degraded by acetylcholinesterase before reaching the dialysis fiber. In the same preparation, local administration of neostigmine enhanced the dialysate catecholamine response to nerve stimulation by about three-fold, but did not influence the responses of heart rate and mean arterial pressure (Akiyama et al., 2003). Total catecholamine release from adrenal gland might not change by the local administration of neostigmine. In the present study, dialysate catecholamine response was correlated with the frequency of splanchnic nerve stimulation. Thus, in the presence of neostigmine, absolute value of dialysate catecholamine response is exaggerated, but could reflect relative changes in catecholamine release from adrenal gland.

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Effects of Ca^{2+} channel antagonists on acetylcholine and catecholamine releases in the in vivo rat adrenal medulla

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Akiyama, Tsuyoshi, Toji Yamazaki, Hidezo Mori, and Kenji Sunagawa. Effects of Ca^{2+} channel antagonists on acetylcholine and catecholamine releases in the in vivo rat adrenal medulla. *Am J Physiol Regul Integr Comp Physiol* 287: R161–R166, 2004. First published March 18, 2004; 10.1152/ajpregu.00609.2003.—To elucidate the types of voltage-dependent Ca^{2+} channels controlling ACh and catecholamine releases in the in vivo adrenal medulla, we implanted microdialysis probes in the left adrenal medulla of anesthetized rats and investigated the effects of Ca^{2+} channel antagonists on ACh, norepinephrine, and epinephrine releases induced by nerve stimulation. The dialysis probes were perfused with Ringer solution containing a cholinesterase inhibitor, neostigmine. The left splanchnic nerves were electrically stimulated at 2 and 4 Hz before and after intravenous administration of Ca^{2+} channel antagonists. ω-Conotoxin GVIA (an N-type Ca^{2+} channel antagonist, 10 μg/kg) inhibited ACh release at 2 and 4 Hz by ~40%, norepinephrine release at 4 Hz by ~50%, and epinephrine release at 2 and 4 Hz by ~45%. A fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) did not further inhibit these releases. ω-Conotoxin MVIIC (a P/Q-type Ca^{2+} channel antagonist, 50 μg/kg) inhibited ACh and epinephrine releases at 4 Hz by ~30%. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited ACh release at 2 and 4 Hz by ~70% and norepinephrine and epinephrine releases at 2 and 4 Hz by ~80%. Nifedipine (an L-type Ca^{2+} channel antagonist, 300 and 900 μg/kg) did not change ACh release at 2 and 4 Hz; however, nifedipine (300 μg/kg) inhibited epinephrine release at 4 Hz by 20%, and nifedipine (900 μg/kg) inhibited norepinephrine and epinephrine releases at 4 Hz by 30%. In conclusion, both N- and P/Q-type Ca^{2+} channels control ACh release on preganglionic splanchnic nerve endings while L-type Ca^{2+} channels do not. L-type Ca^{2+} channels are involved in norepinephrine and epinephrine releases on chromaffin cells.

anesthetized rats; microdialysis; norepinephrine; epinephrine; preganglionic autonomic nerve endings

Ca^{2+} influx through the voltage-dependent Ca^{2+} channels induces the release of transmitters from neuronal or secretory cells by initiating exocytosis from vesicles. Voltage-dependent Ca^{2+} channels have been classified into L-, N-, P-, Q-, R-, and T-types (12, 25, 30). To better understand the mechanism controlling the release of transmitters, it is important to determine the type of Ca^{2+} channels involved in the release of the transmitters on neuronal or secretory cells.

In the in vivo adrenal medulla, catecholamine release is controlled by central sympathetic neurons through preganglionic splanchnic nerves. Splanchnic nerve endings make synaptic-like contacts with chromaffin cells (9). ACh released from splanchnic nerve endings consequently evokes catecholamine release from chromaffin cells by activation of cholinergic receptors. Thus, in vivo catecholamine release requires Ca^{2+} influx through the voltage-dependent Ca^{2+} channels at two different sites in the adrenal medulla: splanchnic nerve endings and chromaffin cells. Numerous studies have investigated the nature of Ca^{2+} channels controlling transmitter release from postganglionic autonomic nerve endings (8, 11, 32, 33, 36, 37). Little information is, however, available on the type of Ca^{2+} channels controlling the ACh release from preganglionic autonomic nerve endings including splanchnic nerve endings. Moreover, although the types of Ca^{2+} channels controlling catecholamine release have been investigated using isolated chromaffin cells in various species (5, 6, 13, 16, 21, 23, 24), it remains unknown whether endogenous ACh induces Ca^{2+} influx through the same types of Ca^{2+} channels on chromaffin cells.

