



# THE ANNALS OF THORACIC SURGERY



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*Ann Thorac Surg* 2002;73:173-179

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Print ISSN: 0003-4975; eISSN: 1552-6259.

# Long-Term Treatment With Nipradilol, a Nitric Oxide–Releasing $\beta$ -Adrenergic Blocker, Enhances Postischemic Recovery and Limits Infarct Size

Yoshihiro Suematsu, MD, Toshiya Ohtsuka, MD, Hitoshi Horimoto, MD, Katsuhide Maeda, MD, Yasunari Nakai, MD, Shigetoshi Mieno, MD, and Shinichi Takamoto, MD

Department of Cardiothoracic Surgery, University of Tokyo, Tokyo, and Department of Thoracic Surgery, Osaka Medical College, Osaka, Japan

**Background.** This study examines whether the chronic administration of nipradilol, a nitric oxide–releasing  $\beta$ -adrenergic blocker, decreases ischemia-reperfusion injury.

**Methods.** Rats were treated with nipradilol (10 mg/kg per day orally) or a vehicle alone for 4 weeks. Isolated rat hearts were assigned to one of five groups (each  $n = 6$ ): global ischemia groups treated with the vehicle or with nipradilol were subjected to 20 minutes of ischemia; ischemic preconditioning groups treated with the vehicle or with nipradilol were subjected to 3 minutes of ischemic preconditioning; and the L-arginine group treated with the vehicle received 1 mmol/L of L-arginine before global ischemia. Hemodynamic variables and coronary flow were recorded continuously. Nitrites and nitrates levels were measured 60 minutes after reperfusion, and the infarct size was determined. In another series (each  $n = 6$ ), lipid peroxidation was investigated.

**Results.** In the nipradilol group, significant preserva-

tion of the left ventricular pressure and coronary flow, as well as the level of nitrates and nitrites, was observed, compared with the global ischemia group. The infarct size was also significantly reduced in the ischemic preconditioning ( $23.5\% \pm 5.47\%$ ), L-arginine ( $25.6\% \pm 5.59\%$ ), and especially the nipradilol ( $10.7\% \pm 1.65\%$ ) groups. However, in the nipradilol plus ischemic preconditioning group, the protective effects were eliminated. Lipid peroxidation after nipradilol treatment was significantly reduced before and after global ischemia, compared with the global ischemia group.

**Conclusions.** The chronic administration of nipradilol improves postischemic functional recovery and infarct size, partly by preventing the formation of lipid peroxides. These cardioprotective effects were, however, abolished by ischemic preconditioning.

(Ann Thorac Surg 2002;73:173–9)

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Nipradilol is a novel  $\beta$ -adrenergic blocker that has a nonselective  $\beta$ -adrenergic blocking action and exerts vasodilating actions by the release of nitric oxide (NO) [1, 2]. Several studies have shown  $\beta$ -adrenergic blockers to reduce the extent of myocardial injury after coronary artery occlusion [3–6]. In addition, we have previously shown that short-term application of nipradilol preconditions the heart by means of a mechanism modulating NO or  $\beta$ -adrenergic receptors [7]. However, the effects of long-term administration of this drug remain unclear. Clinically, many patients undergoing coronary operations are already taking a  $\beta$ -blocker, and these drugs are likely to be administered for very long periods. Therefore, we examined the protective effect of nipradilol against ischemic preconditioning, as well as evaluating the long-term application of nipradilol alone.

## Material and Methods

### Animals

Male Wistar rats (300 to 350 g body weight) were used in this study because of unique metabolic action of nipradilol [8]. The living conditions for the rats were a room temperature of  $23 \pm 3^\circ\text{C}$ , room humidity of  $60\% \pm 10\%$ , a 12-hour light–dark cycle, and food and water ad libitum. All animals were randomly divided into five groups. The first three groups received oral administration of 2 mL/kg per day of 0.5% carboxymethylcellulose (CMC; vehicle), and the other two groups received 10 mg/kg per day of nipradilol (suspended in 2 mL/kg of 0.5% CMC). Oral administration was continued for 4 weeks. The dosage of nipradilol was determined as described previously [9]. The general appearance of the rats was observed daily, and they were weighed every 7 days.

