

ening. However, it was not possible to follow dynamic lattice spacing changes. In the present study, we used a fine-focused x-ray beam to record diffraction patterns of a localized region of the LV of ejecting rat hearts in situ and then determined crossbridge cycling and myosin lattice spacing.

Methods

Animals and Surgical Preparation

Anesthetized (50 mg/kg sodium pentobarbital IP) male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan), 9 to 10 weeks of age (350 to 400 g), were artificially ventilated and thoracotomized. Procedures were performed according to SPring-8 guidelines for the care and welfare of experimental animals. The heart was continuously irrigated while the apex was raised by a manipulator paddle and restrained by 2 superficial sutures in the LV to minimize vertical movements. Pressure-volume loops were recorded from an apically inserted 1.4F micromanometer (SPR-671 Millar Instruments) and a 1.5F conductance catheter (S-I Medico-tech Co Ltd, Osaka)¹⁰ to determine the temporal sequence of cardiac events and heart rate (determined from end-diastole [ED] interval).

X-Ray Diffraction With Collimated Synchrotron Radiation

Measurements were conducted at the 40XU beamline of SPring-8.⁶ A collimated quasimonochromatic beam (wavelength, 0.08 nm) with a beam flux of $\approx 10^{12}$ photons per second (15 keV; ring current, 60 to 100 mA) and dimensions 0.2×0.2 mm was focused at an oblique tangent to the myocardium (≈ 3 m from the detector). The ventilator was stopped at end-expiration to reduce heart movements during measurements (≈ 2.1 seconds). Images were digitally recorded at a 15-ms sampling interval with the use of an image intensifier and a fast CCD camera,⁶ simultaneous with pressure-volume analog signals (1000-Hz sampling frequency). The beam passed through the apical myocardium between the ends of the descending branch of the left coronary artery (LAD) and the posterior interventricular vein. Final burning of the recorded region (higher energy levels) confirmed that the beam only exposed fibers in the epicardium and part of the intermediate layer (histological inspection).

Acute Ischemia Treatment

Heart baseline recordings were established, permanent ligation of the proximal LAD was performed, and recordings were repeated 5 to 10 minutes later.

Intensity Ratio Calculations and Analyses

Integrated intensity of $I_{1,0}$ and $I_{1,1}$ was determined from the areas under the reflection peaks after background subtraction.⁶ Intensity ratio ($I_{1,0}/I_{1,1}$) was used rather than absolute reflection intensities of $I_{1,0}$ and $I_{1,1}$, which are influenced by changes in the quantity of fibers sampled during contractions.³ Myosin mass transfer index was defined as the difference in intensity ratio between ED and end systole (ES).

Results

Mass Transfer and Lattice Spacing in Nonischemic Hearts

Intensity ratio significantly decreased during systole (increase in LV pressure [LVP] and decrease in LV volume [LVV]) and conversely, increased during diastole under the baseline rhythm (Figure 1a). Averaging intensity ratio over multiple beats reduced variability during diastole in the otherwise sinusoidal patterns (black lines, Figure 1b). With regard to time, $d_{1,0}$ spacing increased continuously

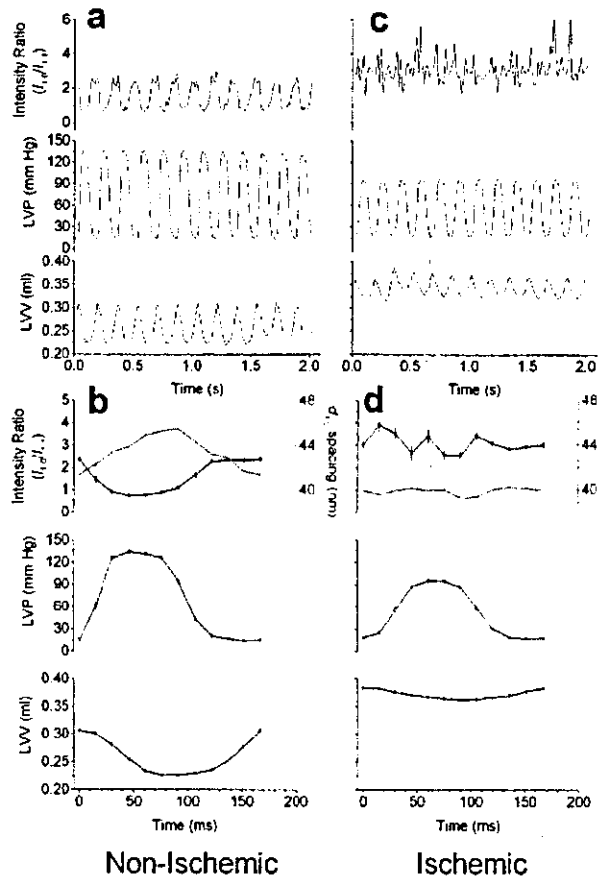


Figure 1. Relations between calculated intensity ratio, $d_{1,0}$ spacing, LVP, and LVV obtained from an LV with the use of x-ray diffraction. a and c, Consecutive records (15-ms intervals) of intensity ratio, LVP, and LVV during the baseline (a) and after LAD occlusion (c) in the same heart. b and d, Average changes in intensity ratio (black lines), $d_{1,0}$ spacing (red lines), LVP, and LVV over the cardiac cycle between the ED events (derived from a and c, respectively; bars indicate SEM).

during systole and then decreased during diastole, suggesting that considerable changes occur in the myofilament spacing (red line, Figure 1b).

Intensity ratio averaged 2.80 ± 0.11 (SEM, $n=13$ hearts) at ED, and the average myosin mass transfer index was 1.71 ± 0.09 . In all hearts, the decrease in intensity ratio during crossbridge formation was completed before the full extent of the $d_{1,0}$ spacing change (2 to 5 nm between hearts, Figure 2a). Furthermore, at any given LVV, the $d_{1,0}$ spacing during systole was 1 to 2 nm larger than diastole (Figure 2b).

Mass Transfer and Lattice Spacing During Regional Ischemia

LAD occlusion reduced the intensity ratio change and prevented normal lattice spacing increase, consistent with reduced contractility of the ischemic region (Figure 1, c and d). Occlusion significantly increased intensity ratios at both ED ($P < 0.05$) and ES ($P < 0.001$) in the same LV region ($n=6$,

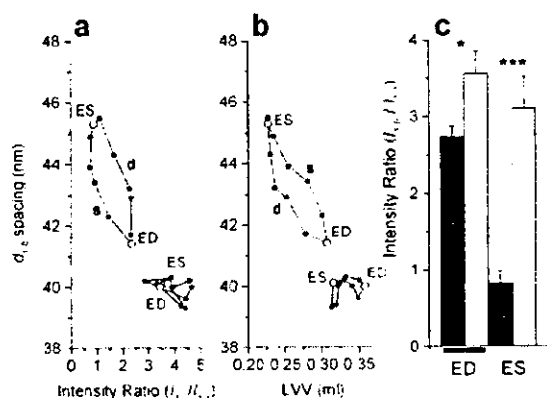


Figure 2. a and b, Average loops formed between $d_{1,0}$ spacing, LVV, and intensity ratio during consecutive cycles (shown in Figure 1) under baseline conditions (black symbols) and regional ischemia (red symbols). Systolic (s) and diastolic (d) trajectories are indicated for baseline. c, Mean intensity ratio at ED and ES during baseline (black columns) and regional ischemia (open columns; group mean \pm SEM). Ischemia versus baseline, paired *t* test * $P < 0.05$, *** $P < 0.001$.

Figure 2c). Although mean heart rate was not depressed (341 ± 16 bpm, 4% increase) and there was only a small decrease in mean LV ES pressure (-26.9 ± 5.8 mm Hg SEM), regional ischemia severely depressed the mass transfer index (55% of baseline).

Discussion

Our data clearly demonstrate that current synchrotron technology can produce sufficient energy to obtain well-defined reflections from single exposures to enable calculation of intensity ratio and $d_{1,0}$ spacing in in situ rat hearts (Figure 1). The mean ED intensity ratio of this study is similar to 2.96 obtained from LV in arrested rat hearts under normoxic perfusion.⁸ Furthermore, in rat papillary muscles, the resting ratio was 3.07.^{1,2} The results presented here were restricted to epicardial recordings (within 0.8-mm depth), consisting of helically orientated fibers, to minimize the contributions from fibers with orientations that vary at greater depth.¹¹ Nevertheless, it was recently established that neither diastolic intensity ratio nor mass transfer varies with depth of x-ray beam penetration in beating, perfused hearts (paced at 2 Hz).⁶

In Situ Changes in Myofilament Spacing

We showed that significant lattice expansion occurs during contraction and that the relation between $d_{1,0}$ spacing and intensity ratio is not linear but a loop in nonischemic hearts (black line, Figure 2a). The $d_{1,0}$ spacing changes during contraction (2 to 5 nm) were larger than the 1-nm difference in $d_{1,0}$ spacing reported between the epicardium and endocardium (at diastole).⁶ Therefore, the larger $d_{1,0}$ spacing change found in ejecting hearts cannot be explained by shifts in the fiber layers exposed to the beam. The loop formed by these indexes might contain valuable information about how crossbridge axial and radial forces alter the dynamics of lattice spacing changes. Crossbridge projections from the myosin backbone produce radial force

perpendicular to that of axial force in the filament direction.¹² Release of isometric tension in intact skeletal myofibers during sarcomere shortening causes a brief and rapid lattice spacing increase, in excess of that predicted by fiber shortening in itself.^{12,13} We therefore conclude that lattice volume is not constant in the dynamic state because myosin lattice spacing is significantly larger (1 to 2 nm) during contraction than ventricular filling at the same LVV ($d_{1,0}$ spacing during systole greater than diastole, Figure 2b). Crossbridge formation probably causes a brief lattice expansion in ejecting hearts mediated by radial forces.

Sensitivity of In Situ Indexes to Regional Ischemia

The relevance of our new findings is that although the intensity ratio of beating hearts in diastole was similar to that of relaxed papillary muscles, there is a very different response of the myocardium in beating hearts to ischemia in terms of crossbridge dynamics and lattice space changes. Higher intensity ratios and more variable intensity ratio changes during systole (Figure 1, c and d) occurred as the result of lower absolute $I_{1,1}$ in systole and a lack of consistent increase in $I_{1,1}$ when $I_{1,0}$ decreased (data not shown). Thus, permanent regional ischemia severely attenuated mass transfer in the epicardium (Figure 2c). Furthermore, ischemia induced increases in ED intensity ratio in vivo, whereas other studies report maximal decreases in the intensity ratio under anoxic perfusion (isolated arrested hearts)⁸ or rigor.^{1,2} An increase in intensity ratio might be related to metabolite accumulation or pronounced passive stretching, because fiber shortening in infarcted regions progressively decreases until fibers eventually become passively stretched (bulging) by fiber shortening in the nonischemic region.¹⁴ In support of the bulging possibility, we found that $d_{1,0}$ spacing no longer increases between ED and ES after occlusion.

The cellular basis of the Frank-Starling law of the heart involves increases in contractility caused by length-dependent increases in Ca^{2+} sensitivity associated with increased ventricular filling.⁹ However, it is still debated whether increased crossbridge activation results from increased probability of crossbridge formation with decreasing lattice spacing associated with fiber stretching (see review in Reference 9). In a future publication, we will examine how I.V. volume loading influences mass transfer in relation to myofilament spacing and length-dependent activation of contraction in situ.