We have recently developed a dialysis technique to simultaneously monitor ACh and catecholamine releases in the in vivo adrenal medulla (2). This method makes it possible to characterize Ca^{2+} channels controlling ACh release from splanchnic nerve endings and catecholamine release from adrenal medulla in the in vivo state. In the present study, we applied the microdialysis technique to the adrenal medulla of anesthetized rats and investigated the effects of Ca^{2+} channel antagonists on dialysate ACh and catecholamine responses induced by the electrical stimulation of splanchnic nerves.

MATERIALS AND METHODS

Animal preparation. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996). Adult male Wistar rats weighing 380–450 g were anesthetized with pentobarbital sodium (50–55 mg/kg ip). A cervical midline incision was made to expose the trachea, which was then cannulated. The rats were ventilated with a constant-volume respirator using room air mixed with oxygen. The left femoral artery and vein were cannulated for monitoring arterial blood pressure and administration of anesthetic, respectively. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (15–25 $mg \cdot kg^{-1} \cdot h^{-1}$ iv). Electrocardiogram was monitored for recording heart rate. A thermostatic heating pad was used to keep the esophageal temperature within a range of 37–38°C. With the animal in the lateral position, the left adrenal gland and left splanchnic nerve were exposed by a subcostal flank incision, and the left splanchnic nerve was transected. Shielded bipolar stainless steel electrodes were applied to the distal end of the nerve, which was then stimulated with a digital stimulator (SEN-7203, Nihon Kohden) with a rectangular pulse (10 V and 1 ms in duration).

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Dialysis technique. The materials of the dialysis probe were the same as those used in our previous dialysis experiments (1, 2). Briefly, each end of the dialysis fiber (0.31 mm OD, and 0.20 mm ID; PAN-1200 50,000 mol wt cutoff, Asahi Chemical) was inserted into the polyethylene tube (25-cm length, 0.5 mm OD, and 0.2 mm ID; SP-8) and glued. The length of the dialysis fiber exposed was 3 mm.

The left adrenal gland was gently lifted, and the dialysis probe was implanted in the medulla of the left adrenal gland along the long axis by using a fine guiding needle. The dialysis probe was perfused with Ringer solution containing a cholinesterase inhibitor, neostigmine (10 μM), at a speed of 10 μl/min using a microinjection pump (CMA/100, Carnegie Medicin). Ringer solution with no buffer consisted of (in mM) 147.0 NaCl, 4.0 KCl, and 2.25 $CaCl_2$. One sampling period was 2 min (1 sample volume = 20 μl). We started the protocols followed by a stabilization period of 3–4 h and sampled dialysate taking the dead space volume into account.

Dialysate ACh, norepinephrine (NE), and epinephrine (Epi) concentrations were measured as indexes of ACh and catecholamine releases in the adrenal medulla. Half of the dialysate sample was used for the measurement of ACh, and the remaining half for the measurement of NE and Epi. ACh and catecholamine assays were separately conducted using each high-performance liquid chromatography with electrochemical detection as previously described (3, 4).

Experimental design. The experiment was performed based on the previous experiment showing that dialysate ACh and catecholamine responses were reproducible on repetition of stimulation (2). The left splanchnic nerves were electrically stimulated for 2 min at 30-min intervals. Three dialysate samples were continuously collected per electrical stimulation: one before, one during, and one after stimulation. Stimulations at two different frequencies (2 and 4 Hz) were performed before and after intravenous administration of Ca^{2+} channel antagonists.

We tested three types of Ca^{2+} channel antagonists (25): the N-type Ca^{2+} channel antagonist ω-conotoxin GVIA, the P/Q-type Ca^{2+} channel antagonist ω-conotoxin MVIIC, and the L-type Ca^{2+} channel antagonist nifedipine. We determined the first doses of Ca^{2+} channel antagonists based on the dose used in the earlier experiments (7, 14, 26, 29, 37) and tested ω-conotoxin GVIA (10 μg/kg) in six rats, ω-conotoxin MVIIC (50 μg/kg) in six rats, and nifedipine (300 μg/kg) in six rats. Second, we tested a fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) in six rats, a combination of fivefold higher doses of ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) in six rats, and a threefold higher dose of nifedipine (900 μg/kg) in six rats. We did not test a higher dose of ω-conotoxin MVIIC singly because a high dose of ω-conotoxin MVIIC loses its selectivity for P/Q-type and inhibits N-type Ca^{2+} channels (18).

