Nicorandil Improves Endothelial Function via

Reduction of Oxidative Stress;

Evaluation of in vivo Endothelial Function by

Measurement of Flow-Mediated Dilation in Rats

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Kenichi SERIZAWA

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Kenichi SERIZAWA

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Abstract

Endothelial dysfunction is a powerful surrogate marker of cardiovascular risk. Flow-mediated dilation (FMD) is an indicator of endothelial function and is identified as an independent predictor of future cardiovascular events in clinical settings. Nicorandil, an anti-angina agent with ATP-sensitive potassium channel (K_{ATP} channel) opening and nitrate-like activity, has been shown to improve the prognoses of patients with stable angina pectoris via several mechanisms, including endothelial protective effects. Although patients with angina have many risks for endothelial dysfunction, such as diabetes and drug-eluting stent, it has not been also fully understood about the risks that nicorandil is effective against. The purpose of this study is to evaluate the endothelial protection provided by nicorandil against two risk factors, diabetes and paclitaxel. Paclitaxel was used for drug-eluting stent. Furthermore, I tried to evaluate the endothelial function by FMD.

In chapter 1, I examined the effect of nicorandil on diabetic endothelial dysfunction. Male Sprague-Dawley rats were intraperitoneally injected with streptozotocin (STZ) to induce diabetes. Nicorandil and tempol were administered in drinking water for one week, starting 3 weeks after STZ injection. Endothelial function was evaluated by measuring FMD in the femoral arteries of anaesthetized rats. Femoral arteries were harvested and protein expression was analyzed by western blotting. High glucose-induced reactive oxygen species (ROS) production was measured in cultured human coronary artery endothelial cells (HCAECs). I could observe the reduction of

FMD in diabetic rats, which was improved by nicorandil or tempol. NADPH oxidase and endothelial nitric oxide synthase (eNOS) uncoupling were increased in femoral arteries of diabetic rats, which were inhibited by nicorandil. High glucose-induced ROS production in HCAECs was inhibited by nicorandil, N-omega-nitro-L-arginine methyl ester (L-NAME) or apocynin.

In chapter 2, I examined the effect of nicorandil on paclitaxel-induced endothelial dysfunction. An osmotic pump was implanted subcutaneously into a small pouch between the shoulder blades of rats to continuously infuse paclitaxel. Nicorandil, diazoxide, isosorbide dinitrate (ISDN) or tempol were administered for 1 week from just after implantation of the osmotic pump. FMD was measured at 1 week after implantation of the osmotic pump. Femoral arteries were harvested for real-time PCR. ROS production by paclitaxel was measured in HCAECs and *in vivo* in the mice. I could observe the reduction of FMD by paclitaxel, and nicorandil, diazoxide and tempol, but not ISDN, improved the reduction of FMD. NADPH oxidase was increased in femoral arteries by paclitaxel, which was inhibited by nicorandil. Paclitaxel-induced ROS production in HCAECs was inhibited by nicorandil. This effect was reduced by 5-HD, but not ODQ. ROS production was also observed *in vivo* mice treated with paclitaxel, which could be prevented by nicorandil.

In the present study, I demonstrated that nicorandil improved the reduction of FMD through its anti-oxidative effect in diabetic and paclitaxel-treated rats. Moreover, the anti-oxidative effect of nicorandil

might be exerted via K_{ATP} channel opening. Therefore, nicorandil may improve the prognoses of angina patients with concomitant diabetes and drug-eluting stent treatment by protecting their endothelial function.

Abbreviations

ADMA: asymmetric dimethylarginine

BH_4: tetrahydrobiopterin

DAF-2DA: Diaminofluorescein-2 diacetate

DCF: 2', 7'-dichlorofluorescein

DES: drug-eluting stent

EDHF: endothelium-derived hyperpolarization factor

eNOS: endothelial nitric oxide synthase

FMD: flow-mediated dilation

GCH-1: GTP cyclohydrolase 1

HCAECs: human coronary artery endothelial cells

H_2DCF-DA: 2', 7'-dichlorodihydrofluorescein diacetate

ISDN: isosorbide dinitrate

K_{ATP} channel: ATP-sensitive potassium channel

L-NAME: N-omega-nitro-L-arginine methyl ester

NF-κB: nuclear factor-kappa B

NO: nitric oxide

NOx: nitrate and nitrite

NTG: nitroglycerin

ODQ: 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one

PAI-1: plasminogen activator inhibitor

PES: paclitaxel-eluting stent

PKC: protein kinase C

PVDF: polyvinylidene difluoride

ROS: reactive oxygen species

STZ: streptozotocin

Tempol: 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl

5-HD: 5-hydroxydecanoate

General Introduction

It has become evident that the endothelium plays a crucial role in regulating vascular tone and structure. Common conditions that predispose to atherosclerosis, such as diabetes, hypertension, hypercholesterolemia, are associated with endothelial dysfunction. They may be linked to proinflammatory and prothrombotic endothelial phenotypes (Landmesser *et al.*, 2004). Alterations in endothelial function play a pivotal role in the development and progression of atherosclerosis and its clinical complications. Therefore, endothelial dysfunction is a powerful surrogate marker of cardiovascular risk, and the severity of endothelial dysfunction correlates with a patient's risk for experiencing an initial or recurrent cardiovascular event (Widlansky *et al.*, 2003).

Clinically, endothelial function generally has been evaluated by endothelium-dependent relaxation. Flow-mediated dilation (FMD) is an established method for assessing endothelial function (Celermajer *et al.*, 1992). It is determined from the dilation of conduit arteries following temporary ischemia and reperfusion by release of a forearm-occluding cuff and. Temporary ischemia induces dilation of the downstream microvasculature, then reperfusion increases blood flow (i.e., reactive hyperemia) and wall shear stress (Kelm, 2002; Pyke and Tschakovsky, 2005). These lead to a rapid activation of endothelial nitric oxide synthase (eNOS) that increases formation of nitric oxide (NO), leading to vasodilation (Rassaf *et al.*, 2006). Endothelial function can be evaluated noninvasively using this technique. FMD also has been used to evaluate endothelial function in dogs

and pigs (Jones *et al.*, 2011; Weber *et al.*, 2011). Recently, Heiss *et al.* reported a method to measure endothelial function by FMD in rats (Heiss *et al.*, 2008).

Nicorandil is an antiangina agent with ATP-sensitive potassium channel (K_{ATP} channel) opening and nitrate-like activity. Nicorandil has been shown to improve the prognoses of patients with stable angina pectoris by preconditioning-like cardioprotective effects in the IONA study and the JCAD study (Horinaka *et al.*, 2010; IONA study group, 2002). In addition to the preconditioning-like cardioprotective effects, some recent reports demonstrated that nicorandil can exert endothelial protective effects. Long-term administration of nicorandil significantly improved endothelial function in patients with ischemic heart disease or with cardiovascular risk factors, as evaluated by FMD in forearm arteries (Ishibashi *et al.*, 2008; Sekiya *et al.*, 2005). In swine heart, nicorandil reduced myocardial no-reflow after ischemia reperfusion by protecting endothelial function (Zhao *et al.*, 2006). In human umbilical vein endothelial cells, nicorandil inhibited apoptosis induced by serum starvation by inhibiting production of reactive oxygen species (ROS) (Date *et al.*, 2005). Therefore, it is believed that nicorandil can improve the prognoses of patients with angina due, in part, to protecting endothelial function. However, the endothelial protective mechanisms of nicorandil have not been fully understood. Moreover, the risk factors of endothelial dysfunction against which nicorandil is effective are not clear.

Patients with angina have multiple risk factors for endothelial

dysfunction, such as diabetes, hypertension, hypercholesterolemia, smoking, aging and obesity (Celermajer *et al.*, 1992; Sorensen *et al.*, 1994; Steinberg *et al.*, 1996; Widlansky *et al.*, 2003). In addition to these traditional risk factors, drug-eluting stent (DES) recently have been considered as another potential cause of endothelial dysfunction. DES, which is used for percutaneous coronary intervention, is coated with anti-proliferative compounds, such as paclitaxel to prevent in-stent restenosis. While DES dramatically reduce in-stent restenosis (Stone *et al.*, 2004), the potent anti-proliferative agents have raised concerns regarding endothelial dysfunction (Shin *et al.*, 2007).

Accordingly, the purpose of this study was to elucidate the effects and mechanisms on the endothelial protective effect of nicorandil against two risk factors. In the chapter 1, I examined the effect of nicorandil on diabetic endothelial dysfunction in rats. In the chapter 2, I examined the effect of nicorandil on paclitaxel-induced endothelial dysfunction in rats. In these studies, I tried to evaluate the endothelial function by measurement of FMD in living rats.

Chapter 1

Studies of effect of nicorandil

on diabetic endothelial dysfunction in rats

1-1. Introduction

Diabetes mellitus is regarded as an independent major risk factor for the development of cardiovascular disease, since long-term survival and freedom from cardiac events were reduced in diabetic coronary angioplasty patients (Kasai *et al.*, 2008; Kip *et al.*, 1996; The Japanese Coronary Artery Disease (JCAD) Study Investigators, 2006). Endothelial dysfunction plays a central role in diabetic vascular diseases (Creager *et al.*, 2003). A common mechanism underlying this endothelial dysfunction could involve increased production of ROS in vascular tissue (Gryglewski *et al.*, 1986). High glucose greatly increases endothelial superoxide production (Cai *et al.*, 2005), leading to an eNOS uncoupling state, followed by lower production of NO and more ROS production, which acts to quench NO (d'Uscio *et al.*, 2003; Landmesser *et al.*, 2003; Laursen *et al.*, 2001; Tiefenbacher *et al.*, 2000; Vasquez-Vivar *et al.*, 2002). Reduced NO availability attenuate its beneficial vascular effects such as vasodilation, regulation of vascular smooth muscle proliferation and expression of cellular adhesion molecules that are involved in initiating atherosclerotic plaque formation (Bian *et al.*, 2008). Therefore, increased ROS production with diabetes has been speculated to reduce endothelial NO availability, leading then to endothelial dysfunction (Landmesser *et al.*, 2004; Nakagawa *et al.*, 2011).

Nicorandil is an anti-angina agent that opens K$_{ATP}$ channels and acts like nitrate compounds. It reportedly improves prognoses in patients with angina pectoris via preconditioning effects (IONA study group, 2002). This beneficial effect of nicorandil is exerted across a broad range of patients with

stable angina, for example in diabetic patients (IONA study group, 2004). In general, however, the preconditioning effect is abolished in diabetic animals (Miki *et al.*, 2009; Katakam *et al.*, 2007) and humans (Ishihara *et al.*, 2001). Therefore, it is believed that the cardioprotective effect of nicorandil is mediated by the other mechanisms. Recently, nicorandil has been shown to exert endothelial protective effects in both clinical settings and animal studies. Therefore, I hypothesized that nicorandil can prevent diabetic endothelial dysfunction.

In the present study, I investigated the protective effect of nicorandil on endothelial function in rats with diabetes induced by streptozotocin (STZ). Endothelial function was evaluated by measuring FMD in femoral arteries using a high-resolution ultrasound system under *in vivo* conditions in which blood flow, many humoral factors and nerve activity were maintained. The mechanism underlying the protective action of nicorandil also was investigated in relation to ROS production in the endothelium, both *in vivo* and *in vitro*.

1-2.　Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Japan, Yokohama, Japan, 6 weeks old, 200-240 g) were used in all experiments. All rats were fed ordinary laboratory chow and allowed free access to water under a constant light and dark cycle of 12 h. Diabetes was induced by intraperitoneal administration of STZ (40 mg/kg, Wako Pure Chemicals Co., Osaka, Japan) once a day for 3 days. Glucose concentrations were measured one week after STZ administration. Diabetes was considered to have been induced when the glucose level was higher than 250 mg/dL. Nicorandil [N-(2-hydroxyethyl)nicotinamide nitrate ester] was synthesized in the Chugai Organic Chemistry Laboratory. Nicorandil (15 mg/kg/day) and 4-hydroxy-2,2,6,6- tetramethylpiperidine-N-oxyl (tempol, 20 mg/kg/day, Sigma-Aldrich, St. Louis, MO, USA) were administered in drinking water for one week, starting 3 weeks after STZ administration.

All animal procedures were conducted in accordance with Chugai Pharmaceutical's ethical guidelines for animal care, and all experimental protocols were approved by the Animal Care Committee of the institution and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National institutes of Health.

Measurement of FMD

Blood pressure (tail-cuff method, BP-98A Softron, Tokyo, Japan) and blood glucose were measured four weeks after STZ administration, and the

rats were anaesthetized with thiobutabarbital with constant monitoring of rectal temperature. A catheter was inserted into the jugular vein to administer drugs. The animals were kept stable with a heated sheet and warming lamps.

