Materials and Methods (for online only)

Hironori Uzuka, MD, PhD;1 Yasuharu Matsumoto, MD, PhD;1 Kensuke Nishimiya, MD, PhD;1,2
Kazuma Ohyama, MD;1 Hideaki Suzuki, MD, PhD;1,3 Hirokazu Amamizu, MD;1
Susumu Morosawa, MD;1 Michinori Hirano, MD, PhD;1 Tomohiko Shindo, MD, PhD;1
Yoku Kikuchi, MD, PhD;1 Kiyotaka Hao, MD, PhD;1 Takashi Shiroto, MD, PhD;1
Kenta Ito, MD, PhD;1 Jun Takahashi, MD, PhD;1 Koji Fukuda, MD, PhD;1
Satoshi Miyata, PhD;1 Yoshihito Funaki, PhD;4 Hatsue Ishibashi-Ueda, MD, PhD;5
Satoshi Yasuda, MD, PhD;6 Hiroaki Shimokawa, MD, PhD.1

Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan,1 Wellman Center for Photomedicine, Massachusetts General Hospital, Boston USA,2 Division of Brain Sciences, Department of Medicine, Imperial College London, United Kingdom,3 Cyclotron and Radioisotope Center, Tohoku University, Sendai, Japan,4 and Departments of Pathology5 and Cardiovascular Medicine,6 National Cerebral and Cardiovascular Center, Suita, Japan.

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Address for correspondence:
Hiroaki Shimokawa, MD, PhD.
Professor and Chairman
Department of Cardiovascular Medicine
Tohoku University Graduate School of Medicine
1-1 Seiryo-machi, Aoba-ku
Sendai, Japan 980-8574
(Tel) +81-22-717-7152, (Fax) +81-22-717-7156
(E-mail) shimo@cardio.med.tohoku.ac.jp
Detailed Materials and Methods

All animal care and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH Publication, 8th Edition, 2011), and were approved by the Institutional Committee for Use of Laboratory Animals of Tohoku University (2015MdA-067).

We conducted 2 experimental protocols. In the protocol 1 (Figure 1A), we examined whether Drug-eluting stents (DES)-induced coronary hyperconstricting responses are associated with enhanced coronary adventitial sympathetic nerve fibers (SNF) formation. In the protocol 2 (Figure 1B), we examined whether catheter-based renal denervation (RDN) affects sympathetic autonomic nervous system and whether RDN suppresses coronary hyperconstricting responses after DES implantation in pigs in vivo.

Protocol 1
Male pigs (weighing 25 to 30 kg, N=6) were pre-treated with aspirin (200 mg/day, per os (PO)) and clopidogrel (225 mg/day, PO) for 2 days before stent implantation (Figure 1A). After sedation with medetomidine (0.1 mg/kg, intramuscular injection (IM)) and midazolam (0.2 mg/kg, IM), followed by inhaled sevoflurane (2–5%) and heparinization (5,000 U, intravenous injection (IV)), each animal received percutaneous coronary intervention with an everolimus-eluting stent into the left anterior descending coronary artery (LAD) or the left circumflex coronary artery (LCX) randomly (N=6 each). The untreated coronary artery was used as a control artery. To avoid the influence of major side branches (e.g. diagonal and posterolateral branches) on the precise measurement of quantitative coronary angiography (QCA), the stents were deployed across those branches. Balloon inflation ratio was adjusted to achieve an overstretch ratio of 1.0–1.1 under the guidance of intravascular ultrasound (View IT, Terumo Corporation, Tokyo, Japan). The dual anti-platelet therapy with aspirin (100 mg/day, PO) and clopidogrel (75 mg/day, PO) was continued until euthanasia. At 1 month after stent implantation, we performed coronary angiography (CAG) to examine coronary vasomotion. After CAG study, the animals were euthanized with a lethal dose of potassium chloride (0.25 mEq/kg, IV) under deep sedation with inhaled 5% sevoflurane. The heart was removed and prepared for histological analysis for coronary adventitial SNF and vasa vasorum.