Nifedipine was administered twice before 2- and 4-Hz stimulation, but ω-conotoxin GVIA and MVIIC were administered once before 2-Hz stimulation because the ω-conotoxin family has long-lasting blocking actions (8, 18, 36). We assessed the responses to nerve stimulation 30, 20, and 10 min after administration of ω-conotoxin GVIA, ω-conotoxin MVIIC, and nifedipine, respectively, when heart rate and arterial pressure had already been stabilized.

At the end of the experiment the rats were killed with pentobarbital sodium, and the implant sites were examined. The dialysis probes were confirmed to have been implanted in the adrenal medulla, and no bleeding or necrosis was found macroscopically.

Drugs. Drugs were mixed fresh for each experiment. Neostigmine methylsulfate (Shionogi), ω-conotoxin GVIA (Peptide Institute), and ω-conotoxin MVIIC (Peptide Institute) were dissolved and diluted in Ringer solution. Nifedipine (Sigma Chemical) was dissolved in ethanol and diluted in Ringer solution.

Statistical methods. To examine the effects of nerve stimulation and Ca^{2+} channel antagonists, we analyzed heart rate and mean arterial pressure and dialysate ACh, NE, and Epi responses by using one-way ANOVA with repeated measures. When statistical significance was detected, the Newman-Keuls test was applied (35). Statistical significance was defined as $P < 0.05$. Values are presented as means ± SE.

RESULTS

Effects of Ca^{2+} channel antagonists on heart rate and mean arterial pressure. ω-Conotoxin GVIA (10 μg/kg) decreased heart rate from 418 ± 9 to 328 ± 13 beats/min ($P < 0.05$) and mean arterial pressure from 115 ± 2 to 74 ± 2 mmHg ($P < 0.05$). ω-Conotoxin GVIA (50 μg/kg) did not further decrease heart rate and mean arterial pressure. ω-Conotoxin MVIIC decreased heart rate from 408 ± 3 to 390 ± 5 beats/min ($P < 0.05$) but did not change mean arterial pressure. Combined ω-conotoxin GVIA and MVIIC decreased heart rate from 415 ± 10 to 327 ± 4 beats/min ($P < 0.05$) and mean arterial pressure from 124 ± 2 to 57 ± 2 mmHg ($P < 0.05$). Nifedipine (300 μg/kg) decreased mean arterial pressure from 113 ± 4 to 86 ± 4 mmHg ($P < 0.05$) but did not change heart rate. Nifedipine (900 μg/kg) decreased mean arterial pressure from 124 ± 3 to 73 ± 2 mmHg ($P < 0.05$).

Effects of Ca^{2+} channel antagonists on ACh and catecholamine releases. ACh could not be detected in dialysate before or after stimulation. Thus we expressed dialysate ACh concentration during stimulation as an index of ACh release induced by stimulation. In contrast, substantial amounts of NE and Epi were observed in dialysate before stimulation. Intravenous administration of Ca^{2+} channel antagonists did not affect these basal NE and Epi releases (Table 1). Dialysate NE and Epi concentrations increased by nerve stimulation and rapidly declined after the stimulation. Thus we subtracted the dialysate NE and Epi contents before stimulation from those during stimulation and expressed these values as indexes of NE and Epi releases induced by stimulation.

Effects of ω-conotoxin GVIA. ω-Conotoxin GVIA (10 μg/kg) significantly inhibited ACh release at 2 Hz from 6.2 ± 0.9 to 3.6 ± 0.5 nM, ACh release at 4 Hz from 12.2 ± 1.7 to 7.9 ± 1.2 nM, NE release at 4 Hz from 34 ± 6 to 17 ± 3 nM, Epi release at 2 Hz from 81 ± 13 to 42 ± 3 nM, and Epi release at 4 Hz from 180 ± 21 to 94 ± 7 nM. However, inhibition of NE release at 2 Hz was not statistically significant (Fig. 1*A*). A fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) did not

Table 1. *Basal dialysate NE and Epi concentrations before and after administration of Ca^{2+} channel antagonists*

