Different Contributions of Endothelin-A and Endothelin-B Receptors in Postischemic Cardiac Dysfunction and Norepinephrine Overflow in Rat Hearts

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Background—Endothelin (ET)-1 and norepinephrine (NE) are involved in myocardial ischemia/reperfusion injury. We investigated the role of ET-1 in ischemia/reperfusion–induced NE overflow and cardiac dysfunction using a selective ETA receptor antagonist (ABT-627), a selective ETB receptor antagonist (A-192621), and the spotting lethal (sl) rat, which carries a naturally occurring deletion in the ETB receptor gene.

Methods and Results—According to the Langendorff technique, isolated hearts were subjected to 40-minute global ischemia followed by 30-minute reperfusion. In Sprague-Dawley rat hearts, ischemia/reperfusion–induced cardiac dysfunctions such as decreased left ventricular developed pressure and coronary flow and increased left ventricular end-diastolic pressure were worsened by treatment with A-192621. This agent enhanced excessive NE overflow in the coronary effluent from the postischemic heart. In contrast, treatment with ABT-627, in the absence or presence of A-192621, significantly improved postischemic cardiac dysfunction and markedly suppressed NE overflow to the same extent. Postischemic cardiac dysfunction and NE overflow in the heart of ETB receptor–deficient homozygous (sl/sl) rats were highly observed compared with cases in wild-type rats, and exaggerated responses to ischemia/reperfusion in sl/sl rats were abolished by ABT-627 treatment. Exogenously applied ET-1 produced severe cardiac dysfunction and a significant increase in NE overflow in a dose-dependent manner, but these responses were markedly suppressed in the presence of 5-N-ethyl-N-isopropyl-amiloride, an inhibitor of the Na+/H+ exchanger (NHE).

Conclusions—Pharmacological blockade or genetic deficiency of ETB receptors is detrimental to the postischemic heart, and exaggerated cardiac pathology under the above conditions is mediated by ETA receptor activation. ETA/NHE-mediated excessive NE overflow is contributive, at least in part, to postischemic cardiac dysfunction in rats. (Circulation. 2005;111:302-309.)

Key Words: endothelin ■ ischemia ■ norepinephrine ■ reperfusion

Endothelin (ET)-1, a 21-amino acid peptide, is produced by vascular endothelial cells,1 vascular smooth muscle cells,2 and cardiomyocytes.3 ET-1 is most abundant in cardiovascular system, and 2 distinct ET receptors, ETA and ETB, have been identified and cloned in mammalian tissues.4,5 Increased plasma ET-1 levels are observed in patients with coronary artery diseases such as myocardial infarction and angina and immediately after PTCA. In isolated perfused rat hearts, endogenous ET-1 is known to be released during ischemia/reperfusion.8 In addition, coronary artery occlusion and reperfusion in pigs increased the local overflow and tissue content of ET-1–like immunoreactivity.9 It has been reported that ischemia increases ET-1 binding sites in cardiac membranes.10 These findings suggest that endogenous ET-1 plays an important role in the pathophysiology of myocardial ischemia/reperfusion. Actually, a monoclonal antibody against ET-1 can reduce infarct size in rats after coronary artery ligation and reperfusion.11 Moreover, both selective ETA receptor antagonists and nonselective ETA/ETB receptor antagonists exhibited protective effects against postischemic cardiac dysfunction,12–14 although others failed to observe such beneficial effects.15