FMD measurement in rats was described previously (Heiss *et al.*, 2008). The diameter of femoral arteries and Doppler flow were measured using a high-resolution ultrasound system (Vevo 770, VisualSonics, Toronto, Canada). The femoral artery was visualized with a 30- or 40-MHz transducer. After identifying the femoral artery by its characteristic flow pattern, the position of the probe was optimized to clearly show the vessel wall/lumen interfaces and then fixed throughout the investigations. Experiments were started over an equilibration period of 15 min and when body core temperature was stable (37 ± 1°C).

Ultrasound measurements of diameter and Doppler-flow were obtained from longitudinal sections of the femoral artery before and after 5 min of hindlimb ischemia. Ischemia and reperfusion of the hindlimb were achieved with a snare occluder positioned upstream from the site to be visualized, around the common iliac artery, through a trans-abdominal access. The snare occluder consisted of a 5-0 nylon surgical suture around the artery that was passed through a 4 cm PE-200 tube, and the skin was closed with surgical clips. Hindlimb ischemia was created by pulling on the filament through the tube and clamping it.

Baseline recordings were taken after an equilibration period, and then the common iliac artery was occluded with the snare occluder. Flow arrest

was confirmed by abrogation of the Doppler signal. After 5 min of ischemia, the hindlimb was reperfused by releasing the occluder. The changes in flow velocity and the diameter of the femoral artery were monitored at 0, 0.5, 1, 2 and 3 min after reperfusion. In this study, FMD was defined as the peak change in femoral artery diameter measured at 1 min after reperfusion. The clinical guideline to assess FMD defined the peak change in brachial artery diameter as an endothelial function, which occurred about 1 min after reperfusion (Corretti *et al.*, 2002).

To evaluate endothelium-independent vasodilation, nitroglycerin (NTG) was administered to the same rats after FMD measurement, with 10 min breaks between measurements. After obtaining baseline recordings of diameter, NTG (5 μg/kg) was administered intravenously via the jugular vein catheter. Changes in femoral artery diameter were monitored at 10, 30, 60 and 120 sec after administration.

Western blot analysis

Femoral arteries were harvested and frozen in liquid N_2 immediately after isolation and stored in a -80°C freezer until protein expression was measured by Western blotting. The femoral artery was homogenized in a homogenization buffer composed of 25 mM Tris-HCl (pH 7.4), 1 mM dithiolthreitol, 25 mM sodium fluoride, 1 mM sodium orthovanadate, protease inhibitor cocktail tablet, phosphatase inhibitor cocktail and 1% Triton X-100. The homogenates were centrifuged at 14,000 g for 20 min at 4°C. Supernatants were collected, and protein concentrations were

determined using a BCA Protein Assay Kit (Thermo Scientific, Woltham, USA). Equal amounts of protein extracts were separated on 10% SDS-polyacrylamide gel and immobilised on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, USA). To investigate the eNOS uncoupling state, low-temperature SDS-PAGE was performed to detect the dimer and monomer of eNOS. Briefly, protein extracts were mixed with sample buffer (without β-mercaptoethanol), and non-boiled samples were separated on SDS-PAGE. The membranes were blocked in a PVDF Blocking Reagent (TOYOBO, Osaka, Japan), and incubated with anti-p47[phox], anti-eNOS (Santa Cruz Biotechnology, Santa Cruz, USA) or anti-GTP cyclohydrolase 1 (GCH-1) antibodies (Abnova, Taipei, Taiwan). After washing, the membranes were incubated with anti-rabbit or anti-goat IgG conjugated with horseradish peroxidise (Santa Cruz Biotechnology). Immunoreactive signals were visualized with the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific), and detected using a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, USA). Each protein signal was normalized to β-actin expression from the same sample.

Measurement of serum NOx

To determine NO excretion, total nitrate and nitrite (NOx) concentrations were measured by the Griess method with a Total Nitric Oxide Assay Kit (Enzo Life Sciences, Farmingdale, USA). Serum samples were diluted 1:2 into reaction buffer and ultra-filtered through a 10,000 molecular weight cut-off filter. Samples were read at 540 nm with a

Microplate Reader (Molecular Devices, Sunnyvale, USA).

Cell culture and treatment

Normal human coronary artery endothelial cells (HCAECs) were purchased from Lonza Inc. (Walkersville, MD, USA). HCAECs at passages 3-5 were cultured with EBM-2 medium supplemented with 5% fetal bovine serum (Lonza). To measure production of ROS, the cells were seeded onto plastic dishes (1×10^5 cells/2 mL/dish) and cultured as monolayers in a 5% CO_2 humidified incubator at 37°C overnight. After incubation, the HCAECs were exposed to normal (5.6 mM) or high glucose (35.6 mM) for 24 h. Nicorandil (100 μM), apocynin (100 μM, Calbiochem, Darmstadt, Germany) or N-omega-nitro-L-arginine methyl ester (L-NAME, 300 μM, St Louis, Sigma-Aldrich) were added over the same period.

Measurement of ROS production using a fluorescent probe in HCAECs

The production of ROS was monitored using the fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Invitrogen, Carlsbad, USA). Briefly, cultured cells were incubated with 10 μM H_2DCF-DA for 45 min in a 37°C incubator, and 2', 7'-dichlorofluorescein (DCF) fluorescence was then quantified by confocal microscopy (Zeiss Axiovert 200) from at least 20 randomly-selected cells/dish, using three dishes for each experimental condition.

Statistical analyses

All data are expressed as mean ± SE. The n values refer to the number of individual animals on which experiments were performed. The statistical significance of differences was determined using Tukey's Multiple Comparison Test. Probability values less than 0.05 were considered significant. Statistical analyses were performed using SAS version 8.2 software (SAS Institute, Cary, USA).

1-3.　Results

Animals

Body weight, blood glucose, blood pressure and heart rate are shown in Table 1. Body weight increased in each group of rats for 4 weeks after STZ administration, although the increase in the STZ rats was smaller than in normal rats. Blood glucose levels in STZ rats were significantly higher than in normal rats. Systolic blood pressure was slightly increased, while heart rate decreased significantly in STZ rats. Nicorandil appeared not to influence any of these parameters in STZ rats.

Table 1. Body weight, blood glucose, blood pressure and heart rate at 4 weeks after STZ administration

	normal	STZ	STZ+nicorandil
N	7	6	6
Body weight (0 wks, g)	213.2 ± 1.6	212.2 ± 3.4	217.1 ± 4.3
Body weight (4 wks, g)	417.8 ± 8.7	299.4 ± 15.4*	327.7 ± 9.8*
Blood glucose (mg/dL)	96.7 ± 1.7	392.3 ± 17.4*	424.5 ± 16.1*
Systolic blood pressure (mmHg)	121.8 ± 1.8	135.4 ± 5.9	133.5 ± 3.2
Heart rate (bpm)	359.4 ± 11.9	312.4 ± 14.5*	334.5 ± 11.4

*$p<0.05$ vs normal

FMD in STZ-induced diabetic rats

Endothelial function was evaluated by the measurement of FMD in rat femoral arteries. Reperfusion after 5-min of hindlimb ischemia instantly increased flow velocity (i.e. reactive hyperaemia) compared with baseline femoral artery flow, followed by a rapid decay to baseline values at around 3 min (Fig. 1A). The increase in flow velocity was associated with a delayed increase in femoral arterial vasodilation that peaked at 1 min after reperfusion (Fig. 1B). This delayed vasodilation in the femoral artery was defined as FMD. In STZ rats, peak velocity in the femoral artery was lower than that in normal rats (normal, 106.7 ± 5.0; STZ, 49.5 ± 2.5 mm/s; $n = 6\text{-}7$, Fig. 1A) at baseline (i.e. before ischemia). However, hyperaemia, expressed as the % change in peak velocity just after reperfusion, did not differ between normal and STZ rats (Fig. 1D). FMD was lower in STZ rats compared with normal rats (normal, 19.5 ± 1.7; STZ, $9.7 \pm 1.4\%$; $n = 6\text{-}7$, Fig. 1B, E). In evaluating endothelium-independent vasodilatory potency, intravenous administration of NTG led to vasodilation of the femoral artery that peaked at 30 sec (Fig. 1C). NTG-induced vasodilation was similar in normal and STZ rats (Fig. 1C, F).

Nicorandil (15 mg/kg/day) was administered in drinking water for one week starting 3 weeks after STZ administration to evaluate its effects on endothelial function. Nicorandil restored the reduced FMD in STZ rats to almost same level as in normal rats (STZ + Nicorandil, $17.7 \pm 2.6\%$; $n = 6$, Fig. 1B, E), whereas nicorandil affected neither NTG-induced vasodilation nor flow velocity. In normal rats, nicorandil produced no significant changes in

FMD (normal, 18.8 ± 4.0; nicorandil, 20.9 ± 1.7%; n = 5-6, Fig. 1G). These results demonstrated that nicorandil could ameliorate endothelial dysfunction in STZ rats, without changing flow velocity or vascular smooth muscle function.
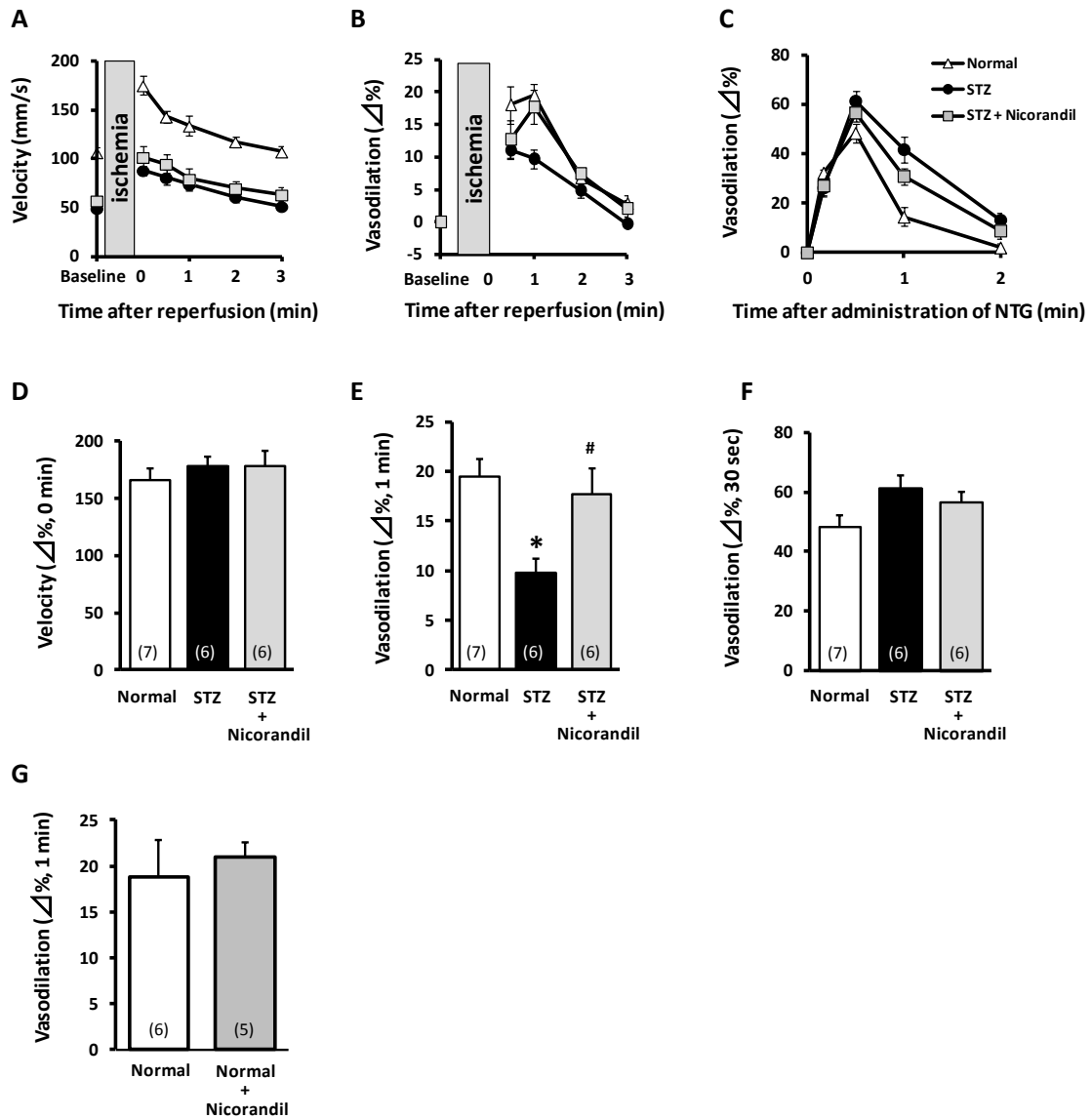
Figure 1. Effect of nicorandil on endothelial dysfunction induced in rats by STZ

Ultrasound measurements were performed at 4 weeks after STZ administration. Time courses of velocity (A), FMD (B) and NTG-induced vasodilation (C) in rat femoral arteries were measured after 5 min ischemia. Percent change in velocity (Δ%, D) and NTG-induced vasodilation (F) were similar among groups. FMD peaked at 1 min after reperfusion and then decreased in diabetic rats, showing it was improved by nicorandil (E). Nicorandil showed no significant changes in FMD of normal rats (G). *$p<0.05$ vs normal, #$p<0.05$ vs STZ (n = 5-7). The numeral in each column is the number of animals.