Acknowledgments

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Extraneuronal enzymatic degradation of myocardial interstitial norepinephrine in the ischemic region

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Abstract

Objective: Catechol *O*-methyltransferase (COMT) is believed to exert degradative action at high norepinephrine (NE) levels. Although COMT exists in cardiac tissues, the contribution of cardiac COMT activity to regional NE kinetics, particularly in ischemia-induced NE accumulation, remains unclear. We investigated the role of cardiac COMT in NE kinetics in the ischemic region. **Methods:** We implanted a microdialysis probe into the left ventricular myocardium of anesthetized rabbits and induced myocardial ischemia by 60-min coronary artery occlusion. We monitored myocardial interstitial levels of NE and its metabolites in the presence and absence of a COMT inhibitor. We intraperitoneally administered entacapone (10 mg/kg) 120 min before control sampling. **Results:** In control, entacapone increased interstitial dihydroxyphenylglycol (DHPG, intraneuronal NE metabolite by monoamine oxidase (MAO)) levels and decreased interstitial normetanephrine (NMN, extraneuronal NE metabolite by COMT) and 3-methoxy-4-hydroxyphenylglycol (MHPG, extraneuronal DHPG metabolite by COMT) levels, but did not change interstitial NE levels. Coronary occlusion increased NE levels to 165 ± 48 nM at 45–60 min of occlusion. This increase was accompanied by increases in DHPG and NMN levels (11.3 ± 1.1 and 9.3 ± 1.3 nM at 45–60 min of occlusion). Entacapone augmented the ischemia-induced NE and DHPG responses (333 ± 51 and 22.9 ± 2.4 nM at 45–60 min of occlusion). In contrast, the ischemia-induced NMN response was suppressed by entacapone (2.0 ± 0.4 nM at 45–60 min of occlusion). Reperfusion decreased interstitial NE levels and increased interstitial DHPG and NMN levels. Entacapone suppressed changes in NE and NMN levels, but augmented the increase in dialysate DHPG. **Conclusion:** Myocardial ischemia evoked increases in myocardial interstitial NE and NMN levels. COMT inhibition augmented the increase in NE (substrate of COMT) levels and suppressed the increase in NMN (metabolite by COMT) levels. In the ischemic heart, COMT contributes to the removal of accumulated NE in the myocardium.

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1. Introduction

It has been reported that myocardial ischemia evokes an excessive norepinephrine (NE) accumulation in the myocardial interstitial space [1,2]. Outward NE transport through the uptake₁ carrier has been proposed as an important mechanism responsible for this ischemia-induced NE accumulation [2–4]. The presence of such high NE levels in the myocardial interstitium may be involved in the progression of myocardial cell injury and a higher incidence of malignant arrhythmia [5,6].

In the non-ischemic heart, released NE is reclaimed by cardiac sympathetic nerve endings via the uptake₁ carrier and repackaged or metabolized to dihydroxyphenylglycol (DHPG) by monoamine oxidase (MAO). NE, which escapes the synapses to the myocardial interstitium, spills over into the bloodstream or is taken up by extraneuronal cells via the uptake₂ carrier and mainly degraded to NE metabolites by catechol *O*-methyltransferase (COMT) [6–9] (Fig. 1). In the ischemic heart, normal transport by the uptake₁ carrier is impaired and NE spills over into the bloodstream, which is decreased due to the reduction of myocardial blood flow [1]. Therefore, extraneuronal enzymatic degradation may be the only mechanism that decreases myocardial interstitial NE. Little information, however, is available on the extraneuronal NE degradation by COMT in the ischemic region [10,11].

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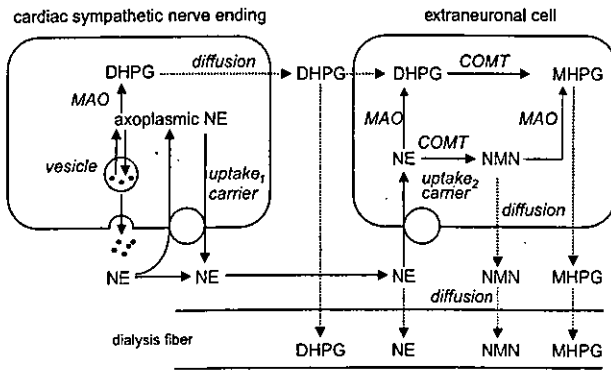


Fig. 1. Schema of putative factors affecting norepinephrine (NE) degradation in the cardiac sympathetic nerve ending and the extraneuronal cell. COMT, catechol *O*-methyltransferase; DHPG, dihydroxyphenylglycol; MAO, monoamine oxidase; NMN, normetanephrine; MHPG, 3-methoxy-4-hydroxyphenylglycol.

Until now, available methodology for examination of organ-specific NE degradation has been limited to the assessment of radiolabelled-catecholamine kinetics [12]. Previously, using the dialysis technique in the *in vivo* heart, we demonstrated that coronary occlusion evokes a marked increase of myocardial interstitial NE levels in the ischemic region [1,2,4] and that outward NE transport through the uptake₁ carrier is involved in this NE efflux [2–4]. Moreover, we recently reported that the dialysis technique makes it possible to simultaneously monitor interstitial levels of NE and extraneuronal metabolites in the rabbit skeletal muscle [13]. Therefore, we consider it possible to elucidate extraneuronal NE metabolism and the role of COMT in its metabolism in the ischemic region. In the present study, we applied the dialysis technique to the heart of anesthetized rabbits and investigated myocardial interstitial levels of NE and its extraneuronal metabolites during coronary occlusion and reperfusion and examined the effect of COMT blockade on myocardial interstitial levels of NE and its metabolites.

2. Methods

2.1. Animal preparation

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Japanese white rabbits (2.5–3.2 kg) were anesthetized with pentobarbital sodium (30–35 mg/kg *iv*). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). The rabbits were intubated and ventilated with room air mixed with oxygen. Body temperature was maintained at around 38 °C with a heating pad and lamp. Heart rate, arterial pressure, and electrocardiogram were monitored and recorded continuously. Heparin sodium (200 IU/kg) was first administered intravenously and then maintained with a continuous infusion (5–10 IU/kg/h) to prevent blood coag-

ulation. With the animal in the lateral position, the fifth or sixth rib on the left side was partially removed to expose the heart. A small incision was made in the pericardium, and the dialysis probe was implanted in the region perfused by the left circumflex coronary artery (LCX) of the left ventricular wall. A snare was placed around the main branch of LCX to act as the occluder for later coronary occlusion. To ensure that the sampling area was in the ischemic region, we examined the color and motion of the ventricular wall during a brief occlusion and confirmed that the dialysis probe was correctly located. To avoid a preconditioning effect, the duration of occlusion was limited to within seconds.

2.2. Dialysis technique

Materials suitable for cardiac dialysis probes have been described in detail elsewhere [14]. Briefly, we designed a handmade long transverse dialysis probe. One end of a polyethylene tube (25-cm length, 0.5 mm OD, and 0.2 mm ID) was dilated with a 27-gauge needle (0.4 mm OD). Each end of the dialysis fiber (8-mm length, 0.31 mm OD, and 0.20 mm ID; PAN-1200 50 000 molecular weight cutoff, Asahi Chemical Japan) was inserted into the polyethylene tube and glued. We used a fine guiding needle (30-mm length, 0.51 mm OD, and 0.25 mm ID) for implantation of the dialysis probes. We connected a guiding needle to a dialysis probe with a stainless rod (5-mm length and 0.25 mm OD). At perfusion speed of 2 μ l/min, *in vitro* recovery rates of NE, DHPG, normetanephrine (NMN), and 3-methoxy-4-hydroxyphenylglycol (MHPG) were $46 \pm 8\%$, $48 \pm 1\%$, $33 \pm 3\%$, and $46 \pm 2\%$, respectively (number of dialysis probes = 3) [15].

Dialysis probes were perfused with Ringer's solution at a speed of 2 μ l/min using a microinjection pump (Carnegie Medicine CMA/100). Ringer's solution consisted of (in mM) 147.0 NaCl, 4.0 KCl, 2.25 CaCl₂. Sampling periods were 30 min (1 sampling volume = 60 μ l) in control and 15 min (1 sampling volume = 30 μ l) during occlusion and reperfusion. Each sample was collected in a microtube containing 3 μ l of 0.1 N HCl to prevent amine oxidation. Based on the results of our previous study [1,2], we commenced the protocol followed by a stabilization period of 2 h. Taking into consideration the dead space between the dialysis fiber and sample tube, we sampled the dialysate.

Dialysate NE, DHPG, NMN, and MHPG concentrations were measured as indices of myocardial interstitial NE, DHPG, NMN, and MHPG levels. Furthermore, dialysate NE and DHPG were used as indices of COMT substrate, and dialysate NMN and MHPG as indices of COMT production. We used three distinct systems of high-performance liquid chromatography (HPLC) with electrochemical detection for the highly sensitive measurements: one for NE, one for DHPG, and one for NMN and MHPG measurement [16–18]. The mobile phase consisted of 1-octane-sulfonic acid sodium salt in phosphate buffer and methanol. In each HPLC system, the concentration of each component and the reference voltage were adjusted to the optimum condition. One-

third each of the dialysate sample was used for the measurement of NE, DHPG, and NMN and MHPG. Dialysate NE concentration was measured by the first HPLC after removing interfering compounds by the alumina procedure [14,16]. Dialysate DHPG concentration was measured by direct injection into the second HPLC [17]. Dialysate NMN and MHPG concentrations were measured by direct injection into the third HPLC [18]. The detection limits of NE, DHPG, NMN, and MHPG were 0.2, 0.2, 1, and 0.9 pg/injection.

2.3. Experimental protocols

After control sampling, we occluded the main branch of LCX for 60 min and then released the occluder. We continuously sampled dialysate from the ischemic region during 60 min of coronary occlusion and 15 min of reperfusion.

2.3.1. Vehicle group ($n=8$)

We administered saline intraperitoneally as vehicle 120 min before control sampling. After control sampling, we observed the time course of dialysate NE, DHPG, NMN, and MHPG levels from the ischemic region during 60 min of coronary occlusion and 15 min of reperfusion.

2.3.2. Entacapone group ($n=8$)

To elucidate role of COMT in the ischemia-induced changes in myocardial interstitial NE and its metabolites, we observed the effect of COMT inhibitor on dialysate NE, DHPG, NMN, and MHPG levels in the ischemic region. We administered intraperitoneally a COMT inhibitor entacapone (10 mg/kg; Orion Parma, Espoo, Finland) 120 min before control sampling. Entacapone was dissolved in phosphate-buffered saline, the pH of the solution was adjusted to 7.4. The route and dose of entacapone were selected to cause the full inhibition of soluble COMT in tissue [19]. After control sampling, we observed the time course of dialysate NE, DHPG, NMN, and MHPG levels with a similar protocol to that used in the vehicle group.

At the end of each experiment, the rabbits were killed with an overdose of pentobarbital sodium, and the implant regions were checked to confirm that the dialysis probes had been implanted within the cardiac muscle.