Protocol 2
Male pigs (N=18) were pre-treated with aspirin (200 mg/day, PO) and clopidogrel (225 mg/day,
PO) for 2 days before stent implantation (Figure 1B). After sedation with medetomidine and midazolam, followed by inhaled sevoflurane and heparinization, arterial blood pressure and heart rate were continuously recorded and arterial blood samples were withdrawn. Each animal received percutaneous coronary intervention with everolimus-eluting stents into the LAD and the LCX. After the DES implantation, pigs were randomly assigned to either RDN or sham group (N=9 each). The anti-platelet therapy with aspirin (100 mg/day, PO) and clopidogrel (75 mg/day, PO) was continued until euthanasia. At 1 month after the procedure, blood pressure and heart rate were again recorded and arterial blood samples were withdrawn. We also performed microneurography to evaluate the muscle sympathetic nerve activity. We subsequently performed CAG and renal artery angiography to examine coronary vasomotion and patency of renal arteries. After the angiographies, the animals were euthanized with a lethal dose of potassium chloride (0.25 mEq/kg, IV) under deep sedation with inhaled 5% sevoflurane. The abdominal tissue, the brain, and the heart were removed and prepared for histological and autoradiographic analysis.

RDN Procedure
Following the DES implantation, RDN or sham procedure was performed. After renal artery angiography was performed with a 6-French guiding catheter, the radiofrequency probe (an open-irrigated 3.5-mm-tip catheter, Thermocool, Biosence-Webster, Inc., Diamond Bar, CA, USA) was advanced to 7 points for each renal artery and was energized for 120 sec each at 8~10 Watts in the RDN group or 0 Watts in the sham group with an irrigation flow of 20 ml/min and limiting impedance decrease of 10 Ω. During the procedure, electrophysiological parameters, such as power output, localized impedance, and localized temperature were monitored at every 10 or 20 secs. The radiofrequency ablation therapy was applied as 4-quadrants (circumferential) along the bilateral renal arteries from the distal site at the bifurcation to the main trunk. Final angiography was performed to exclude vessel wall dissection and to document vessel patency and kidney perfusion. After 1 month, the animals again underwent renal artery angiography to confirm vessel patency before euthanasia.

Histological Analysis of the Renal Arteries
To examine the effects of RDN on renal SNF, we performed histological analysis of bilateral renal arteries, as previously described. Briefly, a tissue block including the dorsal muscles, aorta, bilateral kidneys, and bilateral renal arteries was removed as one block and was set into a surface to ensure tissue fixation and structural integrity. The whole tissue was immersed in 10% neutral buffered formalin for at least 24 hours. After fixation, the aorta was dissected to expose the renal ostia. The renal artery and approximately 1.5~2 cm of the
surrounding retroperitoneal connective tissues were isolated and sampled sequentially from the ostium to the hilus approximately every 3 mm. Three samples per renal artery, which were located at proximal, mid and distal sites, were chosen for serially cutting at 3~5 μm and used for Masson’s trichrome staining and immunohistochemistry of SNF using rabbit anti-tyrosine hydroxylase (TH) (pSer40) antibody (1:200) (AHP912, AbD Serotec, North Carolina, USA). Percentage of renal nerves affected by the RDN procedure was evaluated by the degree of TH staining intensity in the renal nerves (0=negative, fully damaged; 1=weak (blush), highly damaged; 2=mild, mildly damaged; 3=moderate, almost no changed; and 4=strong, no changed). Furthermore, Grades 0 to 2 were defined as affected nerves and Grade 3 and 4 were defined as not affected. In a total of 54 slides (bilateral proximal, mid, and distal parts of renal arteries in 9 pigs), every renal nerve observed was examined. Moreover, one pig received RDN procedure and euthanized 2 hour later for the assessment of acute effect of RDN on variability of the endothelium of the renal arteries using 1,3,5-triphenyl tetrazolium chloride staining.