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Involvement of oxidative stress in decreased FMD in STZ-induced diabetic rats

The effect of tempol was investigated to clarify how oxidative stress was involved in endothelial dysfunction in rats treated with STZ. Tempol (a radical scavenger) significantly reversed the decrease in FMD (STZ, 6.5 ± 1.5; STZ + Tempol, $13.3 \pm 1.4\%$; $n = 6$, Fig. 2). Expression of NADPH oxidase was measured in femoral arteries of STZ rats because it is a potential source of vascular superoxide. In STZ rats, the p47phox protein (a major NADPH oxidase component) increased in the femoral artery. Nicorandil prevented an increase in expression of p47phox (Fig. 3A). Expression of total eNOS was measured in the rat femoral artery to examine how it is associated with endothelial dysfunction. Unexpectedly, total eNOS expression increased in STZ rat femoral arteries, and the increase was prevented by nicorandil (Fig. 3B). However, this increase in total eNOS expression did not raise serum NOx levels in STZ rats, and nicorandil also showed no significant changes (Fig. 4). In oxidative state, reducing tetrahydrobiopterin (BH$_4$) causes eNOS uncoupling state, resulting in production of superoxide by eNOS monomer (Landmesser *et al.*, 2003). In this study, the eNOS dimer/monomer ratio decreased (Fig. 3C), and expression of GCH-I (a synthetase of BH$_4$) also decreased in STZ rat femoral arteries (Fig. 3D). Nicorandil restored the eNOS dimer/monomer ratio and GCH-1 expression to normal values (Fig. 3C, D).
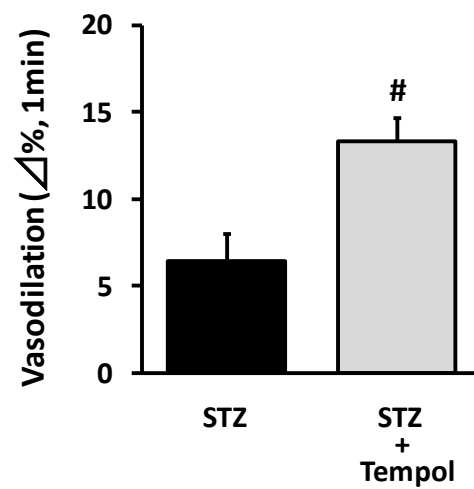
Figure 2. Effect of tempol on endothelial dysfunction induced in rats by STZ

Ultrasound measurements were performed 4 weeks after STZ administration. Data indicate the % changes in femoral diameter at 1 min after reperfusion. Tempol lessened the decrease in FMD in diabetic rats. #p<0.05 vs STZ (n = 6).
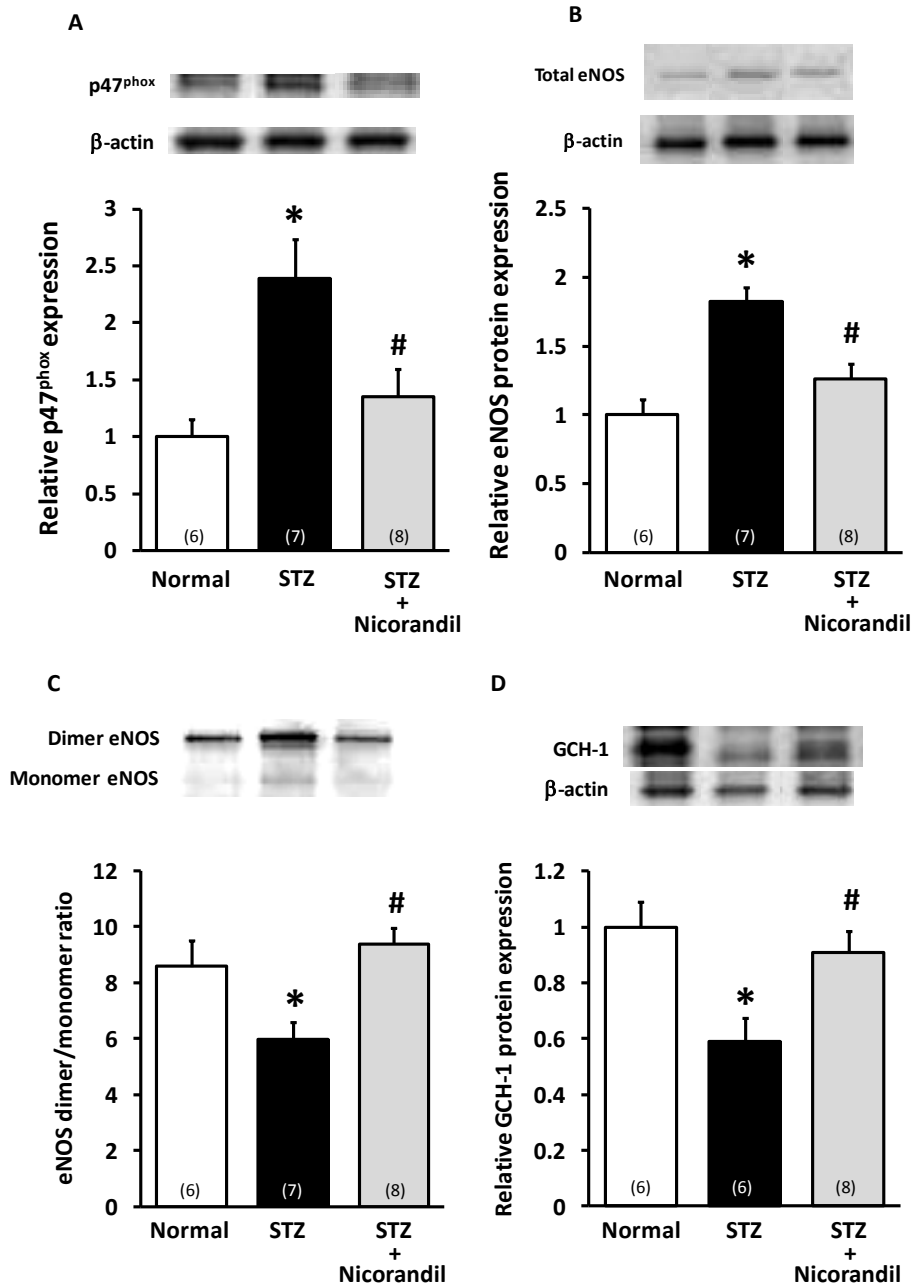
Figure 3. Effects of nicorandil on p47^{phox}, total eNOS and GCH-1 expression, and eNOS uncoupling in rat femoral arteries

Femoral arteries were harvested from diabetic rats at 4 weeks after STZ administration. Nicorandil decreased the increase in p47^{phox} (A) and total eNOS proteins (B) in the arteries. Nicorandil increased the decrease in the eNOS dimer/monomer ratio (C) and GCH-1 protein (D) in the arteries. *p<0.05 vs normal, #p<0.05 vs STZ (n = 6-8). The numeral in each column means the number of animals.
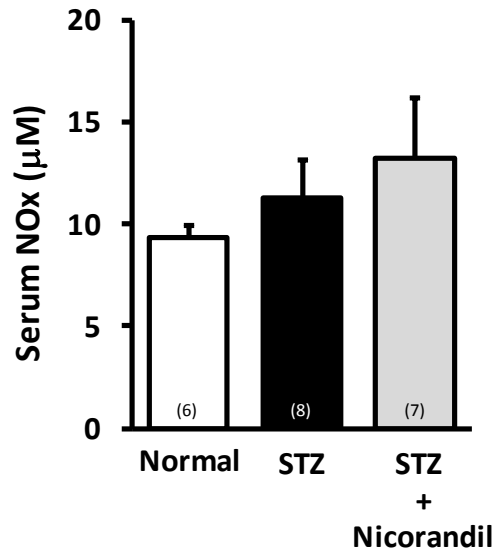
Figure 4. Effect of nicorandil on serum NOx levels in STZ rats

NO excretion was assessed from serum NOx concentration measured by the Griess method. Serum NOx concentrations were not changed among the groups (n = 6-8). The numeral in each column is the number of animals.

ROS production in HCAECs induced by high glucose

To clarify the possibility that eNOS itself might serve as a source of superoxide in the diabetic state, I examined the influence of the NOS inhibitor L-NAME on production of ROS induced by high glucose in HCAECs. High glucose increased ROS production indicated as DCF fluorescence in HCAECs. Interestingly, L-NAME decreased high glucose-induced ROS production in HCAECs (Fig. 5A-D), suggesting that eNOS is an important superoxide source in high glucose conditions. Furthermore, apocynin, an NADPH oxidase inhibitor, also decreased high glucose-induced ROS production in HCAECs (Fig. 5E-H). Therefore, NADPH oxidase is also another important superoxide source in high glucose conditions. Moreover, the anti-oxidative effect of nicorandil was confirmed because nicorandil improved expression of p47^{phox} and eNOS uncoupling state in the femoral arteries of STZ rats (Fig. 3A, C). Nicorandil also prevented high glucose-induced ROS production in HCAECs (Fig. 6A-D). Taken together, these results suggest that nicorandil could exert an anti-oxidative effect by normalizing NADPH oxidase and eNOS uncoupling state in endothelial cells.
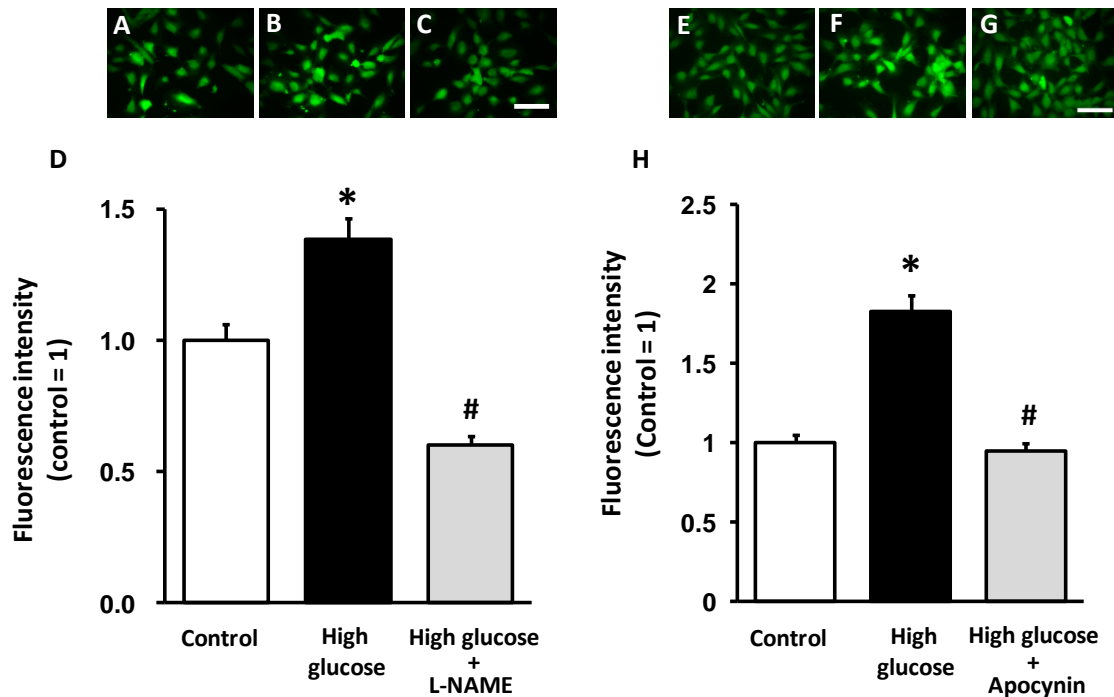
Figure 5. Effect of L-NAME and apocynin on ROS production in HCAECs treated with high glucose