Norepinephrine (NE) release from cardiac sympathetic nerve endings occurs mainly by 2 pathways: Ca2+-dependent exocytotic release and Ca2+-independent carrier-mediated release via activation of the NE transporter (NET) in the outward direction.16,17 In physiological conditions and acute myocardial ischemia (<10 minutes), the NE release is exocytic and dependent on a rise in axoplasmic Ca2+ concentration. The majority of NE released by exocytosis is re-
trived from extracellular space via NET with the Na⁺ gradient. On the other hand, carrier-mediated NE release is known to be induced by protracted myocardial ischemia, which is mediated by the Na⁺/H⁺ exchanger (NHE)–dependent mechanism.⁶,¹⁷ The decreased oxygen supply by ischemia causes ATP depletion and intracellular acidosis due to lactate production. In sympathetic nerve endings in the ischemic condition, free axoplasmic NE accumulates massively owing to the lack of driving force for NE storage, because the vesicular storage of NE depends on the H⁺ gradient and ATP in physiological conditions. Increased axoplasmic H⁺ activates NHE, which consequently leads to an influx of Na⁺ in exchange for H⁺. Furthermore, the inhibition of Na⁺/K⁺ ATPase activity by ATP depletion results in the accumulation of axoplastic Na⁺. This Na⁺ accumulation triggers excessive axoplastic NE release via the reversal of NET from the intracellular space to extracellular space.¹⁸ It has been considered that in protracted myocardial ischemia, this carrier-mediated NE release is the major mechanism for NE overflow from the nerve endings.¹⁸ Enhanced NE release induced by ischemia/reperfusion increases oxygen demand by stimulating heart rate and contractility and decreases oxygen supply by constricting coronary vessels. This vicious circle accelerates the progression of cell damage in ischemic myocardium and potentiates arrhythmogenicity.¹⁹–²¹ In fact, elevation of plasma NE concentration is a predictable factor in the development of ischemic cardiovascular diseases.²² On the other hand, it has been demonstrated that the negative modulation of NE release significantly suppresses postischemic cardiac dysfunction and arrhythmias.²³,²⁴,²⁵

In the heart under physiological conditions, ET-1 decreases the NE efflux evoked by sympathetic nerve stimulation,²⁶ whereas the relationship between ET-1 and cardiac sympathetic nervous system in ischemic conditions is unclear. Therefore, we first evaluated the possible involvement of ET-1 and its receptor subtypes in ischemia/reperfusion-induced NE overflow and cardiac dysfunction using a selective ETA receptor antagonist, ABT-627,²⁶ and a selective ETB receptor antagonist, A-192621.²⁷ In previous studies, we have found that catecholamine secretion in the adrenal gland and NE overflow in response to renal nerve stimulation are suppressed by the activation of ETB receptors.²⁸,²⁹ Second, to determine the role of ETB receptor–mediated ET-1 action in the postischemic heart, we used the spotting-lethal (sl/sl) rat, which carries a naturally occurring deletion in the ETB receptor gene.³⁰ Because homozygous (sl/sl) rats do not live beyond 1 month because of intestinal aganglionosis and the resulting intestinal obstruction, dopamine β-hydroxylase promoter was used to direct ETB transgene expression in sl/sl rats to support normal enteric nervous system development.³¹ These transgenic sl/sl rats live into adulthood and are healthy. They are ETB-deficient in the cardiovascular system, most importantly in vascular endothelium.³² The “rescued” ETB receptor–deficient sl/sl rats are therefore a useful tool in determining the pathophysiological roles of ETB receptors in the cardiovascular system.

### Methods

#### Animals

Two series of experiments were performed. In the first series to evaluate the roles of endogenous or exogenous ET-1 in ischemia/reperfusion-induced cardiac dysfunction, male Sprague-Dawley rats (weight 280 to 350 g, Japan SLC, Inc, Shizuoka, Japan) were used. In the second series, male “rescued” ETB receptor–deficient and wild-type (+/+) rats (weight 270 to 320 g) were used. The creation of transgenic sl/sl rats has been described previously.³¹ The animals were housed in a light-controlled room with a 12-hour light/dark cycle and were allowed ad libitum access to food and water. Animals were maintained at the departmental animal care facility of Osaka University of Pharmaceutical Sciences in accordance with the recommendations in the Declaration of Helsinki. Experimental protocols and animal care methods were approved by the Experimental Animal Research Committee of Osaka University of Pharmaceutical Sciences.

