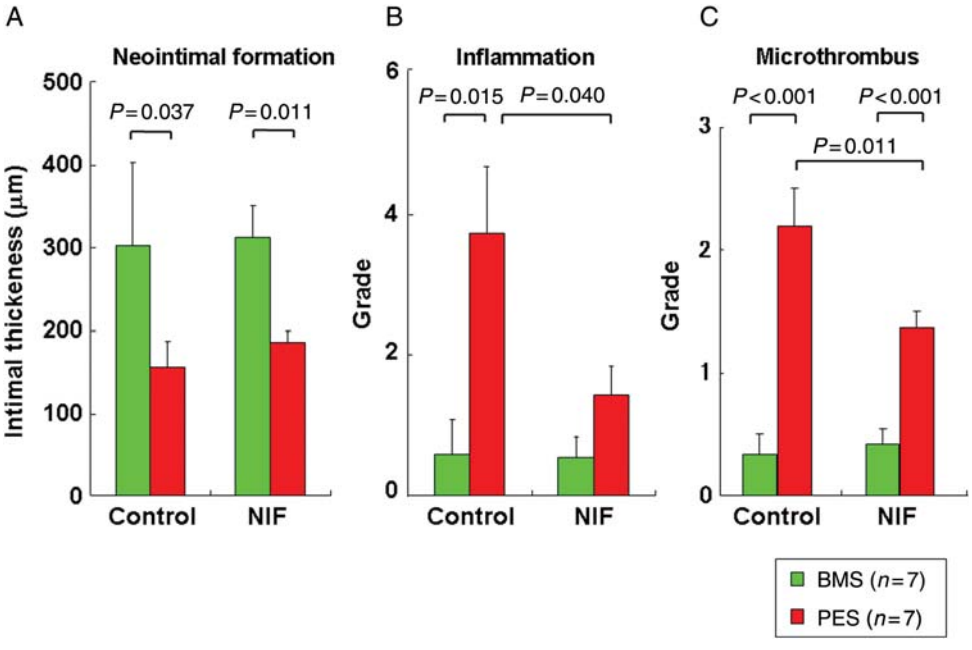

Figure 4 Nifedipine attenuates paclitaxel-eluting stent-induced coronary inflammatory responses. Semi-quantitative analysis of neointimal formation (*A*), inflammatory responses (*B*), and microthrombus formation (*C*) at the bare-metal stent- and paclitaxel-eluting stent-implanted arteries in the control and the nifedipine (NIF) groups ($n = 7$ each). Enhanced inflammatory response at the paclitaxel-eluting stent sites were abolished in the nifedipine group.

Nifedipine suppresses Rho-kinase expression and activity in the coronary artery

Immunohistochemistry showed that the expressions of ROCK1 (*Figure 5A* and *B*), ROCK2 (*Figure 5E* and *F*), and phospho-MYPT1 (*Figure 5I* and *J*) were enhanced at the PES site compared with the BMS site in the control group. The localization of the ROCK immunoreactivities was evident around the struts of PES (see Supplementary material online, *Figure S3*). In contrast, those enhancements of Rho-kinase expression and activity in the PES sites were abolished in the NIF group (*Figure 5C*, *D*, *G*, *H*, *K*, and *L*). Semi-quantitative analysis of ROCK1, ROCK2, and phospho-MYPT1 demonstrated that the PES-induced enhancement of Rho-kinase expression and activity were significantly attenuated in the NIF group compared with the control group (*Figure 6*).

Discussion

The major findings of the present study were that (i) chronic treatment with long-acting NIF suppressed the PES-induced coronary hyperconstricting responses in pigs *in vivo*, (ii) NIF suppressed microthrombus formation and enhanced inflammatory responses at the PES site, and (iii) enhanced expression and activity of Rho-kinase at the PES sites were significantly attenuated in the NIF group. To the best of our knowledge, this is the first study demonstrating the inhibitory effects of long-acting NIF on PES-induced coronary hyperconstricting responses and atherothrombotic changes of the coronary artery, for which suppression of Rho-kinase pathway may be involved.