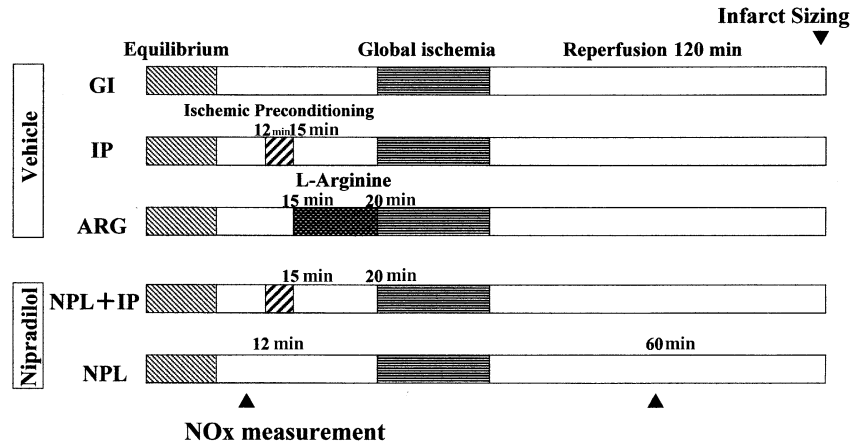
### Langendorff Perfusion

All animals were prepared as described previously [10, 11]. On the day after the final dosing, rats were anesthetized with ether, and heparin (1,000 U/kg, intravenously) was administered through the left femoral vein. The

Accepted for publication Aug 15, 2001.

Address reprint requests to Dr Suematsu, Department of Cardiothoracic Surgery, University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo, 113-8655, Japan; e-mail: suematsu@aurora.dti.ne.jp.

Fig 1. Experimental protocol showing the treatment groups. (GI = global ischemia group; IP = ischemic preconditioning group; ARG = L-arginine group; NPL+IP = nipradilol plus ischemic preconditioning group; NPL = nipradilol group; NOx = nitrates and nitrites.)



heart was rapidly excised and used for Langendorff perfusion. A water-filled latex balloon connected to a P23-XL pressure transducer (Nihon Kohden Co, Tokyo, Japan) was inserted into the left ventricle for measurement of left ventricular pressure (end-diastolic pressure and peak developed pressure). Left ventricular pressure was set to 5 mm Hg by adjusting the volume of the balloon. This balloon volume was maintained for the duration of the experiment. Coronary flow of perfusion fluid was continuously measured with an extracorporeal electromagnetic flow probe. Hemodynamic variables were measured using DTP-200 and WT 625G systems (Nihon Kohden Co, Tokyo, Japan).

The aorta was perfused with oxygenated (95% O<sub>2</sub> + 5% CO<sub>2</sub>, pH 7.4) Krebs-Henseleit buffer (in mmol/L: glucose, 11.0; NaCl, 118.5; KCl, 4.8; MgSO<sub>4</sub>, 1; KH<sub>2</sub>PO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25.0; CaCl<sub>2</sub>, 2.5) at 37°C.

### Experimental Protocol

The experimental protocol used is shown in Figure 1. Hearts were perfused for 20 minutes to establish equilibrium hemodynamics, stopping when left ventricular systolic pressure, diastolic pressure, and coronary flow were maintained at the same level for three continuous periods of measurement timed 5 minutes apart. The vehicle-treated hearts were subjected to the following three protocols: global ischemia hearts (GI, n = 6) were subjected to 20 minutes' ischemia and 120 minutes' reperfusion. Global ischemia was achieved by cross-clamping of the aortic root. Ischemic preconditioned hearts (IP, n = 6) were subjected to 3 minutes of zero-flow global ischemia followed by 5 minutes' reperfusion with Krebs-Henseleit buffer, before 20 minutes of global ischemia and 120 minutes' reperfusion; L-arginine hearts (ARG, n = 6) received 1 mmol/L L-arginine in Krebs-Henseleit buffer 5 minutes before 20 minutes of global ischemia and 120 minutes' reperfusion. A 3-minute episode of ischemia was chosen as the optimal period of ischemic preconditioning, and the dosage of L-arginine was determined as described previously [10, 12]. On the other hand, the hearts of animals receiving oral administration of nipradilol were subjected to one of the following two protocols: nipradilol plus ischemic preconditioning hearts

(NPL+IP, n = 6) received 3 minutes' ischemic preconditioning and 5 minutes' reperfusion, before 20 minutes of global ischemia and 120 minutes' reperfusion; and nipradilol hearts (NPL, n = 6) received 20 minutes of global ischemia and 120 minutes' reperfusion. After the right atrium had been incised to prevent arrhythmia, the hearts were paced continuously through the right atrium at 370 beats/min throughout the experiment using an SEN-3301 (Nihon Kohden Co, Tokyo, Japan).