#### Isolated Rat Heart Preparation

Animals were anesthetized with sodium pentobarbital (50 mg/kg IP). Hearts were rapidly excised, connected via the aorta to Langendorff apparatus (IPH-W2, Labo Support), and perfused in a retrograde manner at a constant pressure of 80 mm Hg with perfusate (Krebs-Henseleit solution) of the following composition (mmol/L): NaCl 118.1, KCl 4.6, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 24.8, glucose 10.³³ The perfusate was bubbled continuously with a gas mixture of 95% O₂/5% CO₂ (pH 7.4), and the temperature was maintained at 37°C throughout the experiment. A latex balloon filled with water was inserted into the left ventricle through the left atrium and attached to a pressure transducer (DX-360, Nihon Kohden). Left ventricular developed pressure (LVPD) and left ventricular end diastolic pressure (LVEDP) were measured by an amplifier for pressure measurement (AP601G, Nihon Kohden); the maximum value of the first derivative of left ventricular pressure was measured with a derivative operation unit (Eq 621G, Nihon Kohden), and these parameters were recorded with PowerLab/4sp (ADInstruments). Coronary flow (CF) was also monitored. The balloon volume was adjusted to provide an LVEDP of 10 mm Hg. After stabilization for 20 to 30 minutes, the experiment was initiated.

#### Experimental Protocol

After stabilization, the hearts were subjected to global ischemia for 40 minutes by clamping of the aortic cannula, followed by reperfusion for 30 minutes. ABT-627 and A-192621 were perfused 30 minutes before ischemia and during reperfusion. The concentrations of ABT-627 (5 μmol/L) and A-192621 (1 μmol/L) were determined based on previous studies²⁶,²⁷ and our pilot study, in which the above concentrations of ABT-627 and A-192621 almost completely suppressed ET-1–induced vasoconstriction and sarafotoxin S6c–induced vasorelaxation in isolated blood vessels, respectively. ET-1 and 5-N-ethyl-N-isopropyl-amiloride (EIPA), an NHE inhibitor,³⁴ were perfused 10 and 20 minutes before the ischemic period, respectively, and during reperfusion. The concentrations of ET-1 (0.03 and 0.1 nmol/L) were determined from preliminary data with dose-response curves using 0.01 to 1 nmol/L ET-1. EIPA was added at 10 μmol/L on the basis of the previous study.³⁵

#### NE Assay

NE in the coronary effluent was measured with high-performance liquid chromatography and an amperometric detector (ECD-100, Eicom), as reported previously.³⁶

#### Drugs

ABT-627 and A-192621 were provided by Abbott Laboratories. They were dissolved in ethanol, and the final concentration of ethanol in the perfusate was 0.005%. ET-1 was purchased from Peptide Institute. ET-1 was dissolved in a saline solution containing 0.1% heat-inactivated bovine serum albumin. EIPA was purchased from Sigma Chemical Company, dissolved in ethanol, and diluted as

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above. Other chemicals were obtained from Nacalai Tesque and Wako Pure Chemical Industries.

**Statistical Analysis**

All values were expressed as mean±SEM. Relevant data were processed by InStat (Graph-PAD Software for Science). For statistical analysis of cardiac function parameters, we used the Friedman nonparametric repeated-measures test followed by a Dunn multiple comparison test for within-group data (time effect). For among-group data (treatment effect at the end of reperfusion period), we used 1-way ANOVA combined with Dunnett (for SD rats) or Bonferroni (for ETB-deficient rats) multiple range tests for multiple comparisons. Differences were considered significant at \( P<0.05 \).

**Results**

**Effects of ABT-627 and A-192621 on Ischemia/Reperfusion–Induced Cardiac Dysfunction**

The perfusion of ABT-627 and A-192621 produced no significant changes in basal cardiac function such as LVDP, LVEDP, and CF. As shown in Figure 1A, the preischemic level of LVDP was markedly reduced by 40 minutes of global ischemia, although the levels gradually recovered after reperfusion. Treatment with A-192621 slightly but significantly worsened the recovery of LVDP after reperfusion. In contrast, treatment with ABT-627, or a combination of ABT-627 and A-192621, markedly improved the reduction of LVDP, to the same level (Figure 1A). A similar pattern was observed in changes in CF after reperfusion, with or without ABT-627 and A-192621 (Figure 1C). As shown in Figure 1B, the elevation of LVEDP observed after reperfusion was enhanced by A-192621 treatment but was significantly attenuated by ABT-627 treatment. Similar attenuation was observed with the combination of both drugs.