Rho-kinase activation and drug-eluting stents-induced coronary hyperconstricting responses

We have previously demonstrated that enhanced Rho-kinase activity plays a central role in the pathogenesis of coronary vasospasm in porcine models,[9,12,13] patients with VSA,[10,11] and DES-induced coronary hyperconstricting responses in pigs.[17] The present study confirms our previous findings that (i) DES enhanced coronary vasoconstricting responses when compared with BMS, (ii) the DES-induced coronary hyperconstricting responses were abolished by a Rho-kinase inhibitor, hydroxyfasudil, and (iii) Rho-kinase expression and activity were increased at the peri-stent sites of DES.[17]

It should be noted that SES and PES have already been deployed in millions of patients worldwide. The impairment of coronary vasomotor function is associated with increased cardiovascular risks,[16,26] and the risk of death was increased with the use of DES to a greater extent than BMS.[3,27] Thus, it is important to develop adjunctive medical treatment to improve coronary vasomotion in patients implanted with DES.

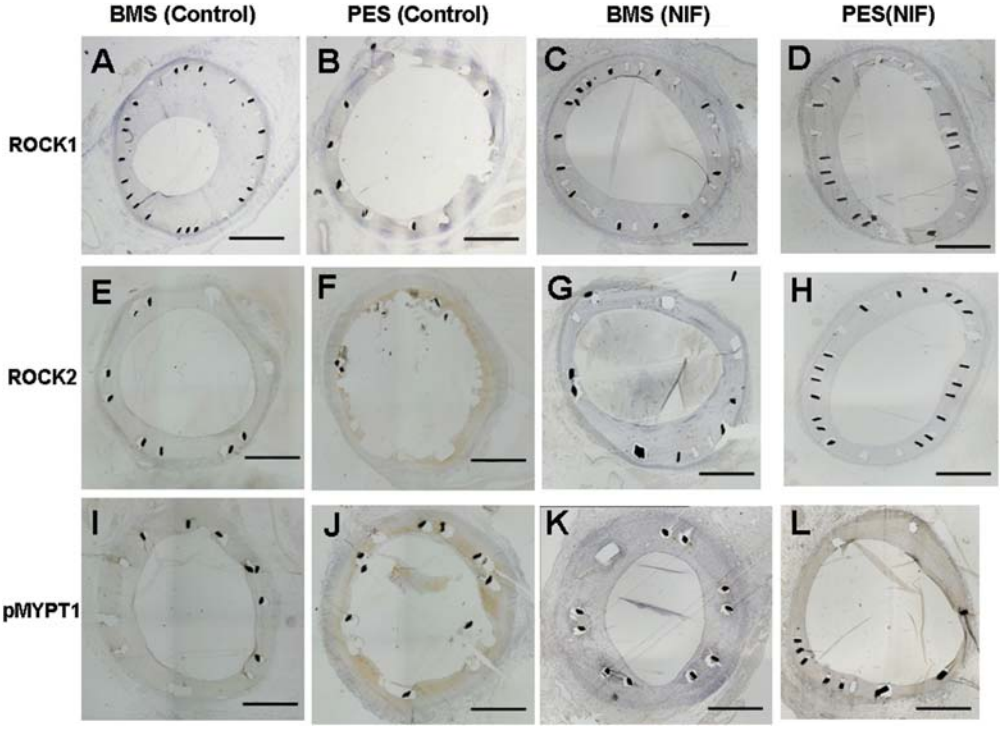

Figure 5 Representative pictures of immunohistochemistry for Rho-kinase expression and activity of stented porcine coronary arteries. Representative immunohistochemistry of ROCK1 (*A*–*D*), ROCK2 (*E*–*H*), and phospho-MYPT1 (*I*–*L*) in the bare-metal stent-treated arteries and paclitaxel-eluting stent-treated arteries in the control and the nifedipine (NIF) groups. Scale bars represent 1 mm.