2.4. Statistical analysis

Hemodynamic and dialysate NE, DHPG, MHPG, and NMN responses to coronary occlusion in the presence and absence of COMT inhibitor were statistically analyzed by two-way analysis of variance with repeated measures on one factor [20]. When a statistically significant effect of coronary occlusion was detected as a whole, the Newman–Keuls test was applied to determine which mean values differed significantly from each other. When statistically significant effect of the COMT inhibitor was detected, the Newman–Keuls test was applied to determine which periods differed significantly between the vehicle and entacapone groups. Statistical significance was defined as $P < 0.05$. Values are presented as means \pm SE.

3. Results

3.1. Time course of heart rate and arterial pressure

The time course of heart rate and mean arterial pressure is shown in Table 1. In the vehicle group, heart rate decreased after 15 min of occlusion, whereas in the entacapone group, heart rate increased after 30 min of occlusion. There was, however, no significant difference in heart rate between groups.

Coronary occlusion significantly decreased mean arterial pressure in both groups. In the entacapone group, mean arterial pressure was higher than those in the vehicle group at each sampling point, while changes in mean arterial pressure were similar to those in the vehicle group.

3.2. Dialysate NE levels in the ischemic region

Coronary occlusion significantly altered dialysate NE levels (Fig. 2). In the vehicle group, dialysate NE levels were 0.39 ± 0.07 nM in the control and increased after coronary occlusion. During 60 min of coronary occlusion, dialysate NE levels markedly increased and reached 165 ± 48 nM at 45–60 min of occlusion. After reperfusion, dialysate NE levels decreased to 62 ± 40 nM, although their

Table 1
Time course of heart rate and mean arterial pressure during coronary occlusion and reperfusion

	Control	Coronary occlusion (min)				Reperfusion (min)
		15	30	45	60	
<i>Heart rate (bpm)</i>						
Vehicle group ($n=8$)	252 \pm 6	236 \pm 7*	238 \pm 7*	239 \pm 6*	237 \pm 6*	238 \pm 6*
Entacapone group ($n=8$)	246 \pm 8	246 \pm 8	251 \pm 9*	253 \pm 7*	253 \pm 8*	251 \pm 9
<i>Mean arterial pressure (mm Hg)</i>						
Vehicle group ($n=8$)	83 \pm 3	71 \pm 4*	75 \pm 3*	76 \pm 3*	78 \pm 2*	76 \pm 3*
Entacapone group ($n=8$)	99 \pm 4 [†]	85 \pm 4* [†]	88 \pm 5* [†]	88 \pm 4* [†]	88 \pm 4* [†]	86 \pm 4* [†]

Values are means \pm SE.

* $P < 0.05$ vs. control value.

[†] $P < 0.05$ vs. concurrent value of vehicle group.

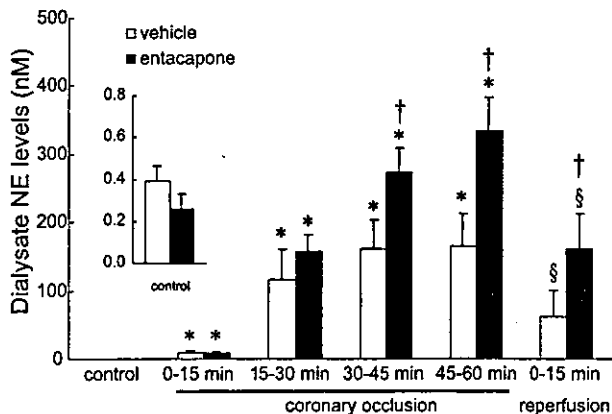


Fig. 2. Dialysate norepinephrine (NE) levels in the ischemic region. Values are means \pm SE. * P <0.05 vs. control value, § P <0.05 vs. value at 45–60 min of occlusion, † P <0.05 vs. concurrent value of vehicle group.

levels were higher than those in the control. In the presence of entacapone, dialysate NE levels also markedly increased and reached 333 ± 51 nM at 45–60 min of occlusion. These increases in dialysate NE levels after 30 min of coronary occlusion were significantly enhanced by entacapone whereas entacapone did not change dialysate NE levels in the control (0.26 ± 0.07 nM). After reperfusion, dialysate NE levels decreased but remained higher than those in the vehicle group.

3.3. Dialysate DHPG levels in the ischemic region

Coronary occlusion significantly altered dialysate DHPG levels (Fig. 3). In the vehicle group, dialysate DHPG levels were 6.5 ± 0.5 nM in the control and did not change within 30 min of coronary occlusion. After 30 min of occlusion, dialysate DHPG levels gradually increased and reached 11.3 ± 1.1 nM at 45–60 min of occlusion. After reperfusion, dialysate DHPG levels further increased to 29.5 ± 2.6 nM. In the presence of entacapone,

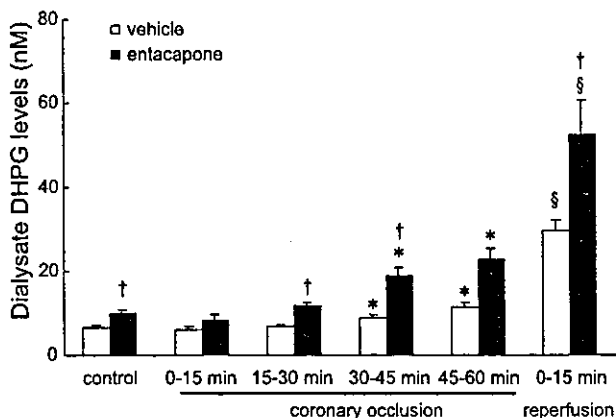


Fig. 3. Dialysate dihydroxyphenylglycol (DHPG) levels in the ischemic region. Values are means \pm SE. * P <0.05 vs. control value, § P <0.05 vs. value at 45–60 min of occlusion, † P <0.05 vs. concurrent value of vehicle group.

dialysate DHPG levels gradually increased during the ischemia and reached 22.9 ± 2.4 nM at 45–60 min of occlusion. After reperfusion, dialysate DHPG levels further increased to 52.6 ± 8.4 nM. In the presence of entacapone, dialysate DHPG levels in the control (9.9 ± 0.8 nM) and after reperfusion were higher than those in the vehicle group.

3.4. Dialysate NMN levels in the ischemic region

Coronary occlusion significantly altered dialysate NMN levels (Fig. 4). In the vehicle group, dialysate NMN levels were 2.9 ± 0.4 nM in the control and increased after 30 min of occlusion and reached 9.3 ± 1.3 nM at 45–60 min of occlusion. After reperfusion, dialysate NMN levels further increased (11.9 ± 2.0 nM). Entacapone decreased dialysate NMN levels in the control to undetectable levels. Then dialysate NMN levels increased after 30 min of occlusion and reached 2.0 ± 0.4 nM at 45–60 min of occlusion. After reperfusion, dialysate NMN levels further increased to 4.1 ± 0.8 nM. Their NMN levels were lower than those in the vehicle group at each sampling point before, during, and after coronary occlusion.

3.5. Dialysate MHPG levels in the ischemic region

Coronary occlusion significantly altered dialysate MHPG levels (Fig. 5). In the vehicle group, dialysate MHPG levels were 3.9 ± 0.3 nM in the control. Dialysate MHPG levels transiently decreased 15–30 min after occlusion (3.6 ± 0.4 nM), but recovered after 30 min of occlusion. After reperfusion, dialysate MHPG levels increased to 5.5 ± 0.3 nM. Entacapone substantially decreased dialysate MHPG levels in the control (1.5 ± 0.4 nM). In the presence of entacapone, dialysate MHPG levels further decreased during occlusion and reached 0.6 ± 0.2 nM at 30–45 min of occlusion. After reperfusion, dialysate MHPG levels increased to 2.3 ± 0.5 nM. Their MHPG levels were lower than those in the

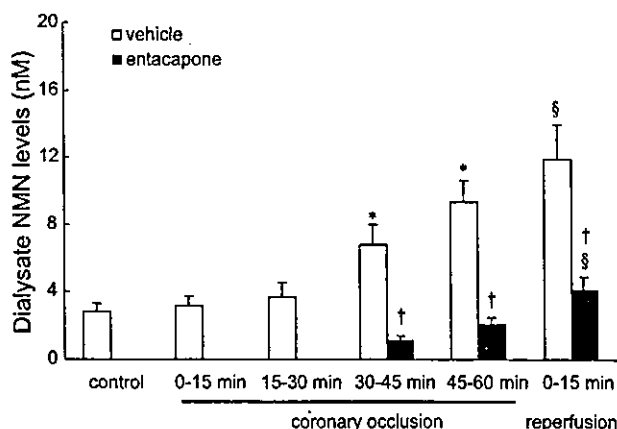


Fig. 4. Dialysate normetanephrine (NMN) levels in the ischemic region. Values are means \pm SE. * P <0.05 vs. control value, § P <0.05 vs. value at 45–60 min of occlusion, † P <0.05 vs. concurrent value of vehicle group.

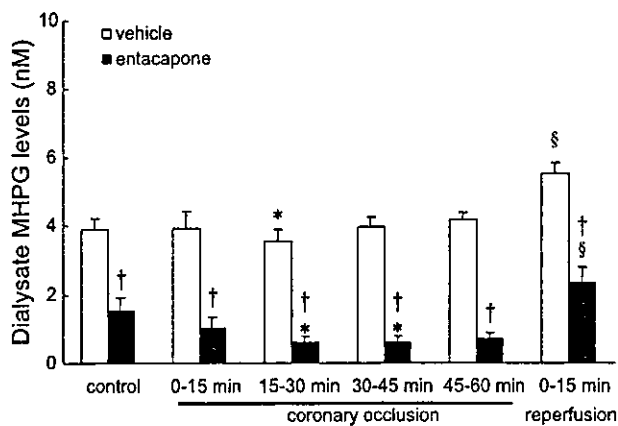


Fig. 5. Dialysate 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in the ischemic region. Values are means \pm SE. * P <0.05 vs. control value, § P <0.05 vs. value at 45–60 min of occlusion, † P <0.05 vs. concurrent value of vehicle group.

vehicle group at each sampling point before, during, and after coronary occlusion.

4. Discussion

Using the dialysis technique in the *in vivo* rabbit heart, we observed myocardial interstitial levels of NE and its neuronal and extraneuronal metabolites in the ischemic region and examined the contribution of extraneuronal NE degradation by COMT to myocardial interstitial NE levels. Our data demonstrate that COMT plays an important role in NE metabolism during 60 min of coronary occlusion and reperfusion.

4.1. Myocardial interstitial NE and its metabolites under control conditions

The administration of entacapone did not alter myocardial interstitial NE levels in the control. Degradation of NE by COMT may play a minor role in the changes in myocardial NE levels [21]. In general, NE that is taken up by cardiac sympathetic nerve endings is repackaged or metabolized to DHPG by MAO. On the other hand, NE that is taken up via extraneuronal NE transport systems by extraneuronal cells is metabolized to NMN or MHPG by COMT [7–10,22,23] (Fig. 1). In the present study, entacapone increased DHPG in the myocardial interstitium but did not alter myocardial interstitial NE levels in the control. Myocardial interstitial levels of DHPG were about 16-fold higher than those of NE and about 2- to 3-fold higher than those of NMN in the control. Therefore, under physiological conditions, released NE could be largely taken up by cardiac sympathetic nerve endings via the uptake₁ carrier and transferred into stored vesicle or metabolized to DHPG by MAO. A smaller percentage of released NE, which escapes the synapses, is taken up by extraneuronal cells via the uptake₂ carrier and is metabolized to NMN by COMT.