### Measurement of Infarct Size

At the end of the experiment, the hearts were sliced across the long axis of the left ventricle into 1-mm-thick transverse sections and incubated in 1% triphenyl tetrazolium chloride (Sigma Chemical Co, St. Louis, MO) in phosphate buffer (pH 7.4) at 37°C for 20 minutes. Infarct areas were enhanced by storage in 10% formaldehyde solution for 24 hours before measurement. All sections were photographed with a digitalized camera and transferred to a personal computer. The area of the left ventricle and that of the infarcted tissue were measured planimetrically by an independent, blinded observer. The volumes of the infarcted zone and the area at risk were calculated by multiplying the planimetered areas by the slice thickness. Infarct volume was expressed as a percentage of left ventricular volume for each heart [13].

### Measurement of Nitrites and Nitrates

The initial coronary flow was collected with a sterilized plastic syringe. The assay was performed with an NO-analyzing system (ECO-20, Eicom Corp., Kyoto, Japan) as described previously [14]. In brief, nitrites and nitrates were separated on a polystyrene polymer column, and the nitrites were mixed with a Griess reagent to form a purple azo dye. Absorbance of the color of the dye product was measured at 540 nm with a flow-through spectrophotometer. The combined nitrites and nitrates (NOx) concentration was measured by assessing the peak area of the absorbance changes with a computer (PowerChrom; Eicom). The lowest NOx concentration detectable was approximately 0.01  $\mu$ mol/L. All results are expressed as a percentage of the preischemic value.

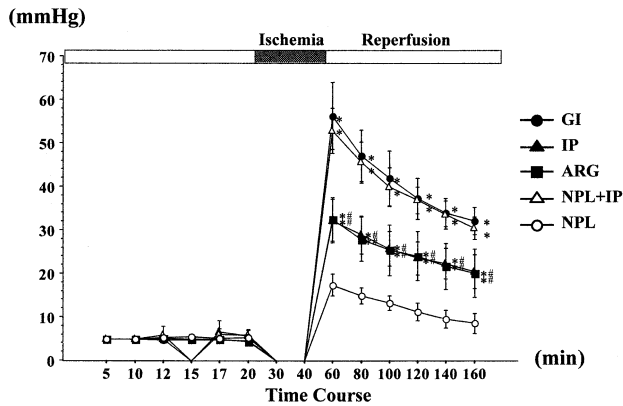


Fig 2. The effects of global ischemia (GI), ischemic preconditioning (IP), L-arginine (ARG), nipradilol plus ischemic preconditioning (NPL+IP), and nipradilol (NPL) on left ventricular end-diastolic pressure. Significant differences during reperfusion at  $p < 0.05$  versus NPL are designated by \* and  $p < 0.05$  versus GI by #.

### Measurement of Lipid Peroxidation

In another series, the vehicle-treated hearts ( $n = 6$ ) and nipradilol-treated hearts ( $n = 6$ ) were excised for measurement of lipid peroxidation before global ischemia, and then each group was subjected to 20 minutes' ischemia and 60 minutes' reperfusion. At the end of the experiment, similarly, lipid peroxidation 60 minutes after reperfusion was measured.