HCAECs were exposed to high glucose (35.6 mM) for 24 h, and L-NAME (300 μM) or apocynin (100 μM) for the same period. These representative images show DCF fluorescence in control (A, E), high-glucose (B, F) and high-glucose + L-NAME (C) or + apocynin (G). High glucose treatment increased ROS production, which could be prevented by L-NAME (D) or apocynin (H). *$p<0.05$ vs control, #$p<0.05$ vs high-glucose (20 cells/dish from 2-3 dishes). Scale bar = 100 μm.
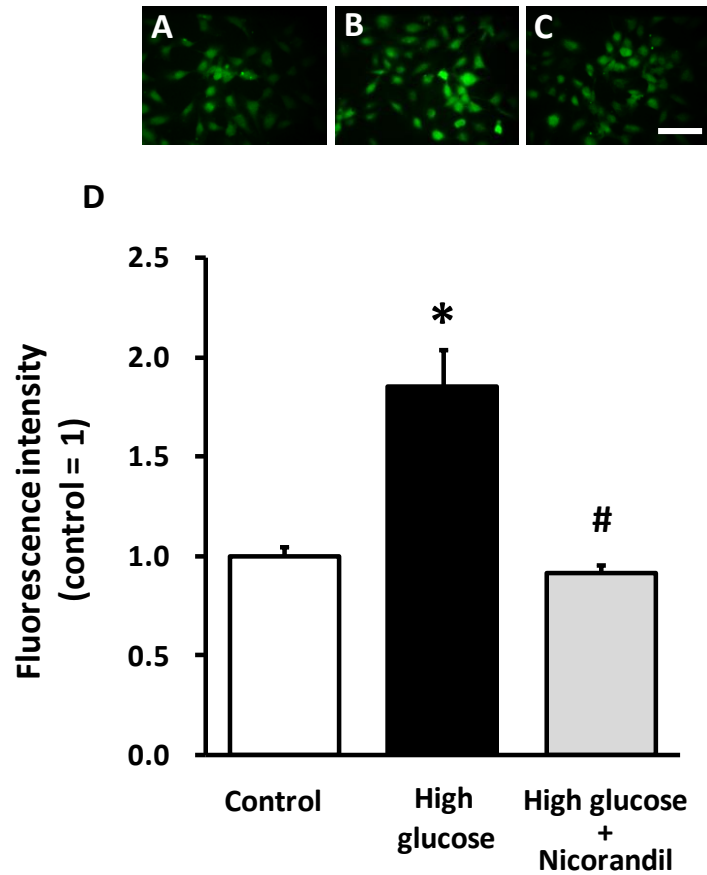
Figure 6. Effect of nicorandil on ROS production induced by high-glucose treatment in HCAECs

HCAECs were exposed to high glucose (35.6 mM) for 24 h, and nicorandil (100 μM) for the same period. Representative images show DCF fluorescence in control (A), high-glucose treatment (B) and high-glucose + nicorandil (C). High-glucose treatment increased ROS production, which was prevented by nicorandil (D). *p<0.05 vs control, #p<0.05 vs high-glucose (20 cells/dish from 3 dishes). Scale bar = 100 μm.

1-4.　Discussion

　　The present study demonstrated that nicorandil protected STZ rats from endothelial dysfunction assessed by the FMD reduction without affecting endothelium-independent vasodilation. Because earlier studies showed that endothelial dysfunction in diabetes is caused by increased ROS (Gryglewski *et al.*, 1986), there should be 2 possibilities for mechanisms underlying endothelial protection. Nicorandil could inhibit the expression and activity of NADPH oxidase, a major source of ROS (Griendling *et al.*, 2000; Muller and Morawietz, 2009). Or, because monomer state of eNOS is a source of ROS but not NO depending on the BH_4 level (Landmesser *et al.*, 2003), nicorandil could reduce ROS production by inhibiting the monomerization of eNOS through increased expression of BH_4 synthase.

　　These results suggested that nicorandil improved endothelial function in STZ rats through an antioxidative effect exerted by normalizing p47[phox] and eNOS uncoupling. Nicorandil may offer a novel therapy for diabetes complications by targeting endothelial dysfunction caused by ROS.

Endothelial dysfunction and NADPH oxidase in diabetes

　　Endothelial dysfunction is attributable to endothelial ROS production derived from vascular NADPH oxidase, which is an important source of superoxide (Griendling *et al.*, 2000; Muller and Morawietz, 2009). The present results generally agree with earlier reports that endothelial-dependent relaxation of arteries is impaired by NADPH oxidase increasing ROS production in both type I (Hink *et al.*, 2001; Ojaimi *et al.*,

2010) and type II diabetic models (Gupte *et al.*, 2010; Mishra *et al.*, 2010).

This study demonstrated that tempol, a radical scavenger, improved FMD of STZ rats (Fig. 2), and that more NADPH oxidase subunit p47[phox] was expressed in femoral arteries (Fig. 3A). As well, apocynin significantly decreased high glucose-induced ROS production in HCAECs (Fig. 5H); indicating that endothelial dysfunction in STZ rats was caused by the oxidative stress produced by NADPH oxidase. Moreover, nicorandil inhibited expression of NADPH oxidase in STZ rat femoral arteries (Fig. 3A), and also prevented high glucose-induced ROS production in HCAECs (Fig. 6). These results suggest that nicorandil ameliorates endothelial dysfunction by decreasing NADPH oxidase which inhibited production of ROS. This agreed with a previous report that nicorandil can inhibit production of ROS by NADPH oxidase activation in response to hypoxia-reoxygenation in human umbilical vein endothelial cells (Tajima *et al.*, 2008).

An increase in ROS production caused by NADPH oxidase in diabetic vessels is at least partially mediated by protein kinase C (PKC) and nuclear factor-kappa B (NF-κB) (Mariappan *et al.*, 2010; Ohshiro *et al.*, 2006). Because nicorandil exerts inhibitory effects on PKC activity (Iliodromitis *et al.*, 2008) and NF-κB activation (Kawamura *et al.*, 2005), nicorandil might prevent the increase in NADPH oxidase by inhibiting PKC or NF-κB in diabetic rats. Moreover, it has been suggested that mitochondrial ROS may activate vascular NADPH oxidase through a PKC-dependent process (Wenzel *et al.*, 2008A). Eguchi *et al* suggested that nicorandil inhibits

ROS-induced ROS release in the mitochondria of endothelial cells (Eguchi *et al.*, 2009). Therefore, nicorandil may inhibit NADPH oxidase by protecting mitochondria.

Contribution of eNOS to ROS production in diabetes

eNOS can produce NO or superoxide. eNOS is uncoupled when BH_4, an essential cofactor of NOS, is reduced in oxidative states (Landmesser *et al.*, 2003). As a result, production of superoxide is increased by the eNOS monomer; whereas, in the presence of abundant BH_4 the eNOS dimer produces mainly NO (Landmesser *et al.*, 2004). In the diabetic state, eNOS is believed to serve as another source of ROS (Hink *et al.*, 2001; Wenzel *et al.*, 2008C). High glucose can induce an eNOS uncoupling state due to BH_4 deficiency (Cai *et al.*, 2005), leading to decrease in NO production and increase in superoxide production (Hink *et al.*, 2001; Satoh *et al.*, 2008). In fact, there are clinical and experimental reports that describe administration of BH_4 to improve endothelial function (Heitzer *et al.*, 2000; Pieper, 1997). In the present study, the expression of GCH-1, a major enzyme for BH_4 synthesis, was decreased in STZ rats (Fig. 3D). This was consistent with the decrease in eNOS dimer/monomer ratio (Fig. 3C). These results suggest that ROS production in STZ rats could be derived from an uncoupled state of eNOS. In fact, the NOS inhibitor L-NAME completely inhibited production of ROS induced by high glucose treatment in HCAECs (Fig. 5D), suggesting that ROS production in STZ rats was derived mainly from eNOS.

Nicorandil is known from several studies to increase the expression

and activity of eNOS. This occurs not only in normal rat hearts (Horinaka *et al.*, 2001) but also in several disease models such as salt-sensitive Dahl rats (Horinaka *et al.*, 2006; Xu *et al.*, 2005), myocardial infarcted rats (Horinaka *et al.*, 2004) and monocrotaline-induced pulmonary hypertensive rats (Hongo *et al.*, 2005). Increased release of NO by nicorandil was correlated with enhancement of eNOS phosphorylation in cultured rat cardiac fibroblasts (Liou *et al.*, 2011). The increase in eNOS expression is related to the improvement of symptoms. On the other hand, the present study demonstrated that nicorandil decreased the increase in total eNOS expression in STZ rats (Fig. 3B) and improved endothelial function. To our knowledge, this is the first demonstration that nicorandil inhibits the increase in expression of eNOS in diabetic rats. There is a possibility that nicorandil inhibited the expression of eNOS. In diabetic rats, hyperglycemia increased superoxide production by activating mitochondrial electron transport chain (Nishikawa *et al.*, 2000), which activated PKC, resulting in upregulation of eNOS in the endothelium (Creager *et al.*, 2003; Hink *et al.*, 2001). Because nicorandil and diazoxide can reduce the mitochondrial ROS production (Ozcan *et al.*, 2002), the inhibition of mitochondrial ROS by nicorandil may serve to inhibit the increase in eNOS expression in STZ rats. Further investigation is required to clarify the mechanisms by which nicorandil regulates eNOS expression.

Chapter 2

Studies of effect of nicorandil

on paclitaxel-induced endothelial dysfunction in rats

2-1. Introduction

It is well known that the endothelium plays an important role in the regulation of vascular tone via release of factors such as NO, prostaglandin I_2, endothelium-derived hyperpolarization factor (EDHF), and endothelin. Endothelial dysfunction predicts adverse cardiac events in patients with coronary artery disease (Kitta *et al.*, 2009). It is mainly caused by loss of NO that occurs from several pathological conditions, such as atherosclerosis, hypertension, and diabetes. Recently, DES has been considered as another potential cause of endothelial dysfunction, despite their successful use in the treatment of ischemic heart diseases by providing dramatic reduction in in-stent restenosis (Dawkins *et al.*, 2005; Stone *et al.*, 2005; Stone *et al.*, 2004). Paclitaxel, one of the major drugs used in DES, is known to cause endothelial dysfunction in humans (Shin *et al.*, 2007) and swine (Pendyala *et al.*, 2009) because it induces apoptosis and cytotoxicity in human coronary artery endothelial cells (Wessely *et al.*, 2007).

Nicorandil, an anti-anginal drug with K_{ATP} channel opening and nitrate-like activity, was shown in the IONA (IONA study group, 2002) and JCAD (Horinaka *et al.*, 2010) studies to improve the prognoses of patients with ischemic heart disease by a pharmacological preconditioning effect (Horinaka, 2011). In both clinical settings and animal studies, it also has been reported that nicorandil reduced cardiac events partially by protecting endothelium. Nicorandil improved FMD (an indicator of endothelial function) in patients with ischemic heart disease (Sekiya *et al.*, 2005) or with cardiovascular risk factors (Ishibashi *et al.*, 2008). And it reduced myocardial

no-reflow in swine by protecting endothelial function (Zhao *et al.*, 2006). In chapter 1, I demonstrated that nicorandil protected endothelial function in diabetic rats. However, there are no reports that nicorandil provides protection from endothelial dysfunction caused by paclitaxel.

In the next study, I investigated the protective effect of nicorandil on paclitaxel-induced endothelial dysfunction. I also sought the mechanisms underlying this protective effect in association with NO bioavailability in vascular endothelial cells.

2-2.   Materials and Methods
Chemicals

Nicorandil was synthesized in the Chugai Organic Chemistry Laboratory. Paclitaxel and the other reagents were analytical grade and purchased from Wako Pure Chemicals Co. Tempol, 5-hydroxydecanoate (5-HD) and 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ), isosorbide dinitrate (ISDN) and diazoxide were obtained from Sigma-Aldrich. Nitroglycerin came from Nippon Kayaku (Tokyo, Japan) and acetylcholine was supplied by Daiichi-Sankyo (Tokyo, Japan).

Paclitaxel was dissolved in 50% dimethylformamide, 25% Cremophor EL, and 25% distilled water. Diazoxide and ISDN were suspended in 3% gum arabic solution. Nitroglycerin and acetylcholine were dissolved in, and diluted with, 0.9 % saline solution. All other drugs were dissolved in distilled water just before the experiment. The concentrations of paclitaxel used in this study were chosen on the basis of studies published previously (Kim *et al.*, 2004; Wessely *et al.*, 2007).

Animals

Male Sprague Dawley rats (250–300 g) were used. All rats were fed ordinary laboratory chow and allowed free access to water under a constant light and dark cycle of 12 h. The rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and adequate anesthetic depth was monitored by pinching the toe. An osmotic pump (model 2ML1; Durect Corp., Cupertino, CA, USA) was implanted

subcutaneously into a small pouch between the shoulder blades to continuously infuse paclitaxel (0.5, 1.5, 5 mg/kg/day). Bupivacaine (5 mg/mL) was applied topically to the skin wound. For the vehicle group, the osmotic pump containing vehicle (50% dimethylformamide, 25% Cremophor EL, and 25% distilled water) was subcutaneously implanted. Nicorandil (15 mg/kg/day) and tempol (20 mg/kg/day) were dissolved in drinking water and administered for 1 week, from just after implantation of the osmotic pump. Diazoxide (15 mg/kg) and ISDN (15 mg/kg) were administered by gastric gavage once a day for 1 week from just after implantation. FMD was measured in rats 1 week after implantation of the osmotic pump.