Compared with MAO, COMT could play a minor role on the degradation of released NE in the control.

After entacapone administration, increases in myocardial interstitial DHPG accompanied decreases in myocardial interstitial MHPG levels in the control. These metabolites of NE penetrate the cell membrane by diffusion [24]. Their values serve as indices of the neuronal and extraneuronal NE metabolism. COMT blockade suppressed the degradation of DHPG to MHPG. Therefore, the decrease in MHPG levels and increase in interstitial DHPG levels could be ascribed to inhibition of COMT by entacapone. In rabbit heart, we confirmed the existence of COMT activity with the main substrate of COMT being DHPG rather than NE.

4.2. Myocardial interstitial NE and its metabolites during coronary occlusion

Myocardial interstitial NE levels markedly increased after 15 min of occlusion. In this phase, outward transport of NE via the uptake₁ carrier takes place from cardiac sympathetic nerve endings [2,3]. The marked increase in myocardial interstitial NE could be due to this non-exocytotic NE release and inhibition of neuronal reuptake via the uptake₁ carrier [2–4].

Entacapone augmented increases in myocardial interstitial NE levels after 30 min of coronary occlusion. This result indicates that COMT contributes to the degradation of myocardial interstitial NE in this phase. Neuronal reuptake via the normal mode of uptake₁ is dependent on the sodium gradient between the intra- and extraneuronal space [25]. Neuronal degradation of released NE via neuronal uptake cannot be expected in this phase because of a reduced sodium gradient [3,25]. Moreover, NE spillover into the bloodstream is decreased due to reduced myocardial blood flow [1]. On the other hand, extraneuronal NE uptake operates independently of the sodium gradient [26]. Burgdorf et al. [27] demonstrated that the extraneuronal monoamine transporter is activated during metabolic distress such as low flow ischemia. Previous investigations with similar preparations suggested that ketamine augments ischemia-induced NE accumulation by inhibition of extraneuronal uptake [28,29]. We consider that substantial NE in the myocardial interstitium is taken up alternatively by extraneuronal cells via the uptake₂ carrier and is metabolized to NMN by COMT in this phase. Thus, COMT activity plays an important physiological role in NE degradation in the ischemic period.

Myocardial interstitial NMN levels increased after 30 min of occlusion. Entacapone decreased basal myocardial interstitial NMN levels and suppressed the ischemia-induced increase in myocardial interstitial NMN levels. This suppression is consistent with the finding that COMT contributes to the degradation of myocardial interstitial NE via extraneuronal NE uptake. Furthermore, even in the presence of entacapone, myocardial interstitial NMN levels increased after 30 min of occlusion. An increase in interstitial NE levels may overcome the inhibition of COMT by

entacapone. Although COMT could play a minor role in the degradation of released NE in the control, a substantial increase in myocardial NE may cause the high affinity of the extraneuronal COMT system. In the control period, an extraneuronal COMT system may contribute to DHPG degradation, whereas in the ischemic period, both neuronal NE uptake and MAO activities may be suppressed by ischemia and alternatively the extraneuronal COMT system may promote NE degradation based on the fact that the myocardial interstitial NE levels in the ischemic period were 10-fold higher than those of DHPG. Therefore, we consider that increases in myocardial interstitial NE levels shift the main substrate of COMT from DHPG to NE.

The relationship between DHPG and MHPG supports our interpretation. In the control, entacapone increased myocardial interstitial DHPG levels and decreased myocardial interstitial MHPG levels. In contrast, in the ischemic period, increases in myocardial interstitial DHPG levels were not associated with increases in myocardial MHPG levels, but increases in myocardial NE levels accompanied increases in myocardial NMN. Thus, both DHPG and NE are metabolized by the extraneuronal COMT system, but the amount of NMN and MHPG may be dependent on the concentration of their substrate. Thus, there was a clear difference in the main metabolite by COMT between the non-ischemic and ischemic periods. The main metabolite by COMT was NMN rather than MHPG in the latter [30]. These results are limited to ischemia within 60 min because prolonged ischemia accompanies the structural membrane defects, and other mechanisms for NE release and degradation may be involved [31].

4.3. Myocardial interstitial NE and its metabolites after reperfusion

Myocardial interstitial NE levels decreased after reperfusion although they were higher than those in control. On the other hand, myocardial interstitial DHPG levels increased after reperfusion. The uptake₁ carrier resumes normal transport function after reperfusion [2,4]. The inward NE transport via the uptake₁ carrier could contribute to the decrease in myocardial interstitial NE levels. The increase in axoplasmic NE by uptake and the recovery of MAO activity could increase myocardial interstitial DHPG levels [2]. Thus, neuronal degradation by MAO contributes to decreasing myocardial interstitial NE after reperfusion.

Reperfusion caused a decrease in myocardial interstitial NE levels and an increase in myocardial NMN levels, both of which changes were suppressed by administration of entacapone. During the early reperfusion period, COMT activity promotes the degradation of NE. Myocardial interstitial NE levels were higher than those in the control. Therefore, during the ischemic and reperfusion periods, these higher NE levels in myocardial interstitium may serve as an effective substrate of COMT for NE degradation via extraneuronal NE uptake.

During the reperfusion period, increases in myocardial interstitial DHPG accompanied increases in myocardial in-

terstitial MHPG levels. Furthermore, administration of entacapone suppressed both of these changes. Myocardial interstitial DHPG levels were similar to myocardial interstitial NE levels. These data suggest that COMT activity promotes the degradation of DHPG. Alternatively, higher NE level in myocardial interstitium may produce MHPG via COMT activity. Recently, we demonstrated in rabbit skeletal muscle that local administration of higher NE increased dialysate NMN but not MHPG levels, whereas local administration of higher DHPG increased dialysate MHPG levels [19]. Therefore, higher NE and DHPG levels serve as the substrate of COMT and independently yield NMN and MHPG during the reperfusion period. Thus, COMT activity plays an important physiological role in the reperfusion period.

4.4. Methodological considerations

In the presence of a high concentration of entacapone, mean arterial pressure was higher than that in the vehicle group at each sampling point before, during, and after coronary occlusion, but changes in mean arterial pressure were similar to those in the vehicle group. In the previous and present studies, intraperitoneal administration of entacapone did not alter control dialysate NE levels from skeletal muscle and myocardium [19]. In humans, entacapone did not alter plasma catecholamine levels or hemodynamics at rest or during exercise [32]. The influence of entacapone on pressure-regulating peptides remains unclear. An increase in mean arterial blood pressure might decrease dialysate NE levels through a baroreflex mechanism. Furthermore, baroreflex-independent and non-exocytotic NE efflux leads to high NE levels in the myocardial interstitium of ischemic regions, making it unlikely that hemodynamic change contributes to the removal of accumulated NE during myocardial ischemia.

Two major classes of COMT have been defined on the basis of their location: a soluble, cytosolic form and a membrane-bound form [33]. Entacapone inhibits both classes of COMT. The soluble, cytosolic form is generally assumed to be the predominant form of the enzyme. The membrane-bound form has been suggested to be responsible for O-methylation at low and physiologically relevant concentrations of the catecholamine neurotransmitters, whereas the soluble, cytosolic form predominates under conditions that lead to saturation of the membrane-bound form [33]. In the present study, myocardial interstitial norepinephrine levels reached 100–1000 times the normal plasma concentrations after 15 min of occlusion. Thus, a soluble, cytosolic form could contribute to the observed decrease in myocardial interstitial NE levels in the ischemic region.

5. Conclusion

Under physiological condition, extraneuronal enzymatic degradation by COMT plays a minor role on the inactivation

of myocardial interstitial NE. Under ischemic conditions, however, myocardial interstitial NE levels are markedly increased by ischemia. Normal transport by the uptake₁ carrier is impaired and NE spillover into the bloodstream is decreased due to the reduction of myocardial blood flow, but extraneuronal enzymatic degradation by COMT contributes to the decrease in myocardial interstitial NE levels in the ischemic region.

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β -Adrenoceptor Blocker Carvedilol Provides Cardioprotection via an Adenosine-Dependent Mechanism in Ischemic Canine Hearts

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Background—Carvedilol is a β -adrenoceptor blocker with a vasodilatory action that is more effective for the treatment of congestive heart failure than other β -blockers. Recently, carvedilol has been reported to reduce oxidative stress, which may consequently reduce the deactivation of adenosine-producing enzymes and increase cardiac adenosine levels. Therefore, carvedilol may also have a protective effect on ischemia and reperfusion injury, because adenosine mediates cardioprotection in ischemic hearts.

Methods and Results—In anesthetized dogs, the left anterior descending coronary artery was occluded for 90 minutes, followed by reperfusion for 6 hours. Carvedilol reduced the infarct size ($15.0 \pm 2.8\%$ versus $40.9 \pm 4.2\%$ in controls), and this effect was completely reversed by the nonselective adenosine receptor antagonist 8-sulfophenyltheophylline ($45.2 \pm 5.4\%$) or by an inhibitor of ecto-5'-nucleotidase ($44.4 \pm 3.6\%$). There were no differences of either area at risk or collateral flow among the various groups. When the coronary perfusion pressure was reduced in other dogs so that coronary blood flow was decreased to 50% of the nonischemic level, carvedilol increased coronary blood flow (49.4 ± 5.6 to 73.5 ± 7.5 mL \cdot 100 g⁻¹ \cdot min⁻¹; $P < 0.05$) and adenosine release (112.3 ± 22.2 to 240.6 ± 57.1 nmol/L; $P < 0.05$) during coronary hypoperfusion. This increase of coronary blood flow was attenuated by either 8-sulfophenyltheophylline or superoxide dismutase. In human umbilical vein endothelial cells cultured with or without xanthine and xanthine oxidase, carvedilol caused an increase of ecto-5'-nucleotidase activity.

Conclusions—Carvedilol shows a cardioprotective effect against ischemia and/or reperfusion injury via adenosine-dependent mechanisms. (*Circulation*. 2004;109:2773-2779.)

Key Words: adenosine ■ stress ■ ischemia ■ reperfusion ■ infarction

Beta-adrenoceptor antagonists (β -blockers) are used for the treatment of ischemic heart disease because these drugs reduce adrenergic activity.^{1,2} Carvedilol is a β -blocker that has shown efficacy for chronic heart failure in several large-scale trials.^{3,4} Carvedilol decreases vascular resistance³ and improves the pathophysiology of chronic heart failure.⁵ This drug dilates both systemic and coronary vessels,⁶ which is not a typical characteristic of β -blockers. Although this vasodilatory action may contribute to the beneficial effects of carvedilol in ischemic or nonischemic heart failure,⁵ it may not be the primary mechanism of cardioprotection, because vasodilators are not always effective at protecting the heart.⁷ Interestingly, carvedilol can also reduce oxidative stress,⁸ which causes cellular damage through inactivation of mem-

brane enzymes, pumps, and proteins, such as Na⁺/K⁺-ATPase,⁹ Ca²⁺ channels,¹⁰ and ecto-5'-nucleotidase.¹¹ Ecto-5'-nucleotidase is the enzyme that produces adenosine, and adenosine is believed to ameliorate chronic heart failure or myocardial ischemia.¹²

To investigate the relationship between the cardioprotective effect of carvedilol and the reduction of oxidative stress on the enhancement of adenosine release, we examined whether carvedilol could reduce infarct size via adenosine- or ecto-5'-nucleotidase-dependent mechanisms in canine hearts. We also investigated whether carvedilol could increase coronary blood flow (CBF) via attenuation of oxidative stress and enhancement of adenosine release in ischemic canine hearts.