Cardiac lipid peroxide contents were determined by using the thiobarbituric acid reactive material method for the estimation of malondialdehyde content [15]. Hearts were homogenized (10% weight/volume) at 4°C in 0.2 mol/L Tris and 0.16 mol/L KCl buffer (pH 7.4), and the homogenate was incubated for 1 hour at 37°C in a water bath. A 2-mL aliquot was pipetted into a 12-mL Corning culture tube, followed by the addition of 2.0 mL of 40% trichloroacetic acid and 1.0 mL of 0.2% thiobarbituric acid. To minimize peroxidation during the assay procedure, 100  $\mu$ L of 2% butylated hydroxytoluene was added to 10 mL of the thiobarbituric acid solution. Tubes were boiled for 15 minutes and then cooled on ice for 5 minutes. Two milliliters of 70% trichloroacetic acid were added, and the contents were briefly vortexed. Tubes were allowed to stand for 20 minutes at room temperature and were then centrifuged at 800 g for 20 minutes. The developed color was read at 532 nm on a spectrophotometer.

### Statistical Analysis

Statistical analysis was performed using the Stat View (version 5) software package (SAS Institute Inc, Cary, NC). Data are expressed as means  $\pm$  standard deviations. Hemodynamic variables were analyzed by two-way repeated-measures analysis of variance (time and group). Infarct sizes and NO<sub>x</sub> levels during reperfusion were analyzed with one-way analysis of variance followed by the Student's *t* test for unpaired data with Bonferroni correction. Values at *p* less than 0.05 were considered significant.

All animals in this study received humane care accord-

ing to the guidelines in "The Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication 86-23, revised 1985). In addition, animals were used in accordance with the guidelines of the University of Tokyo Institutional Animal Care and Protocol of Animal Preparation.

## Results

### Left Ventricular Pressure

No significant differences in left ventricular end-diastolic pressure, peak developed pressure, and coronary flow were observed within or between groups during equilibrium periods. The effects of GI, IP, ARG, NPL+IP, and NPL on left ventricular pressure in isolated hearts during equilibrium, after 20 minutes of normothermic global ischemia, and after 120 minutes' reperfusion are shown in Figures 2 and 3. Significant differences in left ventricular end-diastolic pressure were observed between NPL and the other four groups during the reperfusion period. In addition, the left ventricular end-diastolic pressure in the GI group showed statistically significant differences from the IP and ARG groups, but not the NPL+IP group. Throughout reperfusion, the rise in left ventricular peak developed pressure in the NPL group was significantly greater than those in the other four groups. Although there was no significant difference between the IP and NPL+IP groups, peak developed pressure in NPL+IP decreased compared with that in the IP group throughout reperfusion.

### Coronary Flow

Coronary flow in the IP and NPL+IP groups decreased to 0% of the preischemic level, and returned to 108.0%  $\pm$  6.69% of the preischemic level in the IP group and 106.5%  $\pm$  4.37% in the NPL+IP group, after ischemic precondi-

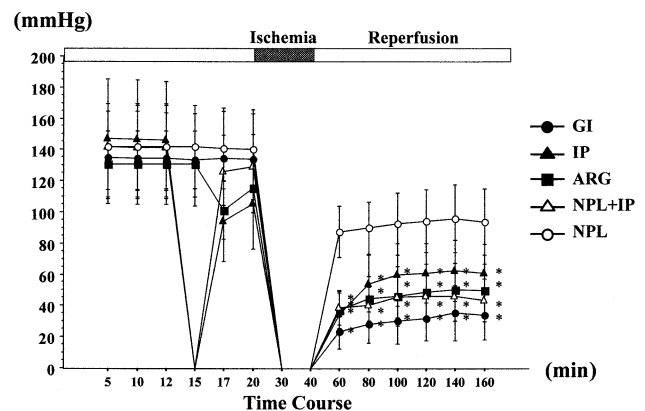


Fig 3. The effects of global ischemia (GI), ischemic preconditioning (IP), L-arginine (ARG), nipradilol plus ischemic preconditioning (NPL+IP), and nipradilol (NPL) on left ventricular peak developed pressure. Significant differences during reperfusion at  $p < 0.05$  versus NPL are designated by \*.