	NE, nM	Epi, nM
ω-Conotoxin GVIA (10 and 50 μg/kg) (n = 12)		
Before administration	4.9±0.9	20.6±2.9
After administration	3.8±0.6	21.0±2.6
ω-Conotoxin MVIIC (50 μg/kg) (n = 6)		
Before administration	4.1±1.0	20.2±2.6
After administration	4.6±0.9	24.0±3.5
ω-Conotoxin GVIA (50 μg/kg) + MVIIC (250 μg/kg) (n = 6)		
Before administration	4.4±1.3	17.5±3.8
After administration	3.1±0.7	20.8±4.4
Nifedipine (100 and 300 μg/kg) (n = 12)		
Before administration	4.0±0.6	17.4±2.2
After administration	3.3±0.9	17.7±2.9

Values are means ± SE; *n*, no. of rats. NE, norepinephrine; Epi, epinephrine.

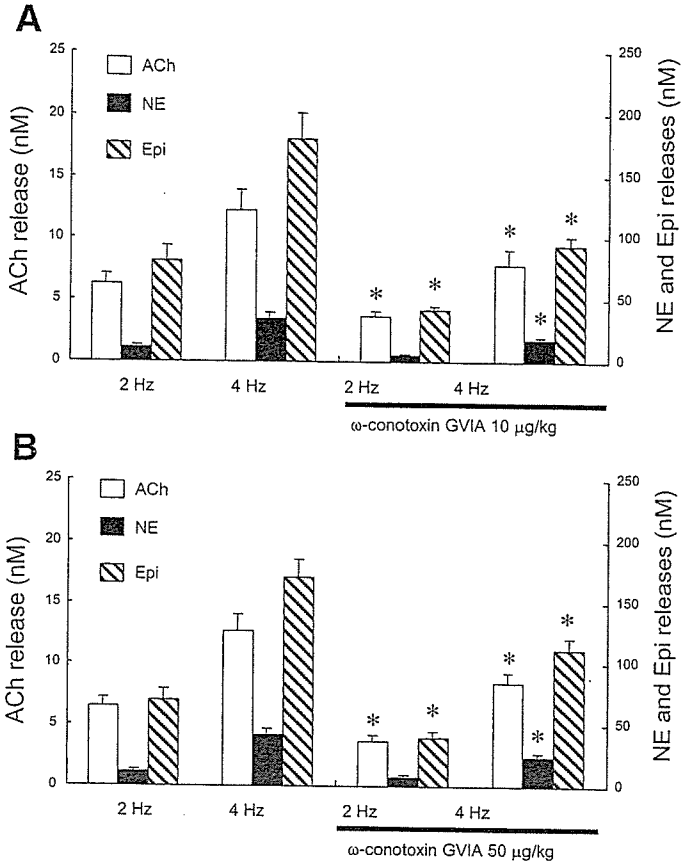

Fig. 1. Effects of ω-conotoxin GVIA on ACh, norepinephrine (NE), and epinephrine (Epi) releases. ω-Conotoxin GVIA (10 μg/kg) inhibited ACh release at 2 and 4 Hz, NE release at 4 Hz, and Epi release at 2 and 4 Hz (*A*). A 5-fold higher dose of ω-conotoxin GVIA (50 μg/kg) did not further inhibit these releases (*B*). Values are means ± SE from 6 rats. $*P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

further inhibit release. ω-Conotoxin GVIA (50 μg/kg) significantly inhibited ACh release at 2 Hz from 6.5 ± 0.7 to 3.7 ± 0.5 nM, ACh release at 4 Hz from 12.6 ± 1.4 to 8.5 ± 0.8 nM, NE release at 4 Hz from 41 ± 6 to 24 ± 4 nM, Epi release at 2 Hz from 70 ± 10 to 40 ± 6 nM, and Epi release at 4 Hz from 170 ± 15 to 112 ± 10 nM (Fig. 1*B*).

Effects of ω-conotoxin MVIIC. ω-Conotoxin MVIIC (50 μg/kg) significantly inhibited ACh release at 4 Hz from 11.7 ± 2.5 to 8.5 ± 2.1 nM and Epi release at 4 Hz from 170 ± 38 to 129 ± 35 nM. Inhibitions of ACh and Epi releases at 2 Hz and NE release at either frequency were not statistically significant (Fig. 2).