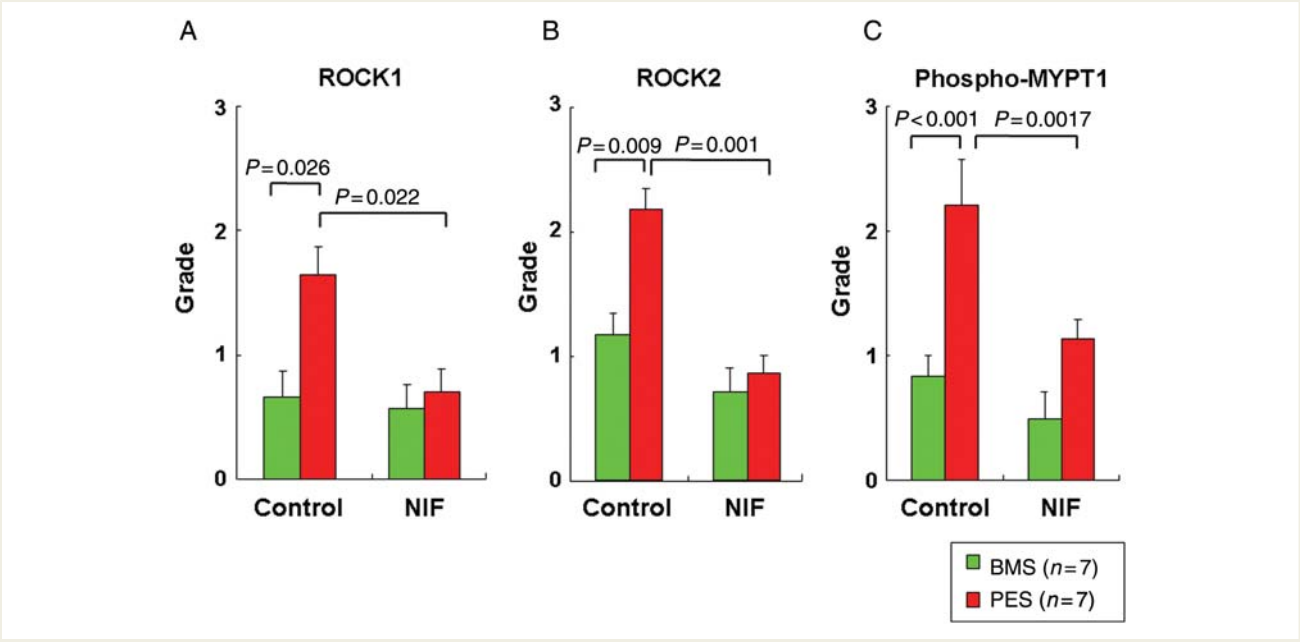

Figure 6 Nifedipine suppresses Rho-kinase expression and activity in porcine coronary arteries at the paclitaxel-eluting stent-implanted site. Immunohistochemical semi-quantitative analysis of ROCK1 (*A*), ROCK2 (*B*), and phospho-MYPT1 (*C*). In the control group, the expression of ROCK1 and ROCK2, and Rho-kinase activity evaluated by the expression of phospho-MYPT1 were all enhanced at the paclitaxel-eluting stent sites. In contrast, in the nifedipine (NIF) group, those changes were abolished.