**Effects of ABT-627 and A-192621 on Ischemia/Reperfusion–Induced NE Overflow**

Basal NE overflow in the coronary effluent before ischemia was extremely low (300 to 500 pg/5 minutes), and ABT-627 and A-192621 did not affect this basal NE overflow. NE overflow in the coronary effluent after 40-minute global ischemia and reperfusion was much higher than the preischemic basal level, increasing to 26394±2114 pg/5 minutes. This massive NE overflow was markedly suppressed by treatment with ABT-627 (7543±1170 pg/5 minutes), whereas A-192621 further enhanced NE overflow (44042±4939 pg/5 minutes). However, A-192621–induced enhancement was completely abolished by concomitant treatment with ABT-627 (13492±1236 pg/5 minutes; Figure 2).

**Cardiac Dysfunction After Ischemia/Reperfusion in Wild-Type and ET\(_B\) Receptor–Deficient Homozygous (sl/sl) Rats**

There was no significant difference in LVDP before ischemia between sl/sl and wild-type rats. As shown in Figure 3, preischemic levels of LVDP were markedly reduced by 40-minute ischemia and reperfusion in both animals, but the extent of reduction was greater in sl/sl than in wild-type rats.
Treatment with ABT-627 significantly improved ischemia/reperfusion–induced contractile dysfunction in both groups, showing a more marked effect in sl/sl rats. Similarly, monitoring of LVEDP (Figure 4) and CF (Figure 5) revealed severe cardiac dysfunction in sl/sl rat hearts exposed to ischemia and reperfusion and its abolition by ABT-627 treatment.

NE Overflow After Ischemia/Reperfusion in Wild-Type and ETβ Receptor–Deficient Homozygous (sl/sl) Rats
Basal NE overflow before ischemia was extremely low, and there were no significant differences between wild-type and sl/sl rats. Forty minutes of global ischemia and reperfusion increased cumulative NE overflow in both animals, and the increment was much more marked in sl/sl than in wild-type rats (wild-type rats 28 ± 128 pg/5 minutes versus sl/sl rats 61 ± 205 pg/5 minutes). ABT-627 suppressed ischemia/reperfusion–induced NE overflow in both animals, to the same level (Figure 6).

Effects of ET-1 and EIPA on Ischemia/Reperfusion–Induced Cardiac Dysfunction
Exogenously applied ET-1 produced no significant changes in basal LVDP, LVEDP, or CF, but the peptide aggravated postischemic cardiac dysfunction in a dose-related manner. However, in the presence of EIPA, ET-1 (0.1 nmol/L) failed to worsen postischemic cardiac dysfunction (Figure 7).

Effects of ET-1 and EIPA on Ischemia/Reperfusion–Induced NE Overflow
Exogenous ET-1 (0.03 and 0.1 nmol/L) had no influence on preischemic NE overflow. As shown in Figure 8, ET-1 significantly increased the cumulative NE overflow for 5 minutes after reperfusion in a dose-dependent manner (ET-1 0.03 nmol/L 55 ± 7139 pg/5 minutes; ET-1 0.1 nmol/L 72 ± 2091 pg/5 minutes versus no addition, 26 ± 2114 pg/5 minutes). EIPA, an NHE inhibitor, efficiently suppressed the ischemia/reperfusion–induced NE overflow (8283 ± 1523 pg/5 minutes). Moreover, ET-1 (0.1 nmol/L)–
induced excessive NE overflow was completely abolished by
the combination with EIPA (17 389±4446 pg/5 minutes).

Discussion
It has been shown that both selective ETA receptor antagonists
and nonselective ETα/ETβ receptor antagonists improve func-
tional recovery after global ischemia/reperfusion and reduce
myocardial infarction induced by coronary occlusion and
reperfusion.12–14,37–39 In the present study, we also observed
that ABT-627 alone and the combination of ABT-627/A-
192621 had similar protective effects against postischemic
cardiac dysfunction in rat hearts. Previous studies have
demonstrated that ET-1 mRNA expression and its peptide
production are increased in cardiomyocytes subjected to
ischemia40 and that plasma ET-1 levels are elevated in both
humans5 and experimental animals11,41 with myocardial in-
farction. Taken together, it is reasonable to consider that
cardiac ET-1 production is enhanced in the ischemic condi-
tion and is contributive to the ischemia/reperfusion–induced
injury by exclusively stimulating ETA receptors.