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Methods

Instrumentation

We have previously reported the details of the instrumentation procedure.¹³ In brief, hybrid dogs (HBD) mated with the beagle, American fox hound, and Labrador retriever for laboratory use (weighing 15 to 21 kg; Kitayama Labes, Gifu, Japan) were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg), intubated, and ventilated with room air mixed with oxygen (100% O₂ at flow rate of 1.0 to 1.5 L/min). The arterial blood pH, PO₂, and PCO₂ before the protocol was begun were 7.38±0.02, 104±3 mm Hg, and 38.7±1.6 mm Hg, respectively. End-diastolic length (EDL) was determined at the R wave on the ECG, and end-systolic length (ESL) was determined at the minimum pressure differential. Then, fractional shortening (FS) was calculated as [(EDL - ESL)/EDL]×100%. Agents were administered into the left anterior descending coronary artery (LAD) via the bypass tube. To constitute the coronary bypass between the carotid artery and the LAD, <30 seconds interruption of the LAD was necessary, but this brief period of ischemia does not provoke either myocardial injury or protection. This study conformed to the *Position of the American Heart Association on Research Animal Use* adopted by the Association in November 1984.

Experimental Protocols

Protocol 1: Effects of Carvedilol on Adenosine Release and CBF in Nonischemic Myocardium

After hemodynamics became stable, coronary arterial and venous blood samples were obtained for the measurement of adenosine concentrations,¹¹ and the difference between the adenosine levels in coronary arterial and venous blood [VAD(Ado)] was then calculated.

Five HBD dogs were used in protocol 1. Hemodynamic parameters (ie, systolic and diastolic aortic blood pressure and heart rate) were monitored. Carvedilol was infused at 1.5 μg · kg⁻¹ · min⁻¹ (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 0.09 mg/mL) for 10 minutes, and then coronary perfusion pressure (CPP), CBF, FS, and VAD(Ado) were measured. Carvedilol was dissolved in a small volume of DMSO (final concentration, <0.15%). In a preliminary study, this dose of carvedilol was shown to be the minimum dose that caused maximal coronary vasodilation in ischemic or nonischemic hearts. We also confirmed that this volume of DMSO did not change either coronary hemodynamics or VAD(Ado) in ischemic or nonischemic hearts.

Protocol 2: Effects of Carvedilol or Propranolol on Adenosine Release and CBF in Ischemic Hearts

After hemodynamics became stable, coronary arterial and venous blood samples were obtained for blood gas analysis and for measurement of adenosine^{11,13} and lactate¹⁴ levels. Lactate extraction ratio (LER) was calculated as the coronary arteriovenous difference of the lactate concentration multiplied by 100 and divided by the arterial lactate concentration.

Twenty HBD dogs were used in protocol 2. Hemodynamic parameters were monitored. To examine whether administration of carvedilol caused coronary vasodilation and reduced the severity of myocardial ischemia and whether adenosine-dependent mechanisms are involved in these actions, saline (n=5), α,β-methyleneadenosine diphosphate (AMP-CP) at 80 μg · kg⁻¹ · min⁻¹ (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 4.8 mg/mL, n=5), or 8-sulphophenyltheophylline (8-SPT) at 30 μg · kg⁻¹ · min⁻¹ (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 1.8 mg/mL, n=5) was infused into the bypass tube. AMP-CP is an inhibitor of ecto-5'-nucleotidase, whereas 8-SPT is a nonspecific adenosine receptor antagonist. Both agents were dissolved in saline before administration. After confirming that systemic and coronary hemodynamics were unchanged for 5 minutes after each drug infusion, CPP was reduced so that CBF decreased to 50% of the baseline level for 5 minutes. Then, infusion of carvedilol was started at 1.5 μg · kg⁻¹ · min⁻¹ (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 0.09 mg/mL) and was continued for 10 minutes,

while CPP was maintained at the reduced level. A preliminary study showed that the above-mentioned dose of 8-SPT was the minimum dose that prevented coronary vasodilation induced by adenosine at 2 μg · kg⁻¹ · min⁻¹, whereas the dose of carvedilol (1.5 μg · kg⁻¹ · min⁻¹) was the minimum level that caused maximal coronary vasodilation.

In addition, propranolol was infused at 30 μg · kg⁻¹ · min⁻¹ (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 1.8 mg/mL) to investigate whether it had effects identical to those of carvedilol (n=5). This dose of propranolol corresponds to 15 μg/mL, and the effective dose of propranolol is >10 μg/mL, indicating that the dose of propranolol in the present study is sufficient to antagonize β-receptors of the hearts.

Protocol 3: Influence of the Antioxidant Activity of Carvedilol on CBF

To examine whether carvedilol eliminates oxidative stress and causes adenosine-dependent coronary vasodilation in ischemic hearts, either saline (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 1.5 mg/mL, n=5) or human recombinant superoxide dismutase (SOD) (5340 IU/mg, >99% purity) at 25 μg · kg⁻¹ · min⁻¹ (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 1.5 mg/mL, n=5) was infused into the bypass tube. CPP was then reduced so that CBF decreased to 50% of the baseline level for 5 minutes. Subsequently, infusion of carvedilol at 1.5 μg · kg⁻¹ · min⁻¹ (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 0.09 mg/mL) was initiated and continued for 10 minutes, while CPP was maintained at the reduced value. As a marker of oxidative stress, the 8-iso-prostaglandin F_{2α} level was measured in coronary arterial and venous blood, and the arteriovenous difference of 8-iso-prostaglandin F_{2α} [VAD(8-Iso-F_{2α})] was calculated. We confirmed that this dose of SOD had no effect on either systemic or coronary hemodynamic parameters.¹¹

Protocol 4: Effects of Carvedilol on Infarct Size After 90 Minutes of Ischemia

In HBD dogs, the bypass tube to the LAD was occluded for 90 minutes, followed by reperfusion for 6 hours, together with administration of either saline (n=7, control) or DMSO (0.0167 mL · kg⁻¹ · min⁻¹, n=5) from 10 minutes before occlusion until 1 hour of reperfusion, except at the time of coronary occlusion. Hemodynamic parameters were monitored during myocardial ischemia and after the start of reperfusion. In the carvedilol group (n=5), carvedilol at 1.5 μg · kg⁻¹ · min⁻¹ (an infusion rate of 0.0167 mL · kg⁻¹ · min⁻¹ at a concentration of 0.09 mg/mL) was infused from 10 minutes before coronary occlusion until 60 minutes after the start of reperfusion, except during occlusion. In the carvedilol+8-SPT group (n=6) and the carvedilol+AMP-CP group (n=6), the effect of carvedilol was tested during concomitant administration of either 8-SPT at 30 μg · kg⁻¹ · min⁻¹ or AMP-CP at 80 μg · kg⁻¹ · min⁻¹. In the 8-SPT group (n=6) and the AMP-CP group (n=7), 90 minutes of ischemia and 6 hours of reperfusion were performed during treatments with 8-SPT and AMP-CP, respectively. Either 8-SPT or AMP-CP was infused from 10 minutes before coronary occlusion until 60 minutes after the start of reperfusion, except during occlusion. In all groups, infarct size was assessed after 6 hours of reperfusion.

Protocol 5: Effects of Carvedilol on 5'-Nucleotidase Activity

In human umbilical vein endothelial cells (HUVECs) cultured with or without xanthine (1×10⁻⁴ mol/L) and xanthine oxidase (1.6×10⁻³ U/mL), 5'-nucleotidase activity was measured by an enzyme assay after exposure to carvedilol (0, 1×10⁻⁸ to 1×10⁻⁵ mol/L) for 15 minutes.¹⁵

Analyses

The methods of measuring plasma adenosine levels,¹¹ myocardial ecto-5'-nucleotidase activity,^{11,16} and plasma lactate levels¹³ have been reported previously.

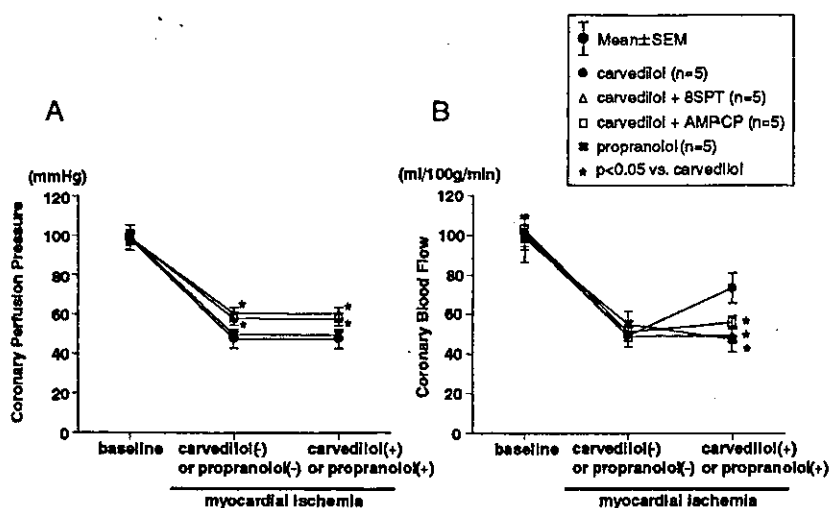


Figure 1. Effects of carvedilol on coronary hemodynamics in ischemic myocardium. A and B show CPP and CBF, respectively. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

Measurement of Infarct Size and Collateral Blood Flow

In protocol 4, the area of myocardial necrosis and the area at risk¹⁶ were measured in all of the dogs upon completion of the protocol by an operator who had no knowledge of the treatment given to each animal. Infarct size was expressed as a percentage of the area at risk.

Regional myocardial blood flow was determined as described previously.¹⁷ Nonradioactive microspheres (Sekisui Plastic Co) made of inert plastic were labeled with bromine. Microspheres were administered at 80 minutes after the start of coronary occlusion. The radio fluorescence of the stable heavy elements was measured with a wavelength dispersive spectrometer (PW 1480, Phillips Co). Because the level of energy emitted is characteristic of specific elements, it was possible to quantify the radio fluorescence of the heavy element with which the microspheres were labeled. Myocardial blood flow was calculated according to the following formula: $\text{time flow} = (\text{tissue count}) \times (\text{reference flow}) / (\text{reference count})$, and was expressed in milliliters per minute per gram wet weight. Endomyocardial blood flow was measured at the inner half of the left ventricular wall.

Exclusion Criteria

To ensure that all of the animals used for analysis of infarct size in protocol 4 were healthy and were exposed to a similar extent of ischemia, the following standards were used for exclusion of unsatisfactory dogs: (1) subendocardial collateral blood flow $>15 \text{ mL} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$, (2) a heart rate $>170 \text{ bpm}$, and (3) >2 consecutive attempts required to terminate ventricular fibrillation using low-energy DC pulses applied directly to the heart.