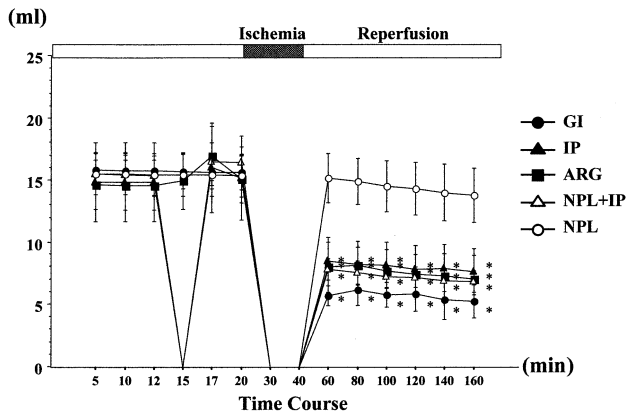


Fig 4. The effects of global ischemia (GI), ischemic preconditioning (IP), L-arginine (ARG), nipradilol plus ischemic preconditioning (NPL+IP), and nipradilol (NPL) on coronary flow. Significant differences during reperfusion at  $p < 0.05$  versus NPL are designated by \*.

tioning (Fig 4). Significant differences were observed in coronary flow between NPL and the other four groups during reperfusion.

#### Myocardial Infarct Size

Infarct size, expressed as a percent of ventricular myocardial volume, was  $31.1\% \pm 4.13\%$  in the GI,  $23.5\% \pm 5.47\%$  in the IP,  $25.6\% \pm 5.59\%$  in the ARG, and  $22.2\% \pm 4.00\%$  in the NPL + IP groups (Fig 5). Infarct size was significantly less in NPL ( $10.7\% \pm 1.65\%$ ) than in the other four groups. Furthermore, infarct size in the IP and ARG groups was significantly less than that in the GI and NPL+IP groups, and there was no significant difference between the NPL+IP and GI groups.

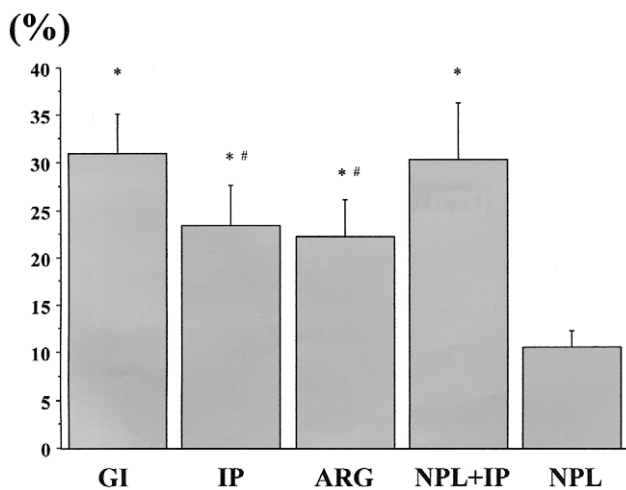


Fig 5. The effects of global ischemia (GI), ischemic preconditioning (IP), L-arginine (ARG), nipradilol plus ischemic preconditioning (NPL+IP), and nipradilol (NPL) on infarct size (percent of left ventricular volume). Significant differences in infarct size at  $p < 0.05$  versus NPL are designated by \* and  $p < 0.05$  versus GI by #.

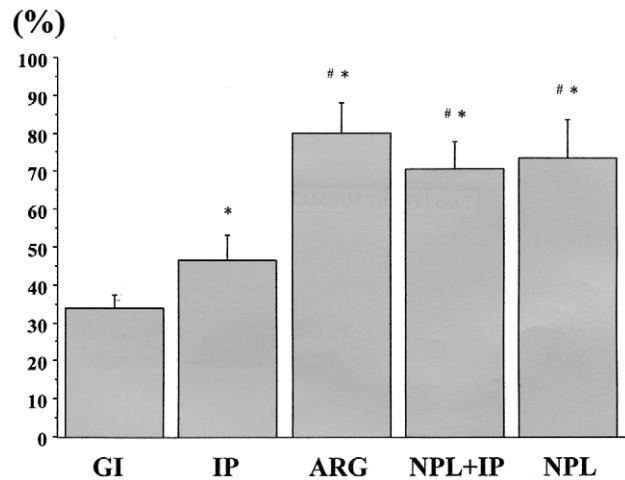


Fig 6. The effects of global ischemia (GI), ischemic preconditioning (IP), L-arginine (ARG), nipradilol plus ischemic preconditioning (NPL+IP), and nipradilol (NPL) on nitrates and nitrites 60 minutes after reperfusion. Each value is presented as a percentage of the preischemic value. Significant differences during reperfusion at  $p < 0.05$  versus GI are designated by \* and  $p < 0.05$  versus IP by #.