All animal procedures were conducted in accordance with Chugai Pharmaceutical's ethical guidelines for animal care, and all experimental protocols were approved by the Animal Care Committee of the institution and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All animals were euthanized by exsanguination under anesthesia after experiments.

Measurements of endothelial function in live rats

FMD was measured in living rats as described in Chapter 1. In this study, vasodilation induced by acetylcholine was also evaluated as another indicator of endothelial function. Acetylcholine (3 ng) was injected into the aorta just proximal to the iliac bifurcation after femoral artery was visualized using a high-resolution ultrasound system in anesthetized rats. The change in diameter of femoral artery was monitored until 1 min after

injection.

Real-time PCR analysis in rat femoral arteries

After measurement of FMD, femoral arteries were harvested and stored in RNAlater solution (Ambion, Austin, TX, USA) until RNA isolation was performed. Tissues were homogenized with a Micro Smash homogenizer (Tomy Digital Biology, Tokyo, Japan), and total RNA was isolated using an RNeasy Fibrous Tissue kit (Qiagen, Dusseldorf, Germany). Real-time PCR was performed using TaqMan Gene Expression Assays for NADPH oxidase components p47[phox] and gp91[phox] in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Gene expression was normalized to the endogenous control, β-actin.

Measurement of ROS in mouse testicular arteries

In vivo ROS production was monitored by the fluorescent probe, dihydrorhodamine 123 hydrochloride (Wako Pure Chemical) according to the method as previously described (Eguchi *et al.*, 2009). Briefly, male ICR mice (10 to 11 weeks old) were anesthetized with pentobarbital (50 mg/kg, i.p.). Endothelial cells were observed by isolectin B4 labeled with FITC: an endothelial cell marker specific to mice (Vector Lab, Burlingame, CA, USA). After the testicular artery (approx. 200 μm in diameter) was carefully exposed, dihydrorhodamine 123 (2.5 mg/kg) was given intravenously, followed by intravenous administration of FITC-labeled isolectin B4 (1.25 mg/kg). The fluorescence intensity was observed by an ultrafast laser

confocal microscope equipped with a piezoelectric motor control unit, and was recorded as arbitrary units (au). Under pentobarbital anesthesia, mice were subcutaneously implanted osmotic pump (model 1007D; Durect Corp.) with body temperature kept stable and monitoring adequate anesthetic depth by pinching the toe. Paclitaxel (5 mg/kg/day), was infused continuously from the osmotic pump. Nicorandil (15 mg/kg/day) was administrated in drinking water from just after infusion of paclitaxel.

Measurements of ROS in HCAECs

To measure production of ROS, HCAECs were seeded onto plastic dishes (1×10^5 cells/2 mL/dish) and cultured in monolayers in a 5% CO_2 humidified incubator at 37°C. After overnight incubation, paclitaxel (10 ng/mL) was added to the culture medium for 24 h. Nicorandil (100 μM), 5-HD (500 μM), and ODQ (20 μM) were added over the same period. The cellular level of ROS production was evaluated as described in Chapter 1.

Western blot analysis in HCAECs

Paclitaxel (10 ng/mL) was added to the culture medium for 24 h. Nicorandil (100 μM) was added over the same period. Then, cells were harvested and used for western blotting. Equal amounts of protein extract were separated on 10% SDS-polyacrylamide gel and immobilized on PVDF membranes. The membranes were incubated with anti-eNOS (Santa Cruz Biotechnology), anti-phospho-eNOS (Cell Signaling Technology, Danvers, USA) and anti-β-actin antibodies. Immunoreactive signals were detected

using a ChemiDoc XRS system (Bio-Rad).

Measurements of NO in HCAECs

Intracellular NO was assessed with diaminofluorescein-2 diacetate (DAF-2DA; Sekisui medical, Tokyo, Japan) fluorescence probe (10 μmol/L) (Wang *et al.*, 2008). Briefly, HCAECs were incubated with PBS containing 10 μM DAF-2 in the dark for 30 min at 37 °C, and then washed twice with PBS. Fluorescence intensity was measured by spectrofluorophotometry (SpectraMax Gemini XS (Molecular Devices, CA, USA) with excitation and emission wavelengths of 485 and 545 nm, respectively.

Statistical analyses

All data are expressed as mean ± SE. The n values refer to the number of individual animals in each experiment. The statistical significance of differences was determined by using the Tukey Multiple Comparison Test. Probability (P) values less than 0.05 were considered significant.

2-3.　Results

Measurements of FMD in rats

Endothelial function was evaluated by measuring FMD in rat femoral arteries. The femoral arterial flow velocity was 121.2 ± 10.3 mm/s at baseline in rats treated with vehicle (Fig. 7A). When the common iliac artery was occluded for 5min and reperfused, the flow velocity increased instantly (i.e. reactive hyperemia) to 176.1 ± 3.7 mm/s (Fig. 7B). The flow velocity decayed rapidly to baseline values at around 3 min (Fig. 7C). The increase in flow velocity was associated with the delayed increase in femoral arterial vasodilation. The femoral arterial diameter increased from 0.40 ± 0.01 mm (Fig 7D) to 0.48 ± 0.01 (Fig 7E) at 1 min after reperfusion. The percentage increase in arterial diameter (vasodilation) peaked at 1 min (Fig. 7F). This delayed vasodilation is FMD.
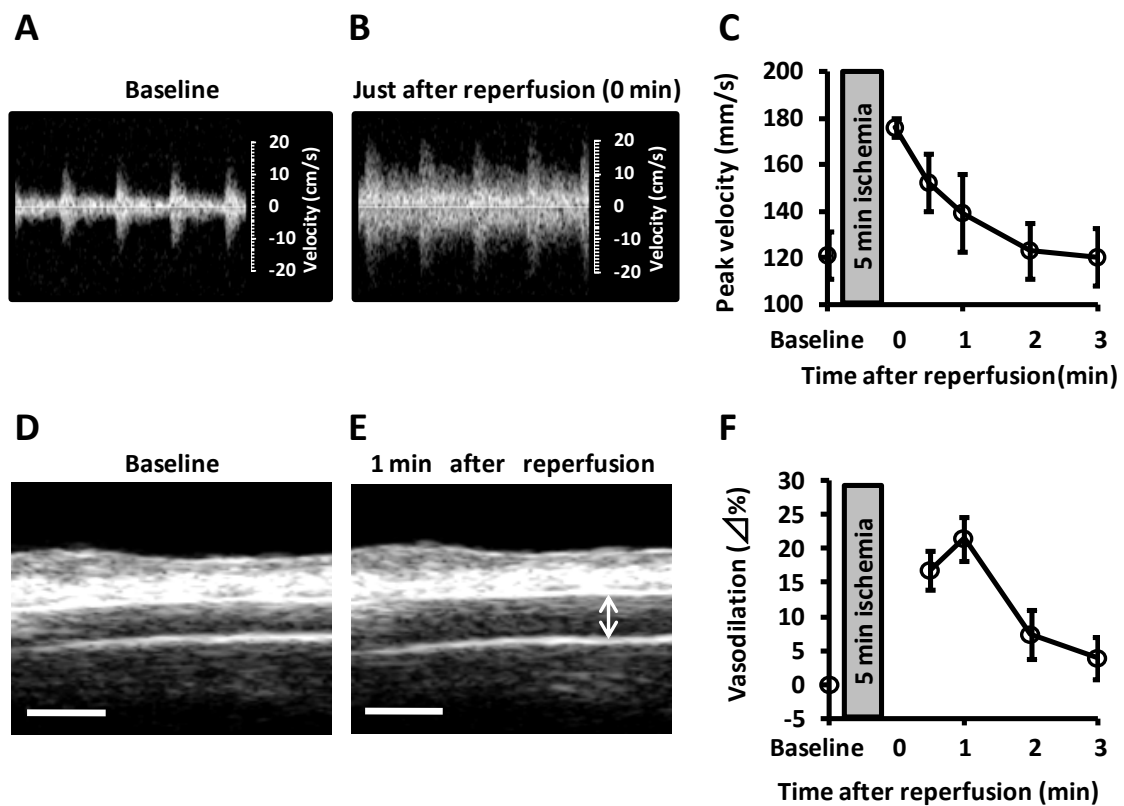
Figure 7. Post-ischemic reactive hyperemia and vasodilation of the rat femoral artery

Original Doppler flow velocity tracings at baseline (A) and at onset of reperfusion after 5 min occlusion of the common iliac artery (B). (C) Time course of peak flow velocity after 5 min ischemia. Representative ultrasound image of the femoral artery at baseline (D) and 1 min after reperfusion (E). Longitudinal sections of the rat femoral artery were visualized with a high-resolution ultrasound probe placed on the upper thigh. (F) Time course of vasodilation (percentage increase in arterial diameter) after 5 min ischemia (n = 5). Arrow indicates femoral artery. Scale bars = 1 mm.

Effects of nicorandil on reduced FMD in rats treated with paclitaxel

I evaluated the influences of paclitaxel and nicorandil on FMD of rat femoral artery. Continuous subcutaneous infusion of paclitaxel (0.5, 1.5, 5 mg/kg/day, 7 days) reduced FMD in a dose-dependent manner (Fig. 8A). Reactive hyperemia was also evaluated as an inducer of FMD in the femoral artery to confirm that stimulation to the endothelium was similar among the groups. As a result, reactive hyperemia was similar among the groups (Fig. 8B). NTG was administered to evaluate endothelium-independent vasodilation because it was possible that paclitaxel impaired vascular smooth muscle function which influenced FMD. NTG-induced vasodilation was similar among the groups (Fig. 8C). These results imply that paclitaxel impaired endothelial function without changing contractility in vascular smooth muscle (Control, 21.6 ± 3.2; Paclitaxel [5 mg/kg/day], 7.1 ± 1.7%, Fig. 8A). On the other hand, co-treatment with nicorandil prevented the decrease in FMD caused by paclitaxel (15.5 ± 2.5%) without changing flow velocity or NTG-induced vasodilation (Fig. 8B, C). In the current study I found that treatment with paclitaxel and nicorandil for 1 week did not affect body weight, blood pressure or heart rate relative to rats treated with vehicle (Table 2).
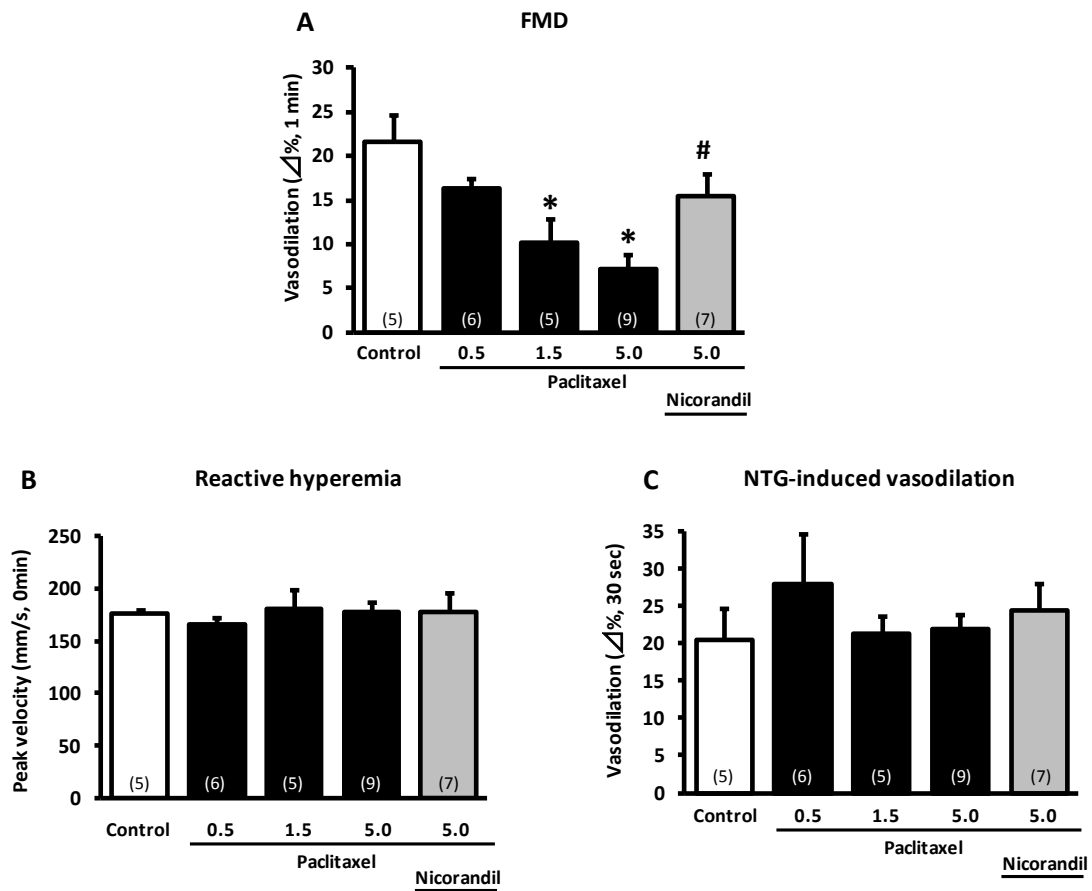
Figure 8. Effects of nicorandil on endothelial function impaired by paclitaxel as evaluated by FMD

(A) Dose-dependent impairment of FMD by paclitaxel (0.5, 1.5, 5.0 mg/kg/day; 1 week) and the effects of nicorandil (15 mg/kg/day; 1 week) on paclitaxel-induced impairment of FMD. Peak velocity (B) and NTG-induced vasodilation (C) were similar among groups. *$P < 0.05$ *vs.* control; #$P < 0.05$ *vs.* paclitaxel (5 mg/kg/day) ($n = 5$–9).