Statistical Analysis

Statistical analysis was performed by use of ANOVA^{18,19} to compare data among the groups. When ANOVA indicated a significant difference, paired data were compared by use of the Bonferroni test. Changes of the hemodynamic and metabolic parameters over time were assessed by ANOVA with repeated measures. Results were expressed as the mean \pm SEM, with a value of $P < 0.05$ being considered significant.

Results

Effects of Carvedilol on VAD(Ado) in Nonischemic Myocardium

Neither systemic hemodynamic parameters (mean blood pressure, 101.0 ± 2.1 versus $98.6 \pm 3.2 \text{ mm Hg}$ and heart rate, 130.2 ± 3.7 versus $128.0 \pm 3.3 \text{ bpm}$) nor FS ($20.1 \pm 1.0\%$ versus $21.5 \pm 1.0\%$) changed during the infusion of carvedilol.

In contrast, CBF was increased (98.4 ± 8.5 versus $112.6 \pm 9.6 \text{ mL} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$), as was VAD(Ado) (40.9 ± 4.0 versus $68.6 \pm 5.5 \text{ nmol/L}$, $P < 0.05$).

Effects of Either Carvedilol or Propranolol on VAD(Ado) During Coronary Hypoperfusion

Administration of either 8-SPT or AMP-CP did not alter the systemic hemodynamics (mean blood pressure, 98.8 ± 6.1 versus $101.8 \pm 5.8 \text{ mm Hg}$ before and after 8-SPT and 99.0 ± 3.0 versus $102.0 \pm 3.2 \text{ mm Hg}$ before and after AMP-CP; heart rate, 132.2 ± 6.9 versus $132.4 \pm 6.1 \text{ min}^{-1}$ before and after 8-SPT and 131.8 ± 4.6 versus $132.8 \pm 3.4 \text{ min}^{-1}$ before and after AMP-CP) or the coronary hemodynamic and metabolic parameters (Figures 1 through 3). Before both CBF and CPP were reduced, there were no significant differences in hemodynamic and metabolic parameters among the 3 groups. In untreated dogs, administration of saline did not affect CPP, LER, or FS. However, addition of carvedilol increased VAD(Ado), CBF, LER, and FS, even in the

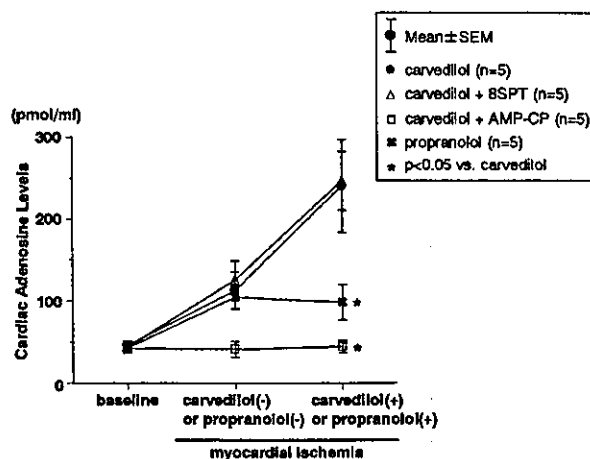


Figure 2. Changes of difference in adenosine levels between coronary venous and arterial blood [VAD(Ado)] in ischemic myocardium. Carvedilol increased VAD(Ado), which was attenuated by an ecto-5'-nucleotidase inhibitor. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

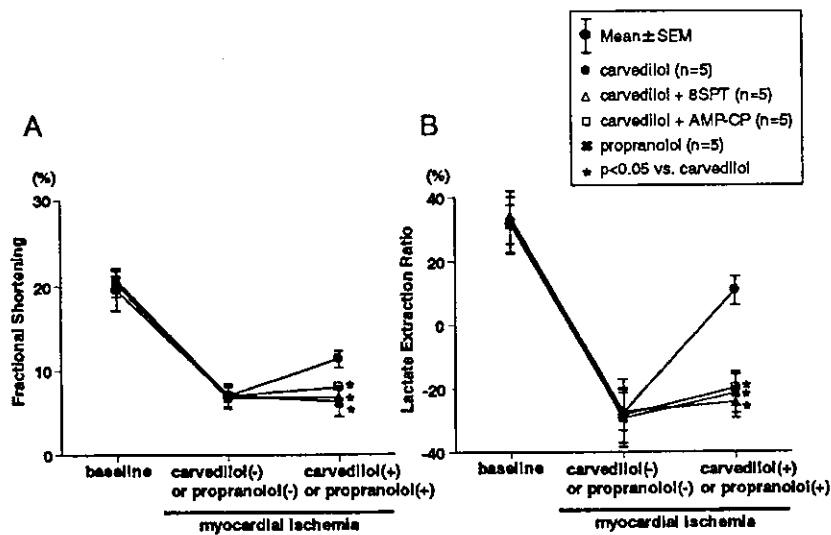


Figure 3. Changes of FS (A) and LER (B) in ischemic myocardium. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

constant low-CPP state, suggesting that myocardial ischemia was improved by carvedilol. These effects of carvedilol were blunted by administration of either 8-SPT or AMP-CP. Unlike carvedilol, an infusion of propranolol did not alter VAD(Ado), CBF, LER, or FS (Figures 1 through 3).

Reduction of Oxidative Stress and Beneficial Effect of Carvedilol in Ischemic Myocardium

In 5 dogs, reduction of CBF caused an increase of VAD(8-Iso-F_{2a}), which was reduced by carvedilol (Figure 4A through 4C). Under these conditions, VAD(Ado) was increased by infusion of carvedilol (Figure 4D). In another 5 dogs, an infusion of SOD did not change either hemodynamic parameters or VAD(8-Iso-F_{2a}) at nonischemic baseline conditions (Figure 4A through 4C). After the reduction of CBF to 50%, VAD(Ado) increased to the level seen in the presence of carvedilol without SOD (Figure 4D), whereas VAD(8-Iso-F_{2a}) did not increase (Figure 4C). Addition of carvedilol did not further attenuate VAD(8-Iso-F_{2a}) or increase VAD(Ado) (Figure 4C and 4D).

Effects of Carvedilol on Infarct Size

Seven of 64 dogs were excluded from analysis because their subendocardial collateral flow was >15 mL · 100 g⁻¹ · min⁻¹, so 57 dogs completed the protocol satisfactorily. Among these 57 dogs, 18 dogs developed ventricular fibrillation at least once, and ventricular fibrillation that matched the exclusion criteria occurred in 15 dogs, so these animals were also excluded from analysis. The numbers of the dogs that met the exclusion criteria of ventricular fibrillation were 2, 2, 0, 2, 3, 3, and 3 in the saline, the DMSO, the carvedilol, the carvedilol+8-SPT, the carvedilol+AMP-CP, the 8-SPT, and the AMP-CP groups, respectively.

Neither aortic blood pressure (≈104 mm Hg) nor heart rate (≈136 min⁻¹) showed any differences among the 7 groups throughout the protocol. The Table shows the area at risk and the endocardial collateral blood flow in the LAD region during myocardial ischemia. There were no significant differences in the area at risk and collateral flow among the 7 groups during myocardial ischemia (Table). Figure 5 shows

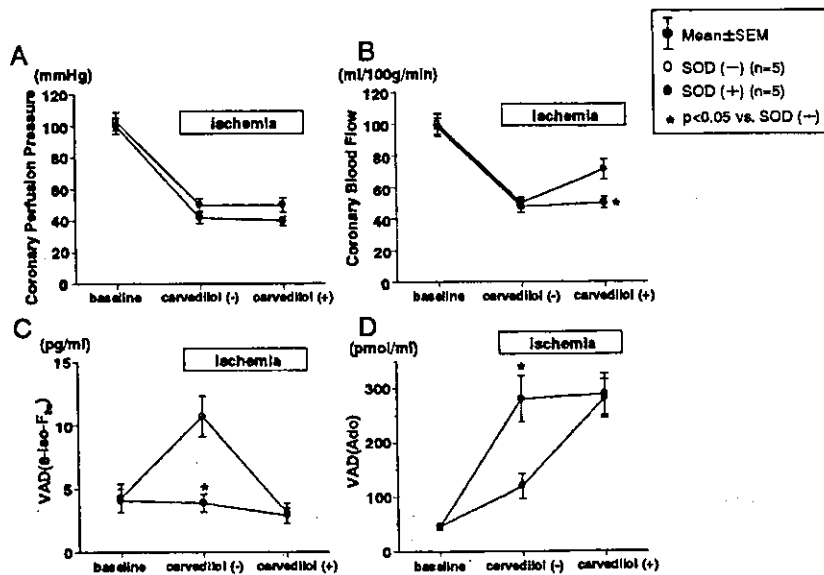


Figure 4. Changes of CPP (A), CBF (B), VAD(8-Iso-F_{2a}) (C), and VAD(Ado) (D) in ischemic myocardium. Statistical significance was tested by ANOVA followed by Bonferroni's test.

Area at Risk and Collateral Blood Flow During Myocardial Ischemia in Each Group

Groups	Risk Area, %	CBF During Myocardial Ischemia, mL·100 g ⁻¹ ·min ⁻¹
1. Control (saline) group	38.9±1.2	7.8±1.3
2. DMSO group	40.2±1.5	8.1±2.3
3. Carvedilol group	41.5±2.2	8.0±2.6
4. Carvedilol group+8-SPT group	39.3±3.3	9.0±2.0
5. Carvedilol group+AMP-CP group	40.9±3.3	8.7±1.9
6. 8-SPT group	43.1±1.9	8.3±1.9
7. AMP-CP group	41.2±2.1	8.2±1.5

Values are expressed as mean±SEM. There were no differences in the area at risk and collateral blood flow in all of the groups. Statistical significance was tested by ANOVA, followed by Bonferroni's test.

that carvedilol decreased infarct size compared with the control groups. This protective effect was completely blocked by either 8-SPT or AMP-CP, suggesting that the reduction of infarct size by carvedilol was attributable to an adenosine-dependent mechanism.

Effect of Carvedilol on Ecto-5'-Nucleotidase Activity in HUVECs

In HUVECs, carvedilol increased ecto-5'-nucleotidase activity by 35.4±8.4% ($P<0.01$) (Figure 6A). Exposure to xanthine and xanthine oxidase decreased ecto-5'-nucleotidase activity, whereas concomitant addition of carvedilol restored ecto-5'-nucleotidase activity to 104.9±8.7% of the baseline levels ($P<0.01$) (Figure 6B). Neither carvedilol nor xanthine and xanthine oxidase had any effect on cytosolic 5'-nucleotidase.

Discussion

In the present study, we demonstrated that carvedilol increases both adenosine release and CBF in ischemic and nonischemic hearts via reduction of oxidative stress and restoration of ecto-5'-nucleotidase activity. We also showed

that carvedilol could limit infarct size and that this effect was attributable to the reduction of oxidative stress and an adenosine- or ecto-5'-nucleotidase-dependent mechanism. These findings suggested that the cardioprotective effect of carvedilol was attributable to an increase of adenosine in ischemic myocardium in addition to its β -blocking action, because propranolol did not mimic this effect.