#### Measurement of Nitrates and Nitrites

The NOx concentration after 60 minutes' reperfusion is shown in Figure 6. Nitrates and nitrites decreased to  $34.1\% \pm 3.51\%$  of the preischemic levels in the GI group. The NOx concentrations in the ARG ( $80.5\% \pm 7.95\%$ ), NPL+IP ( $70.9\% \pm 7.14\%$ ), and NPL ( $73.8\% \pm 9.91\%$ ) groups were significantly greater than those in the GI and IP groups. There was no significant difference between the NPL and NPL+IP groups.

#### Measurement of Lipid Peroxidation

Lipid peroxidation was investigated by assessing the levels of thiobarbituric acid reactive substances. Before ischemia-reperfusion, the values were  $22.3 \pm 3.8$  and  $14.2 \pm 2.1$  nmol/g in hearts from the GI and NPL groups, respectively, showing a statistically significant difference (Fig 7). Ischemia-reperfusion significantly increased thiobarbituric acid reactive substances in both the GI and the NPL groups ( $52.1 \pm 23.7$  and  $25.5 \pm 7.1$  nmol/g, respectively); the NPL group level was significantly lower than that of the GI group.

#### Comment

Our study shows that the NO-releasing  $\beta$ -adrenergic blocker nipradilol can improve postischemic functional recovery, preserve coronary flow, and reduce infarct size. Both L-arginine and ischemic preconditioning had cardioprotective effects in our experiment, consistent with other experimental reports. On the other hand, the effect of ischemic preconditioning was abolished in the hearts of rats that had received oral nipradilol, although the NOx concentration was significantly preserved as compared with ischemic preconditioning alone. Furthermore, lipid peroxidation in nipradilol-treated hearts was signif-

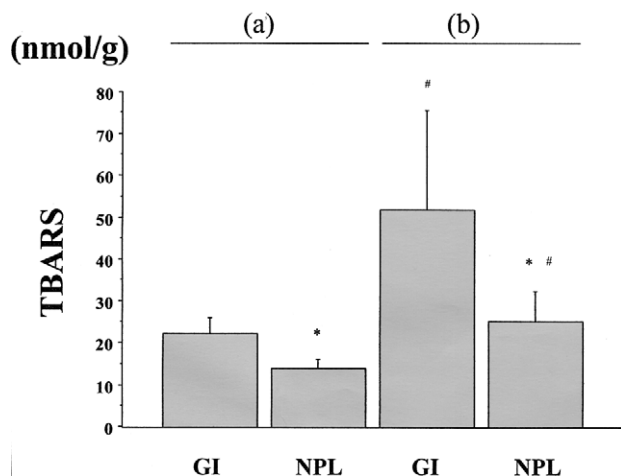


Fig 7. Lipid peroxidation in the global ischemia (GI) and nipradilol (NPL) groups. (a) Baseline level of thiobarbituric acid reactive substances before ischemia-reperfusion. (b) Thiobarbituric acid reactive substances, after 20 minutes' ischemia and 60 minutes' reperfusion. Significant differences at  $p < 0.05$  versus hearts in (a) are designated by # and  $p < 0.05$  versus the GI group by \*.

icantly reduced before and after global ischemia, as compared with vehicle-treated hearts.

Nipradilol is a  $\beta$ -adrenergic blocker that has the structural characteristics of a benzopyran skeleton with a nitroso residue, and reportedly increases cyclic guanosine monophosphate [16]. Organic nitrites are preserved to be denitrated to form the active agent NO by a multistep process, which can increase cyclic guanosine monophosphate. Nitric oxide is well known as an endothelium-derived relaxing factor and is released from vascular endothelial cells, regulating vascular tone. Moreover, several energy-sparing actions have been proposed, including inhibition of calcium influx into myocytes, decreased myocardial contractility, and decreased mitochondrial respiration [17, 18]. In the present study, L-arginine, a precursor of NO, enhanced postischemic functional recovery and significantly decreased myocardial infarct size. The level of NOx was significantly elevated as compared with the GI and IP groups, suggesting that NO generation contributes to cardioprotection. In addition, elevated NOx in the NPL group, which was the same as the ARG group level, demonstrates the cardioprotective effects of nipradilol to involve NO to some extent.