**Autoradiography of the Brainstem**

To examine the brain target of RDN, we performed in vitro autoradiography, as previously described. Briefly, the whole brain was removed and immersed in 10% neutral buffered formalin for at least 24 hours. After fixation, under the guidance of an atlas of the pig brain, 5-mm thick coronal sections for the nucleus tractus solitarius were chosen for this study. Sections were cut at 10-μm thick at -20 °C and stored at -80 °C until use. For autoradiographic analysis, the sections were kept at room temperature for 1 hour and then preincubated for 30 min at room temperature in 50 mmol/L Tris HCl (pH 7.4) containing 10 mmol/L MgCl₂ and 0.1 μmol/L imipramine in glass Coplin jars to remove endogenous catecholamines. Subsequently, sections were incubated for 60 min at room temperature in Tris-HCl (pH 7.4) containing [³H]rauwolscine, which is a specific radioligand for α₂-adrenergic receptor binding sites (32.0 nmol/L, 2.9 TBq/mmol) (NET722, PerkinElmer, Waltham, Massachusetts), 10 mmol/L MgCl₂, 10 nmol/L imipramine, 10 μmol/L pargyline, 0.01% ascorbic acid, and 0.3 μmol/L 5-hydroxytryptamine, which was added to prevent the binding of [³H]rauwolscine to 5-hydroxytryptamine-1A receptors. Non-specific binding of [³H]rauwolscine was measured in the presence of 5 μmol/L (-)-epinephrine. Following intubation, sections were washed three times in ice-cold buffer for 5 min each and then dipped in ice-cold distilled water briefly. The tissue sections were rapidly dried using a stream of cool air and opposed to a tritium-sensitive imaging plates (BAS IP TR 2025 E, Fuji Photo Film, Tokyo) for 72 hours. At the end of the exposure period, autoradiographic images were obtained using an imaging plate system (Fujix Bio-Imaging Analyzer BAS5000, Fuji Photo
Guided by the atlas, a region of interest (ROI) was set at the nucleus tractus solitarius region. The bindings of [\(^3\)H]rauwolscine was estimated by the photo-stimulated luminescence (PSL) within the ROI. The amount of the binding of [\(^3\)H]rauwolscine was expressed as the PSL divided by the ROI area.

### Microneurography for Sympathetic Nerve Activity

At 1 month after the RDN or sham procedure, we performed microneurography to evaluate the muscle sympathetic nerve activity in pigs in vivo. After exposure of the femoral nerve trunk with skin incision at inguinal area, a fine tungsten microelectrode (model UJ-100-0.2-5.0, about 5 MΩ, Unique Medical Inc., Tokyo, Japan) was gently and carefully inserted into the femoral nerve several times by manual operation until a “spontaneous, intermittent burst” signal, which is one of the general characteristics of the peripheral sympathetic nerve signal, was noted. The original microneurographic signal was filtered (0.5~5 kHz band-pass filter constructed with model UA-200, Unique Medical) and digitally stored (PL3516, PoweraLab16/35, ADInstruments, Australia). The original signal was integrated to assess muscle sympathetic nerve activity with LabChart7 (ADInstruments). The extent of muscle sympathetic nerve activity level was expressed as the number of bursts per minute.

### Blood Pressure and Blood Analysis

Arterial blood pressure and heart rate were recorded and arterial blood samples were withdrawn from the femoral artery sheath before RDN or sham procedure and at 1 month after the procedure (Figure 1B). Serum levels of sodium, potassium, chloride, blood urea nitrogen and serum creatinine were measured (BML Inc., Tokyo, Japan). Plasma renin activity and plasma levels of aldosterone were also measured (BML Inc.).

### In Vivo Assessment of Coronary Vasomotion after DES Implantation

At 1 month after DES implantation, we performed CAG to examine coronary vasomotion in pigs in vivo. After control CAG, we examined coronary vasoconstricting responses to serotonin (10 and 100 μg/kg, IC) were examined before and after hydroxyfasudil (30 and 100 μg/kg, IC, for 3 min), a specific Rho-kinase inhibitor (Asahi Kasei Pharma, Tokyo, Japan). Coronary vasodilating responses to nitroglycerin (10 μg/kg, IC) and bradykinin (0.1 μg/kg, IC) before and after NG-monomethyl-L-arginine (1 mg/kg, IC for 10 min) were examined. QCA (INFX-8000V, Toshiba Medical Co., Tokyo, Japan) was performed in a blind manner at the proximal and distal edge segments adjacent to the stent as previously described. Potential errors for QCA analysis may have occurred. Nevertheless, in 10 randomly selected CAG frames, we examined the accuracy of our QCA analysis as previously described.
interobserver and intraobserver variability for the measurement of QCA in the present study was 4.3±0.9% and 2.7±0.7%, respectively, confirming an acceptable reproducibility and validates the results of our QCA analysis.