Table 2. Body weight, blood pressure and heart rate at 1 week after paclitaxel administration

	Vehicle	Paclitaxel			
		0.5	1.5	5.0	5.0 + Nicorandil
N	5	6	5	9	7
Body weight (g)	330.9 ± 13.9	337.3 ± 3.5	343.5 ± 6.6	322.0 ± 16.1	293.3 ± 14.6
Systolic blood pressure (mmHg)	134.9 ± 3.5	136.6 ± 5.0	121.8 ± 5.0	129.2 ± 2.5	122.7 ± 3.4
Heart rate (bpm)	362.6 ± 8.1	369.2 ± 8.5	378.3 ± 8.0	369.1 ± 6.3	394.0 ± 9.5

Effects of nicorandil on vasodilation induced by acetylcholine in rats treated with paclitaxel

Acetylcholine-induced vasodilation serves as an indicator of endothelial function because it can induce NO-dependent vasodilation by eNOS activation in endothelium. Therefore, I next evaluated the influence of paclitaxel and nicorandil on acetylcholine-induced vasodilation in living rats. In vehicle-treated rats, local administration of acetylcholine into the iliac artery led to femoral arterial vasodilation. Peak dilation was at 10 sec and rapidly decayed to baseline by approximately 60 sec (Fig. 9A). In rats treated with paclitaxel (5 mg/kg/day), acetylcholine induced significantly less dilation in the femoral artery (Control, $12.7 \pm 2.1\%$; Paclitaxel, $3.9 \pm 1.1\%$). On the other hand, nicorandil significantly prevented the decrease in acetylcholine-induced vasodilation by paclitaxel (Paclitaxel + Nicorandil, $12.5 \pm 3.5\%$, Fig. 9B). These results were consistent with the FMD measurements (Fig. 8A), indicating that nicorandil surely prevented endothelial dysfunction caused by paclitaxel.
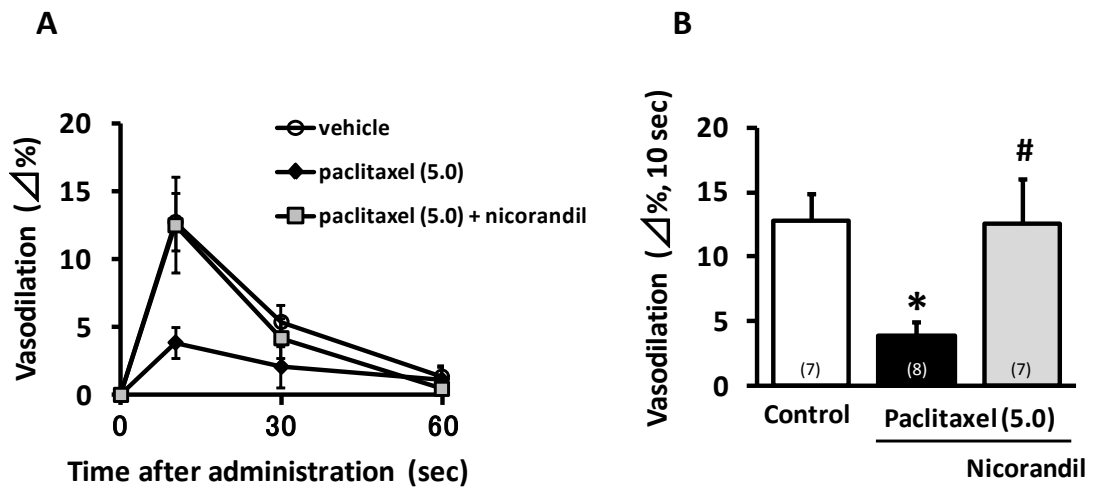
Figure 9. Effects of nicorandil on endothelial function impaired by paclitaxel as evaluated by acetylcholine injection

Acetylcholine was injected into the common iliac artery through an intra-aortic catheter. Time course (A) and mean percentage increase in arterial diameter at 10 sec after acetylcholine injection (B) was evaluated at the femoral artery. *$P < 0.05$ *vs.* control; #$P < 0.05$ *vs.* paclitaxel (5 mg/kg/day) ($n = 7–8$).

Effects of diazoxide, ISDN, and tempol on FMD in rats treated with paclitaxel

Because nicorandil is known to exert multiple actions, such as K_{ATP} channel opening and nitrate-like activity, it was important to determine which action was more important in endothelial protective effect of nicorandil. Diazoxide, a mitochondrial K_{ATP} channel opener, was used instead of nicorandil. As shown in Figure 10, diazoxide prevented significantly the decrease in FMD caused by paclitaxel, suggesting that opening mitochondrial K_{ATP} channels is important in endothelial protection. Conversely, ISDN, a nitrate which serves as an NO donor, had no effect (Fig. 10). These results suggest that mitochondrial K_{ATP} channel opening was more important than nitrate-like activity in endothelial protection. Furthermore, I also examined the involvement of oxidative stress in paclitaxel-induced endothelial dysfunction. Tempol, a ROS scavenger, also prevented the decrease in FMD of rats treated with paclitaxel (Fig. 10). This result could mean that paclitaxel caused endothelial dysfunction by oxidative stress.
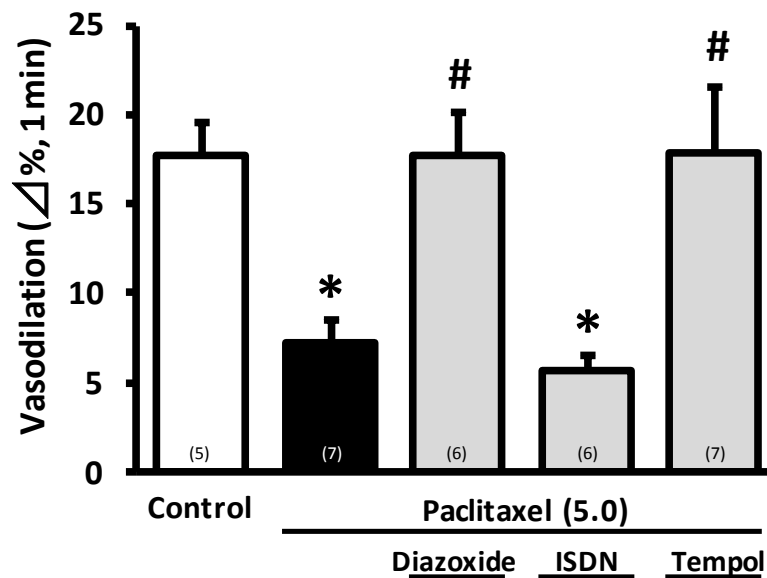
Figure 10. Effects of diazoxide, ISDN and tempol on FMD impaired by paclitaxel

Diazoxide (15 mg/kg/day) and ISDN (15 mg/kg/day) were suspended in 3% gum arabic solution and administrated by gastric gavage once a day for 1 week. Tempol (20 mg/kg/day, p.o.) was administrated in drinking water for 1 week. $*P < 0.05$ vs. control; $\#P < 0.05$ vs. paclitaxel (5 mg/kg/day) ($n = 5$–7).

Effects of nicorandil on gene expression of NADPH oxidase in femoral arteries of rats treated with paclitaxel

Because the involvement of oxidative stress in paclitaxel-endothelial dysfunction was indicated in Figure 10, I examined the effect of nicorandil on the expression of NADPH oxidase which is a major source of ROS. The mRNA expressions of p47^{phox} and gp91^{phox}, the major components of NADPH oxidase, increased significantly in femoral arteries isolated from rats treated with paclitaxel (Fig. 11). On the other hand, nicorandil prevented significantly the increase in p47^{phox} expression (Fig. 11A), and also limited the increase in gp91^{phox} expression (Fig. 11B, $P = 0.09$ *vs.* paclitaxel). These results suggest that nicorandil could prevent oxidative stress induced by paclitaxel through inhibiting NADPH oxidase.
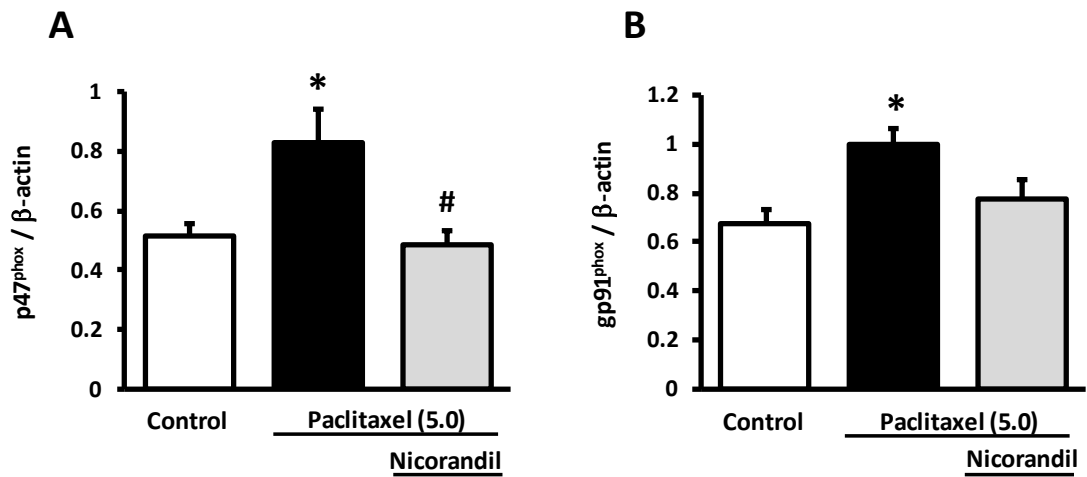
Figure 11. Effects of nicorandil on changes in expression of p47^{phox} and gp91^{phox} mRNA induced by paclitaxel in rat femoral arteries

p47^{phox} (A) and gp91^{phox} (B) expression levels in femoral arteries were measured using real-time PCR. Data are shown as subunit/β-actin ratio. *$P < 0.05$ *vs.* control; #$P < 0.05$ *vs.* paclitaxel (5 mg/kg/day) ($n = 8$).

Effects of nicorandil on production of ROS in testicular arteries of mice treated with paclitaxel

Because nicorandil prevented the increase in NADPH oxidase by paclitaxel, I examined the effect of nicorandil on paclitaxel-induced ROS production. As shown in Figure 12, the fluorescence intensity of dihydrorhodamine 123 hydrochloride in mice treated with paclitaxel was 1.6 times greater than in mice treated with vehicle. On the other hand, co-treatment with nicorandil significantly inhibited any increase in fluorescence intensity (control, 33.0 ± 0.5; paclitaxel, 52.2 ± 1.1; paclitaxel + nicorandil, 44.3 ± 1.0 au). This result suggests that nicorandil could prevent paclitaxel-induced ROS production.
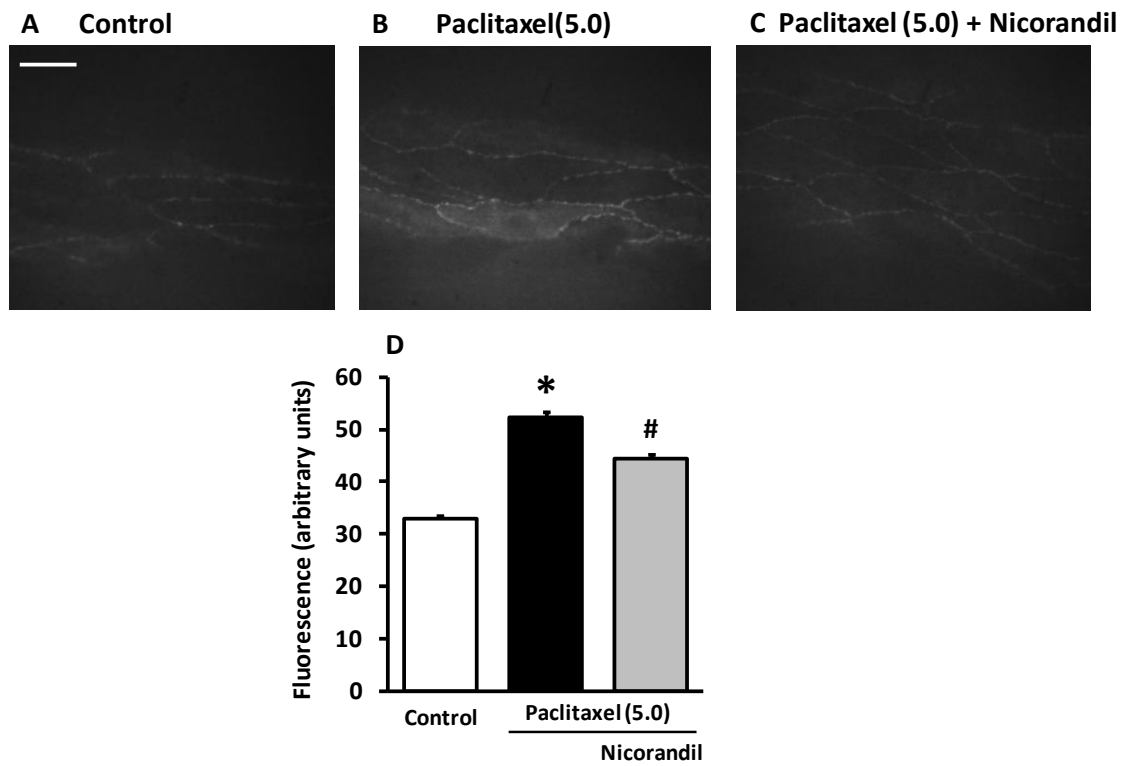
A Control **B** Paclitaxel(5.0) **C** Paclitaxel (5.0) + Nicorandil