Several studies [3-6] have shown  $\beta$ -adrenergic blockers reduce the damage induced by experimental myocardial infarction. Mak and Weglicki [3], in their short-term in vivo as well as ex vivo ischemia-reperfusion experiments, demonstrated that the  $\beta$ -adrenergic blocker reduces the ischemia-reperfusion-induced increase in lipid peroxidation and provides the antiperoxidative protection of ischemic hearts. In addition, Khaper and colleagues [4] showed that long-term propranolol treatment provided an indirect antioxidant effect secondary to  $\beta$ -adrenergic blockade or membrane stabilization and significantly increased contractile function after global

ischemia. Our study also demonstrated lipid peroxidation in nipradilol-treated hearts to be significantly reduced before and after global ischemia, as compared with vehicle-treated hearts. However, NO reportedly exerts a positive cardioprotective effect by inhibiting the release of superoxide radicals or by quenching superoxide radicals produced by neutrophils [19]. Therefore, further experiments are needed to determine whether decreased lipid peroxidation in nipradilol-treated hearts is caused by NO or  $\beta$ -adrenergic antagonism itself.

Ischemic preconditioning is a phenomenon whereby repetitive brief periods of ischemia result in a tolerance to subsequent longer ischemic episodes and reduce ischemia-reperfusion injury. This phenomenon has been reported to limit infarct size [20], attenuate the progression of ischemia-induced metabolic disorders and cell necrosis [21], and reduce the occurrence of arrhythmia after ischemia-reperfusion [22]. In this study, ischemic preconditioning in the hearts of rats that had received oral nipradilol failed to improve cardiac function and limit infarct size. Ischemic preconditioning has been suggested to occur by means of protein kinase C and adenosine triphosphate-sensitive potassium channel-mediated mechanisms [23]. Yabe and associates [24] recently reported that pharmacologic preconditioning by  $\beta$ -adrenergic stimulation activated protein kinase C, which phosphorylates intracellular enzymes and transmembrane ion channels such as the adenosine triphosphate-sensitive potassium channel. On the other hand, Lochner and coworkers [25], in their isolated perfused rat heart experiment, concluded that ischemia-induced activation of the  $\beta$ -adrenergic signaling pathway during preconditioning is a trigger in ischemic preconditioning. Therefore,  $\beta$ -adrenergic blockade can be expected to interfere with ischemic preconditioning.

Meanwhile, the effect of NO on ischemic preconditioning remains unclear. In this study, infarct size was not reduced in the NPL+IP group, although the level of NOx production was the same in the NPL+IP and NPL groups. Balligand and associates [26] demonstrated that inhibition of NO production in adult rat ventricular myocytes was also reversible with L-arginine; they suggested that NO antagonizes  $\beta$ -adrenergic activation. Thus, the cardioprotective effect of nipradilol may be completely eliminated by the  $\beta$ -adrenergic blocking action and NO or by some other underlying mechanism. In our study, however, coronary flow during reperfusion significantly increased in the NPL group compared with the other four groups. The excellent postischemic functional recovery may be related to the increase in coronary flow. Nevertheless, further research is required to reveal the cardioprotective mechanism of nipradilol and the relationship between NO production and  $\beta$ -adrenergic receptor transduction with respect to ischemic preconditioning.

The current study has several limitations. First, this model uses isolated crystalloid-perfused heart preparations to allow for comparison with previous studies that have used these preparations [27, 28]. However, this model does not account for the intervening variables associated with in situ blood-perfused heart prepara-

tions. It is well known that blood-perfused hearts exhibit a greater resistance to ischemia and that infarcts are smaller as compared with crystalloid-perfused hearts [29]. In addition, NO may have many more complex effects in vivo, as it has been shown to inhibit leukocyte adherence to vascular endothelium and to diminish neutrophil and platelet aggregation [30]. Second, a single 3-minute episode of ischemic preconditioning was used in this study. Although infarct sizes in the IP and ARG groups were significantly smaller than those in the GI group, the ischemic episode may have been inadequate to generate a complete preconditioning response. Multiple cycles of ischemic preconditioning have been shown to be effective [31]. Further data are clearly required to examine the relationship of the  $\beta$ -adrenergic blocker and NO on other ischemic preconditioning protocols. Finally, the animals used for our study, aside from the differences among animal species, also had normal hearts as well as being free of coronary artery lesions or collateral circulation. Therefore, further data are clearly required before nipradilol can be applied in a clinical setting.