**Histological Analysis of the Coronary Arteries**

The removed heart was prepared for histological analysis (Figure 1A, B). For fixation of the coronary arteries, saline and 10% neutral buffered formalin were infused into the left coronary arteries via a constant perfusion pressure system (120 cm H₂O). After the fixation, the stented coronary artery and the non-stented control artery were isolated for histological analysis. The vessels were separated into the proximal and distal stent edges for paraffin sections with 3~5 μm thickness. The paraffin sections were stained with Masson’s trichrome staining. Immunohistochemical staining was performed by using rabbit anti-human von Willebrand factor antibody (1:3) (N1505; Dako, Copenhagen, Denmark) for vasa vasorum and rabbit anti-TH antibody for SNF. The densities of vasa vasorum and SNF were expressed as the number of von Willebrand factor-positive microvessels and TH-positive fibers divided by the adventitial area (/mm²), respectively. Immunoreactivities for neuropeptide Y and nerve growth factor in SNF were also examined by using rabbit anti-nerve growth factor antibody (1:200) (Ab6199; Abcam) and rabbit anti-neuropeptide Y antibody (1:16,000) (N9528; Sigma, Saint Louis, Missouri). Semi-quantitative analysis regarding the extents of neuropeptide Y and nerve growth factor expressions was evaluated for each TH-positive SNF by using the following scale; 1=slight; 2=moderate; and 3=high.16

In the protocol 2, we performed additional histological analysis as shown below (Figure 1B). Hematoxylin-eosin, toluidine blue for mast cells, and immunohistochemical stainings were also performed by using mouse anti-CD68 antibody [ED1] (1:50) (Abcam, Cambridge, UK) for macrophages, goat anti-human interleukin-1β (1:50) (AB-201-NA; R&D Systems, Minneapolis, Massachusetts), mouse anti-human Rho-kinase β (ROCK1) antibody (1:50) (61136; BD Biosciences, San Jose, California), mouse anti-Rho-kinase α (ROCK2) antibody (1:50) (610624; BD Biosciences), rabbit anti-human phosphorylated myosin phosphatase target subunit-1 antibody (1:50) (07-251; Millipore, Billerica, Massachusetts), a substrate of Rho-kinase.1,2,4

The density of mast cells was expressed as the number of toluidine blue-positive cells divided by the adventitial area (/mm²). The densities of macrophages and interleukin-β-positive cells were expressed as the number of CD68 and interleukin-β-positive-cells divided by the observation field number (/HPF), respectively.1,2,4 Semi-quantitative analysis regarding the extent of ROCK1, ROCK2 and phosphorylated myosin phosphatase target subunit-1 was evaluated for each radial subpart per 1 section by using the following scale;
0=none, 1=slight, 2=moderate, and 3=high, as previously described.\textsuperscript{2,4,14} Each section was divided into a total 6 radial subparts. For morphometric analysis, each parameter was manually measured by using Image-J (U.S. National Institute of Health, Bethesda, Maryland). Adventitial area was calculated by the following formula; [area outside the external elastic lamina (EEL) within a distance of the thickness of neointima plus media – EEL area].\textsuperscript{2,17}

**Statistical Analysis**

Results are expressed as mean±standard error of mean (SEM) or Tukey boxplots with median values. Throughout the text and figures, N represents the number of pigs, while “n” represents the number of DES edges in the protocol 1, and the number of applications of RDN and renal nerves in the protocol 2. In the protocol 1, we performed hierarchical clustering with Pearson’s correlation as similarity coefficient and the Ward clustering method in order to examine the individual difference in the proximal and distal stent edges in a pig. In this analysis, the values of coronary vasoconstricting responses and histological analysis at the edges were used for clustering, and no evident cluster of proximal/distal pairs of same pig was detected (Figure I in the online-only Data Supplement). Based on this analysis, various changes at the proximal and distal edges in a pig were treated as individual factors in the protocol 1. In the protocol 2, since RDN is a systemic intervention, we evaluated the values by taking average of four sites at the proximal and distal edges in the LAD and LCX with regard to vasoconstricting/vasodilating responses and histology. A comparison of the QCA and histomorphometry was performed by using an unpaired, 2-sided Student’s t-test. Comparison of the semi-quantitative analysis was performed by using a Mann-Whitney U-test. The correlation between the continuous variables was analyzed using a linear regression model. Statistical analysis was performed with IBM SPSS Statistics 20 (IBM, New York, NY, USA). A value of P<0.05 was considered to be statistically significant.
Supplemental References