On the other hand, the pathophysiological role of ETβ
receptors in myocardial injury after ischemia/reperfusion has
not been fully elucidated. We noted that ETβ receptor
blockade with A-192621 worsened the systolic and diastolic
dysfunction of the myocardium exposed to ischemia/reperfu-
sion. Moreover, we found that ischemia/reperfusion–induced
cardiac dysfunction was much more severe in ETβ receptor–
deficient sl/sl rats than in wild-type rats. However, treatment
with a selective ETA receptor antagonist, ABT-627, could
abolish the above detrimental effects induced by pharma-
ological blockade or the genetic deficiency of ETβ receptors.
Brunner and Doherty42 have demonstrated that BQ-788, an
ETB receptor antagonist, increases ET-1 release and elevates
coronary resistance in isolated rat hearts. They suggested that
cardiac ETβ receptors were involved in the local sequestration
and clearance of ET-1, both in ischemic and nonischemic
situations, and that ET-1 displaced by the ETβ antagonist
stimulated ETA receptors, which resulted in coronary con-
striction. In the present study, A-192621 tended to enhance
the decrease in CF induced by ischemia/reperfusion, whereas
ABT-627 with or without A-192621 equally attenuated it. In
addition, exaggerated CF reduction after ischemia/reperfu-
sion in sl/sl rats was remarkably improved by ABT-627
treatment. Thus, our findings fundamentally agree with the

Figure 5. Effects of ABT-627 on ische-
mia/reperfusion–induced changes in CF
in wild-type (A) and ETβ receptor–defi-
cient sl/sl (B) rat hearts. Values are
mean±SEM. *P<0.01 vs wild-type with-
out ABT-627. †P<0.01 vs sl/sl without
ABT-627.

Figure 6. Effects of ABT-627 on ische-
mia/reperfusion–induced cumulative NE
overflow for 5 minutes after reperfusion
in wild-type and ETβ receptor–deficient
sl/sl rat hearts. Values are mean±SEM.
*P<0.05 vs wild-type without ABT-627.
†P<0.01 vs sl/sl without ABT-627.
view suggested by Brunner and Doherty. Because exaggerated cardiac dysfunction due to genetic deficiency or the pharmacological blockade of ET<sub>B</sub> receptors was completely abolished by ABT-627, it appears likely that an increase in ETA receptor–mediated action, rather than a decrease in ETB receptor–mediated action, is responsible for the detrimental effects on postischemic cardiac dysfunction. The antagonism of ETA receptors appears to have a protective effect on ischemia/reperfusion–induced cardiac dysfunction, irrespective of the presence of ETB receptors.

In myocardial ischemia, sympathetic overactivity accompanied by excessive NE release is also associated with cardiac dysfunction and arrhythmia, and it increases metabolic demand, thereby exaggerating the primary ischemia and initiating a malignant cycle that can cause further myocardial damage and high-risk cardiac dysfunction. On the other hand, it has been suggested that the negative modulation of NE release or blockade of its effects efficiently improves postischemic dysfunction and arrhythmia. Most recently, we found that the attenuation of NE overflow after ischemia/reperfusion resulted in a marked improvement in postischemic cardiac dysfunction in isolated rat hearts. Several investigations have also indicated that there is a direct correlation between NE release and the severity of reperfusion arrhythmia in posts ischemic guinea pig, rat, and mouse hearts. Furthermore, it has been demonstrated that increased plasma NE levels in patients with asymptomatic left ventricular dysfunction appear to predict all-cause and cardiovascular mortality and the development of clinical events related to the onset of heart failure or acute ischemic syndromes.

Although the pathological role of ET-1 in myocardium and coronary vascular bed under ischemic conditions has been described, the relationship between ET-1 and the cardiac sympathetic nervous system in ischemic conditions is unclear. Therefore, we evaluated the possible involvement of endogenous ET-1 in postischemic massive NE release. Treatment with ABT-627 with or without A-192621 markedly suppressed the ischemia/reperfusion–induced NE overflow to the same level. In contrast, exaggerated NE overflow was observed by treatment with A-192621 alone. Thus, changes in NE release induced by the pharmacological blockade of the ETA receptor, ETB receptor, or both were closely associated with the drug-induced improvement or deterioration of postischemic cardiac dysfunction. Moreover, similar relationships were also observed with ETB receptor–deficient s<sup>sl</sup>/s<sup>sl</sup> rats, in which NE overflow induced by ischemia/reperfusion was much higher than in wild-type animals, and ABT-627 treatment completely abolished the augmentation of NE overflow induced by the genetic deficiency of ETB receptors. Taken together, it appears likely that endogenous ET-1 causes excessive NE release from sympathetic nerve endings in