Influence of Carvedilol on Adenosine Release in Ischemic Hearts

The β -adrenoreceptors in coronary smooth muscle are involved in coronary vasodilation, and their stimulation is thought to increase CBF via the relaxation of vascular smooth muscle and increased myocardial oxygen demand. Therefore, it may seem unusual that a β -blocker like carvedilol would cause coronary vasodilation. There are several possible explanations for the present findings. First, carvedilol itself may cause vasodilation separately from its β -blocking activity. Indeed, although carvedilol does not have a nitroxy moiety, its chemical structure predicts that the drug could also block α_1 -adrenoceptors,²⁰ which would cause vasodilation. We cannot exclude this possibility, but the role of α_1 -adrenoceptor blockade in the vasodilatory effect of carvedilol seems likely to be minor, because we have previously reported that blockade of α_1 -adrenoceptors attenuates adenosine release in ischemic myocardium,²¹ whereas we found that carvedilol caused an increase of adenosine production. Second, carvedilol may increase vasodilatory substances such as NO or adenosine. We demonstrated that carvedilol could increase cardiac adenosine production independently of its β -blocking effect in the present study, because propranolol did not increase CBF under the same circumstances (Figures 1 through 3). Intriguingly, the carvedilol-induced increases in both adenosine release and coronary vasodilation were greater in ischemic heart than in nonischemic heart. There was a significant difference between the influence of carvedilol on coronary vasodilation under nonischemic and ischemic conditions in the present study, because the percent

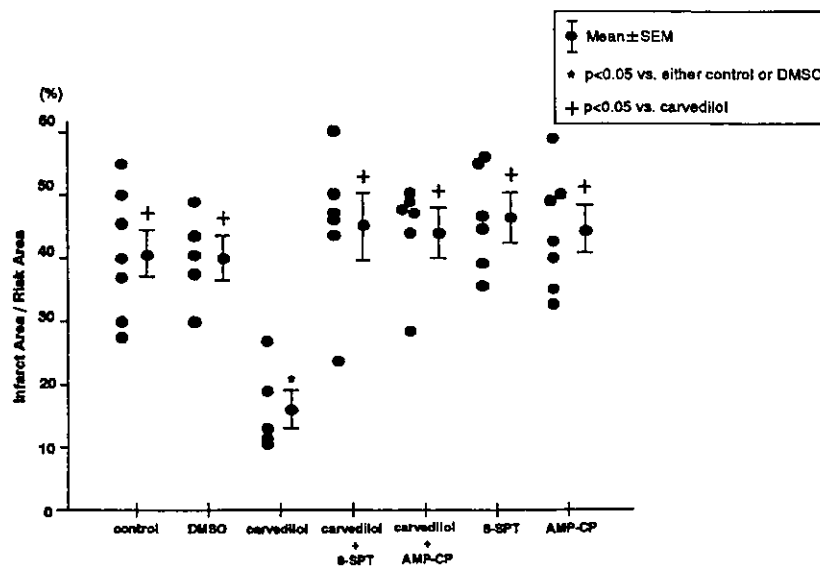


Figure 5. Infarct size as a percentage of area at risk. Infarct size was decreased in carvedilol group compared with control group, and this improvement was blocked by either 8-SPT or AMP-CP. Statistical significance was tested by ANOVA followed by Bonferroni's test.

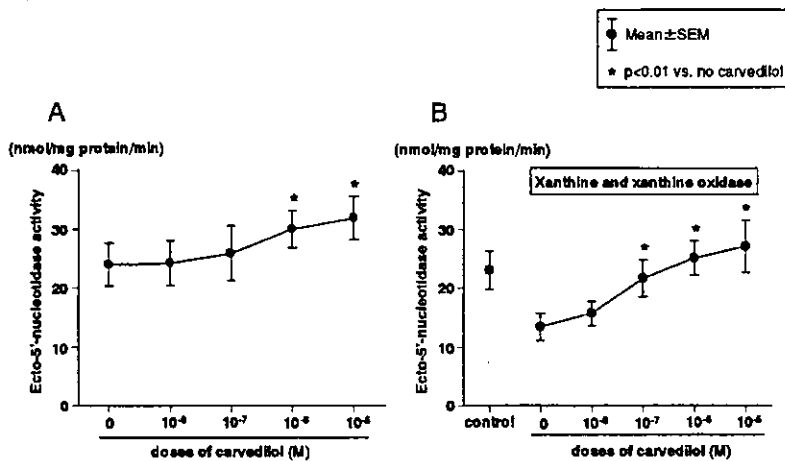


Figure 6. Ecto-5'-nucleotidase activity of HUVECs in presence or absence of carvedilol or xanthine and xanthine oxidase. Statistical significance was tested by ANOVA followed by Bonferroni's test.

increases of CBF in nonischemic and ischemic myocardium were $14.4 \pm 1.1\%$ and $50.6 \pm 10.1\%$ ($P < 0.05$), respectively. One possible explanation is that carvedilol may bind more tightly to β -adrenoreceptors under ischemic conditions than nonischemic conditions, and β -adrenoreceptors are also up-regulated in the ischemic heart,²² which may enhance the adenosine-producing effect of carvedilol. Alternatively, even if carvedilol decreases coronary artery tone in nonischemic heart as well as ischemic heart, the activity of other endogenous vasodilators may decrease to maintain coronary autoregulation. Conversely, the effects of other vasodilators may already be maximal in ischemic hearts, so that carvedilol-induced adenosine release becomes a major determinant of coronary artery tone when adenosine-dependent coronary vasodilation is submaximal. A third possibility is that carvedilol may reduce the levels of substances that attenuate adenosine release and are increased in ischemic myocardium. Because carvedilol is reported to decrease oxidative stress and such stress reduces adenosine production, antioxidant activity of carvedilol may be involved in adenosine-dependent coronary vasodilation and cardioprotection. We showed such evidence in the present study.

In this context, several lines of evidence support the concept that adenosine can markedly attenuate ischemia/reperfusion injury,^{12,23} and we suggest that carvedilol-induced adenosine release is important for cardioprotection.

Mechanism of the Carvedilol-Induced Increase of Cardiac Adenosine

In ischemic hearts, carvedilol caused reduction of oxidative stress and increases in both adenosine release and CBF. Also, in HUVECs under oxidative stress, carvedilol restored ecto-5'-nucleotidase activity to the control level. These findings suggest that carvedilol may eliminate the factors that impaired ecto-5'-nucleotidase activity under ischemic conditions. Oxidative stress is one of these factors. Because oxygen-derived free radicals attenuate the ischemia-induced activation of ecto-5'-nucleotidase, elimination of oxidative stress may increase adenosine release in the ischemic myocardium. We observed that carvedilol could reduce oxidative stress, so this action may explain the present findings. Because ecto-5'-nucleotidase is susceptible to impairment by

oxygen-derived free radicals, it is likely that the beneficial effect of carvedilol on myocardial ischemia in the present study was attributable to its antioxidant activity.

Clinical Relevance and Limitations

Carvedilol has been shown to be effective for treating heart failure.⁵ Its effective clinical dose is about 0.1 to 0.2 $\mu\text{g}/\text{mL}$, and the calculated cardiac concentration of carvedilol in the present study is $\approx 1 \mu\text{g}/\text{mL}$. In dogs, carvedilol at 1 and 4 $\mu\text{g}/\text{mL}$ decreased blood pressure by 9% and 32%, respectively (data not shown), suggesting that the concentration of 1 $\mu\text{g}/\text{mL}$ of carvedilol in canine hearts was comparable to a clinical dose of carvedilol. This difference may be also attributable to species differences, the route of administration of carvedilol, or conscious/anesthetic conditions.

The present study hinted that the mechanism by which carvedilol potentially ameliorates heart failure, especially ischemic heart failure, may be related to adenosine.¹² Carvedilol may have the ability to both antagonize β -adrenoreceptors and increase adenosine release.

Tumor necrosis factor- α is inhibited by both carvedilol and adenosine^{24,25} and has been indicated to have a role in the pathology of congestive heart failure. Because the present study hints that the cardioprotection afforded by carvedilol is adenosine-dependent, it follows that the clinical effects of carvedilol may also be adenosine-dependent. If this hypothesis receives further validation, adenosine and potentiators of adenosine production or adenosine receptor agonists may become candidates for the treatment of heart failure.

Acknowledgments

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Methotrexate and MX-68, a New Derivative of Methotrexate, Limit Infarct Size via Adenosine-Dependent Mechanisms in Canine Hearts

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Abstract: Methotrexate, an anti-rheumatic agent, has recently been reported to show an anti-inflammatory action via ecto-5'-nucleotidase- and adenosine-dependent mechanisms. Because ecto-5'-nucleotidase contributes to the production of adenosine and adenosine has a potent cardioprotective effect against ischemia/reperfusion injury, we investigated whether methotrexate or MX-68 [N-1-((2,4-diamino-6-pteridiny)l methyl)-3,4-dihydro-2H-1,4-benzothiazine-7- carbonyl]-N-2- amino adipic acid] could reduce infarct size via adenosine-dependent mechanisms. In beagle dogs, the left anterior descending coronary artery was perfused through a bypass tube, which was occluded for 90 minutes followed by 6 hours of reperfusion. The size of infarcts was assessed by TTC staining. MX-68 reduced infarct size compared with that in untreated dogs (13.7 ± 1.9 versus $38.6 \pm 5.3\%$, $P < 0.01$). This effect was completely blunted by either the adenosine receptor antagonist 8-sulphophenyltheophylline (8-SPT) ($45.0 \pm 4.6\%$ and $46.8 \pm 5.8\%$ in the 8-SPT and MX-68 + 8-SPT groups, respectively) or by the ecto-5'-nucleotidase inhibitor α, β -methyleneadenosine 5'-diphosphate (AMP-CP) ($44.0 \pm 4.5\%$ and $46.7 \pm 5.8\%$ in the AMP-CP and MX-68 + AMP-CP groups, respectively). Methotrexate also reduced infarct size to a level comparable with that in the MX-68 group, and its effect was also blunted by 8-SPT. There were no significant differences of collateral blood flow or risk area between the groups. We conclude that methotrexate and its derivative (MX-68) both limit infarct size via adenosine-dependent mechanisms.

Key Words: adenosine, ischemia, infarction, inflammation, methotrexate, ecto-5'-nucleotidase, heart

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Although the limitation of infarct size is an important strategy to overcome ischemic heart failure,^{1,2} which is one of the major causes of death today, the most effective adjunctive therapy for ischemic heart disease remains unclear.³ The infarct size-limiting effects of many drugs with anti-inflammatory actions has been tested in an attempt to find effective treatments for myocardial infarction,^{4,9} because the pathophysiology of ischemia/reperfusion injury resembles that of inflammation, including such features as leukocyte infiltration and cytokine production. However, the effect of anti-inflammatory drugs on infarct size seems to vary. Some studies have shown that steroids are effective at reducing infarct size and preventing cardiac remodeling.^{7,8} However, other reports have suggested that steroids cannot reduce infarct size⁹ and these drugs have even been suggested to exacerbate myocardial ischemic injury.^{4,6} On the other hand, other anti-inflammatory agents such as statin and estrogen seem to have a cardioprotective effect.^{10,12} Therefore, attenuation of inflammation does not necessarily have a protective effect against ischemia/reperfusion injury, but has the possibility of mediating cardioprotection. Interestingly, the anti-inflammatory effect of methotrexate (MTX) has been demonstrated to be mediated via adenosine- and ecto-5'-nucleotidase-dependent mechanisms both in vitro and in vivo.¹³ Adenosine inhibits the activation of leukocytes and platelets and also decreases cytokine production.^{14,16} Adenosine has recently been recognized to show a cardioprotective effect against ischemia/reperfusion injury.^{17,18} MTX promotes adenosine release by a variety of different cells and tissues, particularly in the presence of physiological stress. After MTX is taken up by cells, MTX or its metabolites inhibit 5-aminoimidazole-4-

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carboxamide ribonucleotide (AICAR) transformylase, leading to an increase of the AICAR level. We have previously reported that AICAR activates ecto-5'-nucleotidase in the myocardium and increases adenosine release, which may limit infarct size.¹⁹ These reports suggest that MTX, a potent anti-inflammatory agent, may prevent ischemia/reperfusion injury via adenosine-dependent mechanisms.