In summary, the results of the present study indicate that nipradilol significantly improves postischemic functional recovery and limits infarct size by decreasing lipid peroxidation, relative to vehicle treatment. The mechanism of the cardioprotective effect may partly involve a combination of effects on NO and  $\beta$ -adrenergic antagonism, which have feedback mechanisms against ischemic preconditioning. Nipradilol is currently marketed as a therapy for coronary artery disease and hypertension, and  $\beta$ -adrenergic blockers are prescribed for many patients undergoing coronary surgical procedures. These results suggest that nipradilol therapy may have cardioprotective benefits and may be useful for patients requiring a coronary artery operation.

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The statistical analysis described in this study was performed in consultation with Hajime Sato, MD, MPH, DMSc, DrPH, Department of Public Health and Occupational Medicine, University of Tokyo, Tokyo, Japan.

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## INVITED COMMENTARY

It is a privilege to review and comment on this interesting article. The experimental study presented by Dr Sue-matsu and coworkers demonstrates the beneficial effects of chronic administration of Nipradilol, a nitric oxide-releasing beta-adrenergic blocker, on myocardial injury following ischemic injury using an isolated rat heart model. The authors have postulated that chronic pretreatment with Nipradilol may attenuate the ischemic myocardial injury by enhancing coronary endothelial nitric oxide (NO) production and reducing lipid-peroxidation, and the results were mostly supportive of this hypothesis. This important work, after their previous work with short-term treatment with nipradilol, may differentiate a possible mechanism for Nipradilol protection of the myocardium against ischemic injury from the mechanism of NO production and lipid peroxidase inhibition. The conclusions are sound and mostly acceptable, however, there are some concerns about the interpretation of results. The following issues should be discussed to verify the conclusions derived from this study.

The result from combined pretreatment with Nipradilol and ischemic preconditioning raises concerns, because the combination almost abolished the beneficial effects of Nipradilol pretreatment or produced worse results compared to the group with ischemic preconditioning alone. This may be a unique finding; however, the result might have been different if the authors had chosen a different time course for ischemic preconditioning. As the authors mentioned, the time protocol for the ischemic preconditioning employed here may not be optimal for a maximal protective effect. The optimal time

protocol for ischemic preconditioning is still controversial, but an appropriate protocol is mandatory in this study to examine protective behavior against ischemic injury with and without Nipradilol.

The result also introduces a problem in the clinical setting. If patients have been pretreated with a beta-blocker, is ischemic preconditioning not recommended during intervention or surgery? The effect of ischemic preconditioning in this study should be clearly described and more detailed data may be required. Since the mechanism of the ischemic preconditioning is thought to include ATP sensitive K<sup>+</sup> channels, endogenous NO synthesis, adenosine metabolism and more, further investigation to examine the protective mechanism of Nipradilol using possible modifications of these mechanisms is necessary in future.

In summary, although the study design and the results have some controversial points, the authors deserve congratulations for their stimulating work in pharmacological myocardial protection and its mechanism from the standpoint of ischemic preconditioning and endogenous NO synthesis.

*Hikaru Matsuda, MD, PhD*

*Department of Surgery  
Course of Interventional Medicine (E1)  
Osaka University Graduate  
School of Medicine  
Osaka 565-0871, Japan  
e-mail: matsuda@surg1.med.osaka-u.ac.jp.*

**Long-term treatment with nipradilol, a nitric oxide-releasing  $\beta$ -adrenergic blocker, enhances postischemic recovery and limits infarct size**

Yoshihiro Suematsu, Toshiya Ohtsuka, Hitoshi Horimoto, Katsuhide Maeda, Yasunari Nakai, Shigetoshi Mieno and Shinichi Takamoto

*Ann Thorac Surg* 2002;73:173-179

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