Figure 7. A, Effects of ET-1 and EIPA, an Na<sup>+</sup>/H<sup>+</sup> exchange inhibitor, on ischemia/reperfusion–induced changes of LVDP (A), LVEDP (B), and CF (C) in Sprague-Dawley rat hearts. Values are mean±SEM. *P<0.05 and **P<0.01 vs no addition.

Figure 8. Effects of ET-1 and EIPA on ischemia/reperfusion–induced cumulative NE overflow for 5 minutes after reperfusion in Sprague-Dawley rat hearts. Values are mean±SEM. *P<0.01 vs no addition.
postischemic rat hearts via the exclusive activation of ETα receptors and that this large amount of NE is contributive to the detrimental effects on functional recovery after ischemia/reperfusion.

In contrast to our findings, Dagassan et al.\(^\text{15}\) suggested that endogenous ET-1 does not play a major role in induction of reperfusion injury in isolated perfused rat heart using the Langendorff technique, on the basis of the results that bosentan, a nonselective ETα/ETβ receptor antagonist, did not influence recovery of cardiac function and did not ameliorate postischemic hemodynamic variables. The reason for this discrepancy is unclear, but methodological differences should be considered. In the above study, isolated hearts were subjected to only 20-minute global ischemia. This short-term ischemia might not cause an excessive NE overflow. Actually, in the experimental systems used in the present study, NE overflow after 20-minute ischemia and reperfusion was much less than in the case of 40-minute ischemia and reperfusion.

In the present study, exogenously applied ET-1 also enhanced ischemia/reperfusion–induced NE overflow in a dose-dependent manner and worsened the cardiac dysfunction observed after reperfusion, thereby suggesting that postischemic dysfunction results at least in part from ET-1–induced excessive NE release, as well as peptide-induced coronary vasoconstriction. In addition, the above ET-1 actions were completely suppressed by ABT-627 treatment (data not shown), which indicates that exogenous ET-1–induced actions are also mediated exclusively by ETα receptors. Ischemia/reperfusion–induced NE overflow from ischemic hearts is thought to depend on activation of the NHE system.\(^\text{16,17}\) Consistent with this view, we observed that treatment with EIPA, an NHE inhibitor, efficiently reduced NE overflow induced by ischemia/reperfusion, which ameliorated postischemic cardiac dysfunction. Furthermore, the ET-1–induced enhancement of NE overflow immediately after reperfusion and the deterioration of cardiac dysfunction were completely reversed by concomitant treatment with EIPA, which suggests that excessive NE release and subsequent cardiac dysfunction induced by exogenous ET-1 occurs through the NHE system in ischemic hearts. Further studies are required to determine whether ETα receptor–mediated ET-1 action can activate the NHE system in cardiac sympathetic nerves, because this peptide stimulates NHE via protein kinase C–dependent mechanisms in rat ventricular myocytes.\(^\text{45}\) On the other hand, activation of ETβ receptors by exogenously applied sarafotoxin S6c (1 nmol/L) did not affect the ischemia/reperfusion–induced excessive NE overflow, thereby suggesting that the ETβ receptor itself does not play an important role for NE release from the postischemic heart (authors’ unpublished data, 2004).

We conclude that ET-1 promotes postischemic NE overflow via the activation of ETα/NHE and induces ETα-mediated coronary vasoconstriction, both of which lead to subsequent cardiac dysfunction in rat hearts. The exaggeration of postischemic excessive NE overflow and cardiac dysfunction by genetic deficiency or the pharmacological blockade of ETα receptors is also mediated by ETα receptor stimulation. However, because we used an isolated perfused heart model, further studies with whole-body models will be required to clarify the relationships between the sympathetic nervous system and the ET-1 system in the pathology of postischemic cardiac dysfunction.

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