D

Figure 12. Effect of nicorandil on oxidative stress in mice caused by paclitaxel

Production of reactive oxygen species in mouse testicular arteries by paclitaxel was measured using dihydrorhodamine 123 hydrochloride. Representative images show fluorescence in control (A), 7 days after administration of paclitaxel (B), and 7 days after nicorandil was co-administered with paclitaxel (C). Scale bar =25 µm. (D) Each column denotes mean fluorescence intensity from 3 mice. Paclitaxel especially increased the fluorescence intensity at the edge of the spindle-shaped vascular endothelial cells (B). *$P < 0.05$ vs. control; #$P < 0.05$ vs. paclitaxel (5 mg/kg/day).

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Effects of nicorandil on production of ROS, NO release and eNOS phosphorylation induced by paclitaxel in HCAECs

I also examined the effect of nicorandil on paclitaxel-induced ROS production in HCAECs. Paclitaxel (10ng/mL) significantly increased DCF fluorescence compared with control. Nicorandil, in turn, significantly inhibited the increase in DCF fluorescence by paclitaxel (Fig. 13). This result indicated that nicorandil prevented paclitaxel-induced ROS production. A further experiment determined whether K_{ATP} channel opening or nitrate-like activity was more important in the anti-oxidative effect of nicorandil. The anti-oxidative potential of nicorandil was abolished by co-treatment with 5-HD, a mitochondrial K_{ATP} channel inhibitor (Fig. 14A), but not by co-treatment with ODQ, a specific inhibitor of soluble guanylate cyclase (Fig. 14B). Treatment with 5-HD alone increased DCF fluorescence compared with control (Fig. 14A), whereas nicorandil or ODQ alone did not affect DCF fluorescence (Fig. 13, 14B). Opening mitochondrial K_{ATP} channels may be important for the anti-oxidative effect of nicorandil.

Next, I examined the influence of paclitaxel and nicorandil on NO production in HCAECs. Paclitaxel (10 ng/mL) significantly decreased DAF-2 fluorescence intensity compared with control. This result indicated that paclitaxel decreased NO production in HCAECs. Nicorandil tended to inhibit the decrease in DAF-2 fluorescence intensity induced by paclitaxel (Fig. 15A). Nicorandil alone had no effect on fluorescence intensity. On the other hand, there was no significant difference in the phosphorylation of eNOS protein among the groups in HCAECs (Fig. 15B).
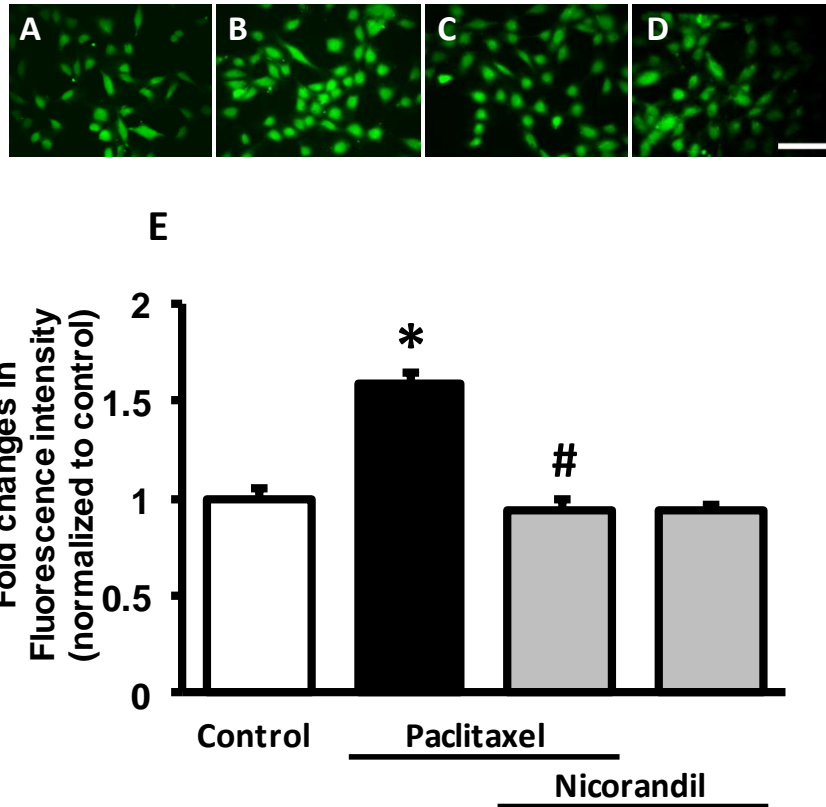
Figure 13. Effects of nicorandil on oxidative stress induced by paclitaxel in HCAECs

The images show representative fluorescences in control (A), after 24 h treatment with paclitaxel (10 ng/mL) (B), paclitaxel + nicorandil (100 μM) (C), and nicorandil alone (100 μM) (D). Fold changes in DCF fluorescence intensity are provided as the normalized ratio to control (E). Intracellular production of reactive oxygen species was measured with confocal microscopy after 45 min DCF (10 μM) label as described in the Methods. Nicorandil inhibited paclitaxel-induced fluorescence intensity in HCAECs. *$P < 0.05$ *vs.* control; #$P < 0.05$ *vs.* paclitaxel. Each column denotes mean fluorescence intensity of 20 cells/dish from 3 dishes. Scale bar = 100 μm.
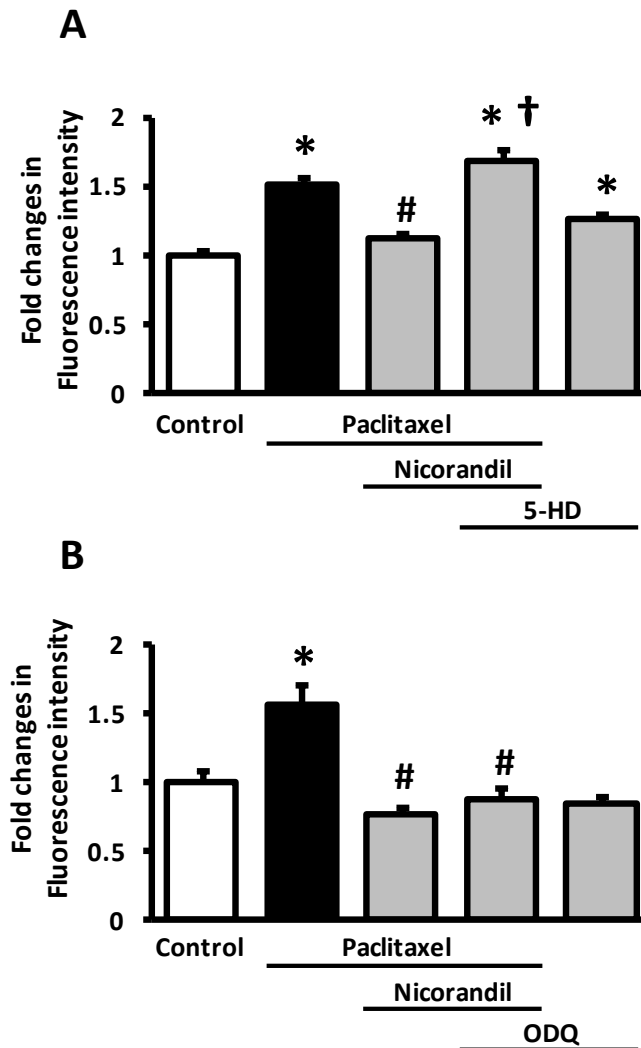
Figure 14. Effects of 5-HD and ODQ on inhibition of nicorandil against oxidative stress induced by paclitaxel in HCAECs

Co-treatment with 5-HD (500 μM; 24 h), a mitochondrial K_{ATP} channel inhibitor (A), but not by co-treatment with ODQ (20 μM; 24 h), a specific inhibitor of soluble guanylate cyclase (B) abolished the inhibitory effects of nicorandil (100 μM) on the increase in oxidative stress induced by paclitaxel (10 ng/mL). Fold changes in DCF fluorescence intensity are provided as the normalized ratio to vehicle control. Intracellular production of reactive oxygen species was measured after 45 min DCF (10 μM) label with confocal microscopy as described in the Methods. *P< 0.05 *vs.* control; #P< 0.05 *vs.* paclitaxel; † P < 0.05 *vs.* paclitaxel with nicorandil. Each column denotes mean fluorescence intensity of 20 cells/dish from 3 dishes.
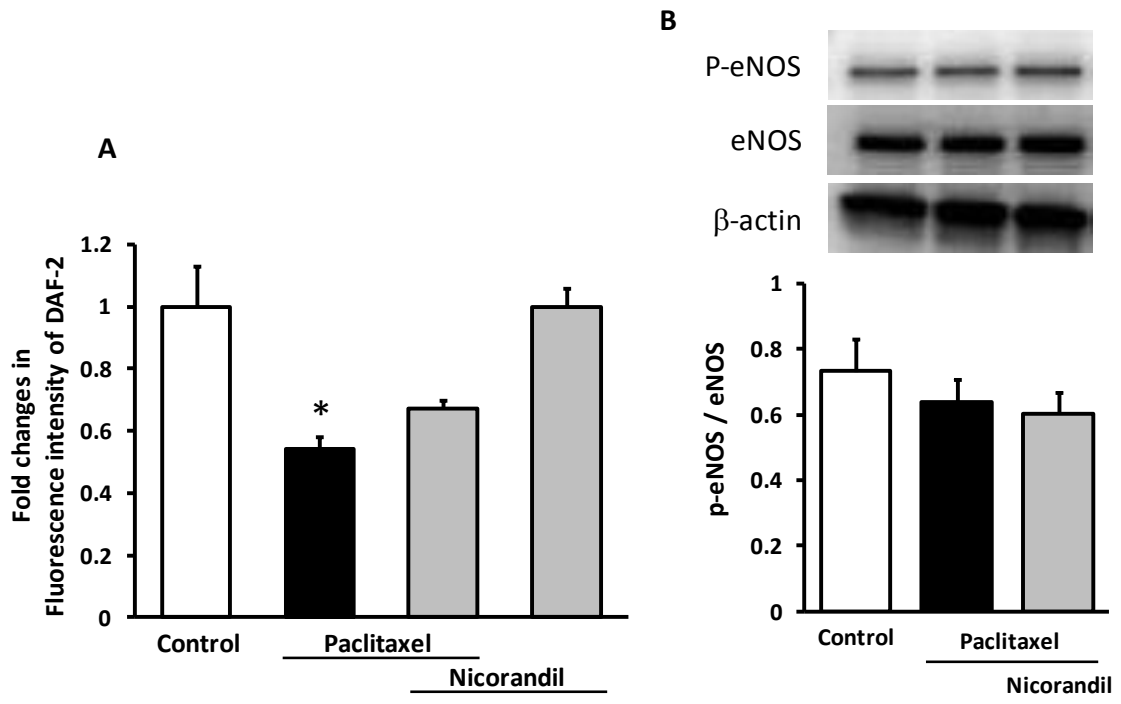
Figure 15. Effects of nicorandil on fluorescent intensity of DAF-2 and phospho-eNOS expression in cultured HCAECs

(A) Each column denotes mean fluorescence intensity of DAF-2 from 6 dishes. (B) Phospho-eNOS expression levels in HCAECs were measured by western blotting. Data are shown as phosphor-eNOS/eNOS ratio from 3 dishes. *P< 0.05 *vs.* control.