To test this hypothesis, we examined whether MTX or a new MTX derivative, N-1-((2,4-diamino-6-pteridinyl)methyl)-3,4-dihydro-2H-1,4-benzothiazine-7-carbonyl)-N-2-amino adipic acid²⁰ (MX-68) could limit infarct size via adenosine- or ecto-5'-nucleotidase-dependent mechanisms.

MATERIALS AND METHODS

Instrumentation

Beagle dogs weighing 10 to 13 kg were anesthetized with pentobarbital sodium (30 mg kg⁻¹, iv). The experimental setup was reported previously.²¹ After opening the chest and administration of heparin (500 U kg⁻¹, iv), the left anterior descending coronary artery (LAD) was cannulated for perfusion with blood from the left carotid artery through an extracorporeal bypass tube, and the aortic blood pressure was monitored in this tube. Another cannula was placed in the left atrium for injection of microspheres to measure collateral blood flow during coronary occlusion. The baseline pH, PO₂, and PCO₂ of systemic arterial blood were 7.40 ± 0.02, 106 ± 3 mm Hg, and 38.5 ± 2.0 mm Hg, respectively.

All studies conformed to the "Position of the American Heart Association on Research Animal Use", as adopted by the Association in November 1984.

Experimental Protocols

The systolic and diastolic aortic blood pressures and the heart rate were monitored as hemodynamic parameters.

After hemodynamics became stable, coronary arterial and venous blood samples were obtained for blood gas analysis. In the control group (n = 7), the bypass tube to the LAD was occluded for 90 minutes, followed by 6 hours of reperfusion with infusion of the vehicle (PBS) from 15 minutes before bypass occlusion until the end of reperfusion (except during bypass occlusion). Hemodynamic parameters were monitored during myocardial ischemia and after the start of reperfusion. In the MX-68 group (n = 6), MX-68 dissolved in PBS was infused as a bolus (0.1 mg kg⁻¹) and then infused continuously (0.1 mg kg⁻¹ h⁻¹) into a systemic vein from 10 minutes before bypass occlusion until the end of the 6-hour reperfusion period (except during bypass occlusion). The effect of MX-68 was also tested during intracoronary infusion of either 8-sulphophenyltheophylline (8-SPT, 50 µg kg⁻¹ min⁻¹) or α,β-methylenadenosine 5'-diphosphate (AMP-CP, 30 µg kg⁻¹ min⁻¹) from 15 minutes before bypass occlusion until 6 hours

of reperfusion (except during bypass occlusion) in the MX-68+8-SPT group (n = 5) and the MX-68+AMP-CP group (n = 5), respectively. In the MTX group (n = 5), MTX was given as an intravenous bolus (0.05 mg kg⁻¹) and then as a continuous infusion (0.05 mg kg⁻¹ h⁻¹) according to the same schedule as that for MX-68. In the MTX+8-SPT group (n = 5), 8-SPT and MTX were administered by the same schedule as for the MX-68+8-SPT group. We also tested whether MX-68 was effective after the onset of reperfusion (the MX-68 (post) group); MX-68 was given as a bolus infusion (0.1 mg kg⁻¹) into a systemic vein at the start of reperfusion and then as a continuous infusion (0.1 mg kg⁻¹ h⁻¹) for 6 hours (n = 7). In all of these groups, the bypass tube was occluded for 90 minutes, followed by 6 hours of reperfusion, and the infarct size was assessed at 6 hours as reported previously.²¹ Microspheres were infused at 45 minutes after the onset of coronary occlusion to measure collateral blood flow.

Measurement of Regional Blood Flow

Regional myocardial blood flow was determined by the microsphere technique as described previously.²¹ Microspheres (Sekisui Plastic Co., Ltd., Tokyo, Japan) made of an inert plastic were labeled with bromine (Br). The mean diameter of the microspheres was 15 µm and the specific gravity was 1.34 for Br. The microspheres were suspended in isotonic saline with 0.01% Tween 80 to prevent aggregation, and were sonicated for 5 minutes followed by vortexing for 5 minutes immediately before injection. Just before administration of the microspheres, a reference blood sample was withdrawn from the femoral artery at a constant rate of 8 mL/min for 2 minutes. Approximately 1 mL of the microsphere suspension (2 to 4 × 10⁶ microspheres) was injected into the left atrium, and then was flushed with warm (37°C) saline (5 mL). Microspheres were administered 80 minutes after occlusion of the bypass tube.

The radiograph fluorescent activity of the stable heavy element was measured with a wavelength dispersive spectrometer (PW 1480, Phillips Co., Ltd., Almelo, The Netherlands). The microspheres were irradiated by a primary radiograph beam, causing electrons to fall back to a lower orbit and emit measurable energy. Because the energy emitted is characteristic of a specific element, it is possible to quantify the radiograph fluorescence of any heavy element with which the microspheres are labeled. Myocardial blood flow was calculated according to the following formula: Flow = (Tissue count) × (Reference flow)/(Reference count), and was expressed as milliliters per minute per gram wet weight. Endomyocardial blood flow was measured in the inner half of the left ventricular (LV) wall.

Measurement of Infarct Size

After reperfusion for 6 hours, the LAD was reoccluded and perfused with autologous blood. Evans blue dye was in-

jected into a systemic vein to identify the area at risk and the nonischemic area.²¹ The heart was then immediately removed and sliced into serial transverse sections that were 6 to 7 mm in width. The nonischemic area was defined as the tissue showing blue staining. The ischemic region was harvested and incubated at 37°C for 20 to 30 minutes in 1% 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma Chemical Company) in 0.1 mol/L phosphate buffer adjusted to pH 7.4. TTC stains the noninfarcted myocardium a brick-red color, indicating the presence of a formazan product created through the reduction of TTC by dehydrogenases in viable tissues. The infarct size was expressed as a percentage of the area at risk.

Selection Criteria

To ensure that all of the animals included in analysis of infarct size were healthy and exposed to a similar extent of ischemia, the following criteria were used to exclude unsatisfactory dogs: (1) subendocardial collateral flow > 15 mL 100 g⁻¹ min⁻¹, (2) heart rate > 170 bpm, and (3) more than 2 consecutive attempts required to convert ventricular fibrillation with low-energy DC pulses applied directly to the heart.

Statistical Analysis

Statistical analysis was performed with ANOVA for comparisons among the groups. If ANOVA indicated a signifi-

cant difference, paired data were compared using Bonferroni test.^{22,23} Changes of the hemodynamic parameters over time were compared by ANOVA with repeated measures. Using endocardial collateral blood flow in the inner half of the LV wall as the covariate, ANCOVA was performed to assess the influence of collateral flow on infarct size. Results are expressed as the mean ± SEM, and *P* < 0.05 was considered significant.

RESULTS

Seventy dogs were randomly assigned to 9 different protocols and the infarct size was determined in each group. Eight dogs were excluded from analysis because subendocardial collateral flow was greater than 15 mL 100 g⁻¹ min⁻¹. Among the remaining 62 dogs, 17 developed ventricular fibrillation at least once and fibrillation that fulfilled the exclusion criteria occurred in 9 dogs, which were also excluded from the study.

Mean aortic blood pressure and heart rate (Fig. 1) did not vary among the 9 groups throughout the study. The percent area at risk in the left ventricle and the endocardial collateral blood flow during myocardial ischemia were also not significantly different among the 9 groups (Fig. 2). Figure 3 shows that MX-68 markedly reduced the infarct size compared with that in the control group. This effect of MX-68 was completely

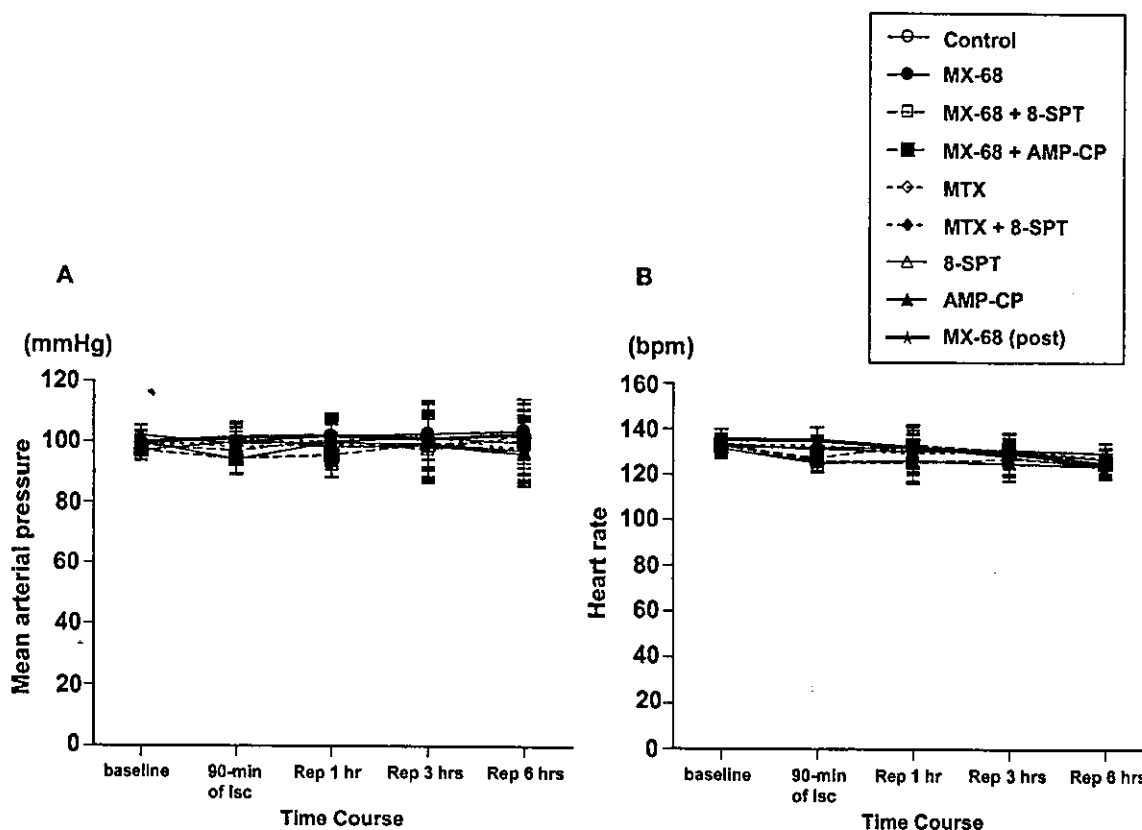


FIGURE 1. Systemic hemodynamic parameters (mean arterial pressure (A) and heart rate (B)) throughout the study. There were no significant changes of these parameters in all the 9 groups. Statistical significance was tested by ANOVA.