Inhibitory effect of long-acting nifedipine on paclitaxel-eluting stent-induced coronary hyperconstriction

In the present study, plasma levels of NIF were within its therapeutic range at 3 weeks and were negligible at the CAG study. Although we did not directly measure the tissue levels of NIF in the stented coronary artery, it was previously reported that the accumulation in the aorta or femoral artery a day after discontinuing NIF was quite low (<0.02 μg/g tissue).[28] Thus, the beneficial effect of NIF was not related to its direct inhibitory effects on coronary vasoconstriction, but rather related to its chronic vasculoprotective effects. Importantly, the vasculoprotective effects of NIF have been reported in the clinical studies with patients with coronary artery disease (ENCORE I and II).[20,29] Indeed, NIF has been shown to inhibit vascular inflammation, up-regulation of pro-inflammatory cytokines, and reactive oxygen species production[30–32] and to up-regulate NO synthesis.[19,33,34]

Possible mechanisms of the inhibitory effects of nifedipine on Rho-kinase activation

Excess inflammatory response and thrombus formation were noted at the DES-implanted coronary arteries in pigs [17,35] and humans.[36] In the present study, these pathological changes at the DES sites were significantly suppressed by the chronic treatment with long-acting NIF associated with reduced expression and activity of Rho-kinase in the coronary artery. Taken together with the inhibitory effect of hydroxyfasudil on DES-induced hyperconstricting responses,[17,35] the vasculoprotective effects of NIF are mediated, at least in part, by inhibition of Rho-kinase pathway. Indeed, Rho-kinase pathway is activated by inflammatory stimuli, such as angiotensin II and interleukin-1β through protein kinase C/nuclear factor-κB pathway,[37] suggesting that DES-induced inflammatory responses enhance Rho-kinase activity. Coronary microthrombus formation may also be caused by Rho-kinase activation through local platelet activation with a resultant release of serotonin and platelet-derived growth factors and subsequent interaction with thrombin.[8,38] In addition to the indirect effects, the direct effects of NIF on Rho-kinase pathway may also be involved. An increase in intracellular Ca^{2+} by L-type voltage-gated Ca channels may also activate Rho-kinase pathway.[39,40] Importantly, it has been recently demonstrated that infusion of CCBs reduces blood pressure with suppression of vascular PI3K-C2α-Rho activity and MYPT1 phosphorylation in hypertensive spontaneously hypertensive rats[41] and that CCBs could inhibit ROCK activity in circulating neutrophils in patients with hypertension.[42] Further studies are needed to elucidate the detailed mechanisms of the interactions between CCBs and Rho-kinase pathway.

Clinical implications

First generation of DES exerts high anti-restenotic efficacy when compared with BMS.[1,2] However, long-term polymer residue due to its non-bioabsorbing durable nature is implicated in a delay in structural and functional healing of stented coronary segment.[43] Thus, biocompatible and bioabsorbable polymers have been developed.[44] In fact, the recent studies demonstrated

that new generation biolimus-eluting stent with bioabsorbance polymers preserves coronary vasomotion when compared with SES and PES.[45,46] In addition to developing innovative devices, Rho-kinase inhibitors[8,11,15] and vasculoprotective CCBs, which are the mainstay of the current clinical practice for coronary artery disease in general and coronary vasospasm in particular,[18] may help us to optimize the efficacy and safety of DES, especially for patients implanted with the first generation of DES.

Study limitations

Several limitations should be mentioned for the present study. First, the present study consisted of a relatively small number of experimental animals and was performed in normal juvenile pigs without pre-existing atherosclerotic coronary lesions, in which vascular function at the remote distal segment of stent was preserved. Secondly, in the present study, we used intracoronary serotonin administration in order to examine coronary vasomotor responses because of the species dependence in endothelium-dependent agonists.[47,48] It is known that in porcine coronary arteries, acetylcholine does not cause endothelium-dependent relaxations due to the lack of cholinergic receptors on the endothelium,[49] whereas serotonin causes the responses through endothelial serotonergic receptors.[50] Finally, we only used a single dose of NIF in the present study. Thus, dose-dependent effects of NIF on the DES-induced hyperconstricting responses remain to be examined with a special reference to its clinical use for DES-implanted patients.

Conclusion

In conclusions, the present study demonstrates that chronic treatment with long-acting NIF suppresses PES-induced coronary hyperconstricting responses and inflammatory changes, at least in part, through Rho-kinase pathway inhibition in pigs *in vivo*.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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