2-4. Discussion

Although paclitaxel-eluting stents (PES) dramatically reduces in-stent restenosis, paclitaxel leads to endothelial dysfunction. This Chapter investigated the protective effects of nicorandil on endothelial dysfunction induced by paclitaxel and the underlying mechanisms using FMD in anesthetized rats. The main finding was that paclitaxel decreased FMD in anesthetized rats by both increasing oxidative stress and decreasing NO production. Furthermore, based on the results of functional FMD, ROS production, mRNA expression, NO synthesis and eNOS phosphorylation, the present study also demonstrated that nicorandil protects against endothelial dysfunction mainly by inhibiting of production of ROS. It is also suggested that this beneficial effect of nicorandil could be exerted through opening mitochondrial K_{ATP} channel.

Contribution of oxidative stress to endothelial dysfunction induced by paclitaxel

It has been postulated that paclitaxel causes endothelial dysfunction by reducing NO availability. A critical determinant of endothelial function is the balance between NO and ROS. The availability of endothelium-derived NO can be limited by enhanced formation of ROS (Landmesser *et al.*, 2004; Muller and Morawietz, 2009). NO can be trapped with ROS in a very rapid reaction, forming peroxynitrite and reducing available NO (Gryglewski *et al.*, 1986). An earlier report indicated that NADPH oxidase is a significant source of the superoxide produced following treatment with paclitaxel, and

that an NADPH oxidase inhibitor or suppression of gp91[phox] by siRNA significantly attenuated cell death induced by paclitaxel (Jang *et al.*, 2008). Thrombotic processes may also be enhanced when paclitaxel reduces NO availability, because less NO availability not only attenuates vasodilation but also adversely affects anti-thrombotic processes such as reduced leukocyte adhesion, platelet adhesion, aggregation, and expression of plasminogen activator inhibitor (PAI-1) (Landmesser *et al.*, 2004). Indeed, paclitaxel has increased the expression of PAI-1 mRNA in HCAECs (Muldowney *et al.*, 2007). Our findings were consistent with those earlier reports. In this study, paclitaxel increased ROS production in both *in vivo* mice (Fig. 12) and HCAECs (Fig. 13, 14), with concurrent increases in mRNA expression of NADPH oxidase (p47[phox] and gp91[phox], Fig. 11) in rat femoral arteries. Moreover, tempol prevented the decrease in FMD by paclitaxel in rat femoral arteries (Fig. 10). On the other hand, paclitaxel did not change eNOS phosphorylation in HCAECs despite the decrease in NO release (Fig. 15). These data clearly suggest that paclitaxel induces endothelial dysfunction by limiting the availability of NO derived from endothelium by increasing ROS production. Earlier reports indicated that paclitaxel induced endothelial dysfunction in humans (Shin *et al.*, 2007), animals (Pendyala *et al.*, 2009; Pires *et al.*, 2007), and cultured cells (Pasquier *et al.*, 2004; Wessely *et al.*, 2007) through apoptosis by increasing the level of pro-apoptotic mRNA transcripts, although the relationship between reduction of NO availability induced by paclitaxel and endothelial dysfunction was not fully revealed. Tawa et al. found that hypoxia-induced ROS production in smooth muscle

cells impaired NO donor-mediated relaxation in endothelium-denuded monkey coronary arteries (Tawa *et al.*, 2011). This means that ROS has the potential to impair not only the NO bioavailability but also vascular smooth muscle function. However, in this study, relaxation induced by nitroglycerin was unimpaired by paclitaxel (Fig. 8C), implying that paclitaxel-induced ROS production did not impair smooth muscle function in this study. The reasons for this discrepancy may lie in differences in amount of ROS production or in the methods used to measure vascular relaxation.

Contribution of K_{ATP} channel to endothelial protective effect of nicorandil

Earlier studies suggested that nicorandil protects endothelium through its anti-oxidative effect. Based on experimental results showing that nicorandil reinforced the anti-aggregatory activity of endothelial cells by reducing ROS production under hypoxia–reoxygenation conditions, Tajima *et al.* suggested that the protective effect of nicorandil on endothelium was mediated by inhibiting NADPH oxidase activity by opening mitochondrial K_{ATP} channel (Tajima *et al.*, 2008). The present study further indicated that nicorandil prevents paclitaxel-induced endothelial dysfunction by reducing oxidative stress by mitochondrial K_{ATP} channel opening. Diazoxide, another mitochondrial K_{ATP} channel opener, prevented reduction of FMD by paclitaxel (Fig. 10), and the reduction of ROS by nicorandil was inhibited in HCAECs by 5-HD, a mitochondrial K_{ATP} channel blocker (Fig. 14A). Interestingly, treatment with 5-HD alone increased oxidative stress (Fig. 14A), suggesting that the mitochondrial K_{ATP} channel is important in

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regulating oxidative stress in the endothelium that is induced by paclitaxel. In fact, ISDN did not prevent the decrease in FMD by paclitaxel (Fig. 10), and ODQ did not inhibit the decrease in ROS by nicorandil in HCAECs (Fig. 14B). Thus, nicorandil might improve endothelial dysfunction caused by paclitaxel through K_{ATP} channel activation. The action of nicorandil was not dependent on its nitrate-like activity, although it cannot be ruled out completely.

General Discussion

In the Chapter 1, I demonstrated that nicorandil ameliorated endothelial dysfunction as evaluated by FMD in STZ rats through an anti-oxidative effect exerted by normalizing p47^{phox} and eNOS uncoupling. In the Chapter 2, I demonstrated that nicorandil ameliorated endothelial dysfunction caused by paclitaxel as evaluated by FMD and may improve NO bioavailability by reducing oxidative stress via K_{ATP} channel activation. In the course of these experiments, it became clear that endothelial dysfunction could be quantified by measuring FMD, and that nicorandil prevented endothelial dysfunction via its anti-oxidative effect by activating mitochondrial K_{ATP} channel. These results suggest that nicorandil might be effective in relieving endothelial dysfunction and improve the prognoses of angina patients with diabetes or DES implantation.

Action mode of nicorandil

Because nicorandil is known to open K_{ATP} channel and have nitrate like activity, it was of interest which action contributed to its anti-oxidative and endothelial protective effects. Earlier reports indicated the importance of K_{ATP} channel in anti-oxidative properties and endothelial protection. Nicorandil, but not ISDN improved FMD in patients with ischemic heart disease (Sekiya *et al.*, 2005). Eguchi et al. suggested that nicorandil prevented thrombi by activating mitochondrial K_{ATP} channels and inhibiting ROS-induced ROS release in the mitochondria of endothelial cells (Eguchi *et al.*, 2009). Ozcan et al. also found that nicorandil prevents oxidative stress by

opening mitochondrial K_{ATP} channels (Ozcan *et al.*, 2002). In the Chapter 2, I also demonstrated that nicorandil and diazoxide improved FMD in rats treated with paclitaxel. Furthermore, ROS production typically reduced by nicorandil was inhibited by 5-HD in HCAECs treated with paclitaxel. This affirmed that K_{ATP} channels are important to the anti-oxidative effect and endothelial protection afforded by nicorandil. Inefficient transport of electrons due to altered mitochondrial membrane potentials can increase electron leakage and ROS production (Gupta *et al.*, 2009). Consequently, mitochondrial ROS can activate vascular NADPH oxidase (Wenzel *et al.*, 2008B). Therefore, nicorandil might exert its anti-oxidative effect by protecting mitochondria by mitochondrial K_{ATP} channel activation. Indeed, nicorandil and diazoxide are known to limit production of mitochondrial ROS (Ozcan *et al.*, 2002). Tajima *et al.* revealed that the protective effect of nicorandil on the endothelium was mediated by inhibiting NADPH oxidase activity via opening mitochondrial K_{ATP} channels (Tajima *et al.*, 2008). Although it cannot be ruled out completely, the action of nicorandil in this study did not depend on its nitrate-like activity. As described in Chapter 2, ISDN did not prevent a decrease in FMD caused by paclitaxel, and ODQ did not inhibit anti-oxidative effect of nicorandil in HCAECs.

Utility of FMD measurement in rats

Herein, I observed that FMD was clearly reduced in STZ rats without a change in endothelium independent relaxation induced by NTG (Chapter 1). This was consistent with earlier reports showing that acetylcholine-induced

relaxation was diminished in isolated vascular beds from STZ rats without affecting endothelium-independent relaxation (Gupte *et al.*, 2010; Pieper, 1997; Pieper *et al.*, 1996). In Chapter 2, moreover, FMD was reduced in rats treated with paclitaxel, which was consistent with reduction of acetylcholine-induced vasodilation. It is possible measuring FMD in living rats is a useful experimental tool to estimate endothelial dysfunction in serious vascular diseases.

Evaluation of endothelial function by measuring FMD in living rats seems to have at least 3 potential advantages. First, FMD measurements can evaluate endothelial function under both physiological and pathological conditions. Endothelial function can be influenced either by NO availability or by sensing of shear stress (Yamamoto and Ando, 2011). NO availability is determined by NO production that depends on the expression level and the enzyme activity of eNOS, both of which are modulated by various humoral factors such as increased ROS (Landmesser *et al.*, 2004), increased asymmetric dimethylarginine (ADMA) (Cooke, 2000; Landmesser *et al.*, 2004), depletion of tetrahydrobiopterin (Crabtree *et al.*, 2011; Landmesser *et al.*, 2003), and an increase in advanced glycation end products (Rojas *et al.*, 2000). In addition, NO can be trapped with ROS in a very rapid reaction, forming peroxynitrite and reducing available NO. All these lines of evidence combined to indicate that the results from isolated arterial strips may underestimate the precise *in vivo* situation of endothelial dysfunction, because these humoral factors are absent for experiments in isolated tissues. Moreover, shear stress induced by blood flow stimulates NO production from

the concerted action of multiple mechanotransducer molecules, including the glycocalyx, integrins, and caveolae (Yamamoto and Ando, 2011), which can be reduced under certain genetic or pathological conditions. Second, rats are better experimental material than large animals in many situations, even though FMD can be applied not only for clinical diagnosis but also in experiments in large animals such as dogs (Jones *et al.*, 2011) and pigs (Weber *et al.*, 2011). Rats are smaller, so experiments can be conducts with fewer amount of drug. Furthermore, many rat disease models are available. Finally, FMD makes it possible to measure endothelial function repeatedly in individual rats. Time-dependent changes in pathological conditions or chronic effects of drugs on endothelial function can be traced in the same rat by permanently implanting an iliac artery occluder. Therefore, in addition to classical endothelial function measurement methods in isolated arteries, FMD in living rats should provide useful information to not only elucidate endothelial physiology and pathology but also to ascertain the roles of endothelial protective agents.

Clinical significance

In the IONA trial, nicorandil significantly improved prognoses by reducing the incidence of major coronary events (IONA study group, 2002). One of the mechanisms underlying the improvement was explained as pharmacological preconditioning (Tsuchida *et al.*, 2002). Recent clinical studies have suggested that the protective effects of nicorandil against endothelial dysfunction would be another possible mechanism because

endothelial dysfunction is a risk factor for cardiovascular disease. It has been reported in studies of patients with cardiovascular risk factors that long-term administration of nicorandil improves FMD (Ishibashi *et al.*, 2008; Ono *et al.*, 2004; Sekiya *et al.*, 2005) and that this improvement was by reducing oxidative stress (Ishibashi *et al.*, 2008; Ono *et al.*, 2004).

In diabetic patients, morbidity and mortality rise due to vascular complications such as atherosclerosis, retinopathy and nephropathy, triggered by endothelial dysfunction. Glycaemic control agents, statins and renin-angiotensin system inhibitors ameliorate endothelial dysfunction (Potenza *et al.*, 2009), thereby improving prognosis. In this study of diabetic rats, nicorandil protected against endothelial dysfunction by inhibiting production of ROS without glycaemic control. This may mean that nicorandil can improve the prognoses of patients with ischemic heart disease and diabetic complications not only through pharmacological preconditioning, but also by protecting the endothelium. In fact, the IONA (IONA study group, 2004) and JCAD (Horinaka *et al.*, 2010) studies demonstrated that nicorandil improves the prognoses of patients with ischemic heart disease and diabetes.

As described in Chapter 2, nicorandil was found to prevent paclitaxel-induced endothelial dysfunction by reducing oxidative stress. Therefore, nicorandil may have favorable effects on endothelial dysfunction in PES-implanted patients as well as in patients with other cardiovascular risk factors. Clinical studies are needed to assess the clinical implications of our findings.

Conclusions

Nicorandil could limit endothelial dysfunction caused by diabetes or paclitaxel through anti-oxidative effects by K_{ATP} channel activation. Therefore, nicorandil might improve the prognoses of angina patients with diabetes or DES implantation.

Acknowledgements

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