Effects of combined ω-conotoxin GVIA and MVIIC. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) significantly inhibited ACh release at 2 Hz from 6.7 ± 0.6 to 1.9 ± 0.3 nM, ACh release at 4 Hz from 12.1 ± 1.3 to 3.8 ± 0.6 nM, NE release at 2 Hz from 11.1 ± 1.1 to 1.2 ± 0.3 nM, NE release at 4 Hz from 36 ± 5 to 8 ± 2 nM, Epi release at 2 Hz from 88 ± 9 to 13 ± 3 nM, and Epi release at 4 Hz from 187 ± 20 to 49 ± 9 nM (Fig. 3).

Effects of nifedipine. Nifedipine (300 μg/kg) did not change ACh release at either frequency but significantly inhibited Epi release at 4 Hz from 172 ± 31 to 135 ± 23 nM. Inhibitions of Epi release at 2 Hz and NE release at either frequency were not statistically significant (Fig. 4*A*). A threefold higher dose of

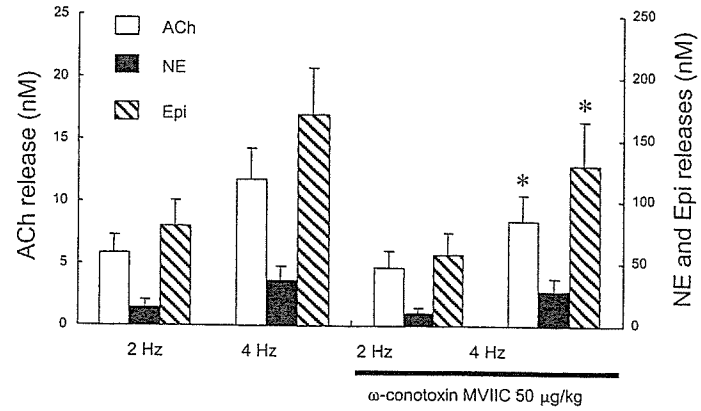

Fig. 2. Effects of ω-conotoxin MVIIC on ACh, NE, and Epi releases. ω-Conotoxin MVIIC (50 μg/kg) inhibited ACh and Epi releases at 4 Hz. Values are means ± SE from 6 rats. $*P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

nifedipine (900 μg/kg) did not change ACh release but significantly inhibited Epi release at 4 Hz from 188 ± 24 to 128 ± 15 nM and NE release at 4 Hz from 33 ± 5 to 24 ± 4 nM. Inhibitions of NE and Epi releases at 2 Hz were not statistically significant (Fig. 4*B*).

DISCUSSION

Effects of Ca^{2+} channel antagonists on ACh release from splanchnic nerve endings. In the present study, ω-conotoxin GVIA (10 μg/kg) inhibited ACh release at both 2 and 4 Hz by approximately 35–40%. A fivefold higher dose of ω-conotoxin GVIA (50 μg/kg) did not further inhibit ACh release. ω-Conotoxin MVIIC (50 μg/kg) inhibited ACh release at 4 Hz by ~30%. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited ACh release at both 2 and 4 Hz by ~70%. N- and P/Q-type Ca^{2+} channels could be present on the splanchnic nerve endings and be involved in ACh release. P/Q-type Ca^{2+} channels may play a role in ACh release at a high frequency of stimulation. ACh release response was resistant to nifedipine (300 and 900 μg/kg) at both 2 and 4 Hz. L-type Ca^{2+} channels could not be present on splanchnic nerve

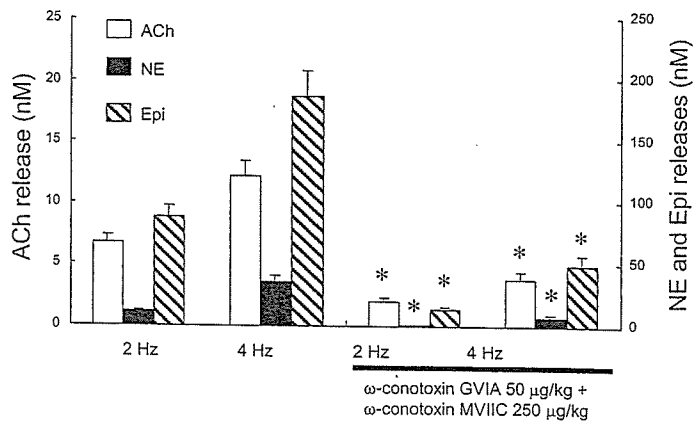

Fig. 3. Effects of combined ω-conotoxin GVIA and MVIIC on ACh, NE, and Epi releases. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited ACh, NE, and Epi releases at 2 and 4 Hz. Values are means ± SE from 6 rats. $*P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

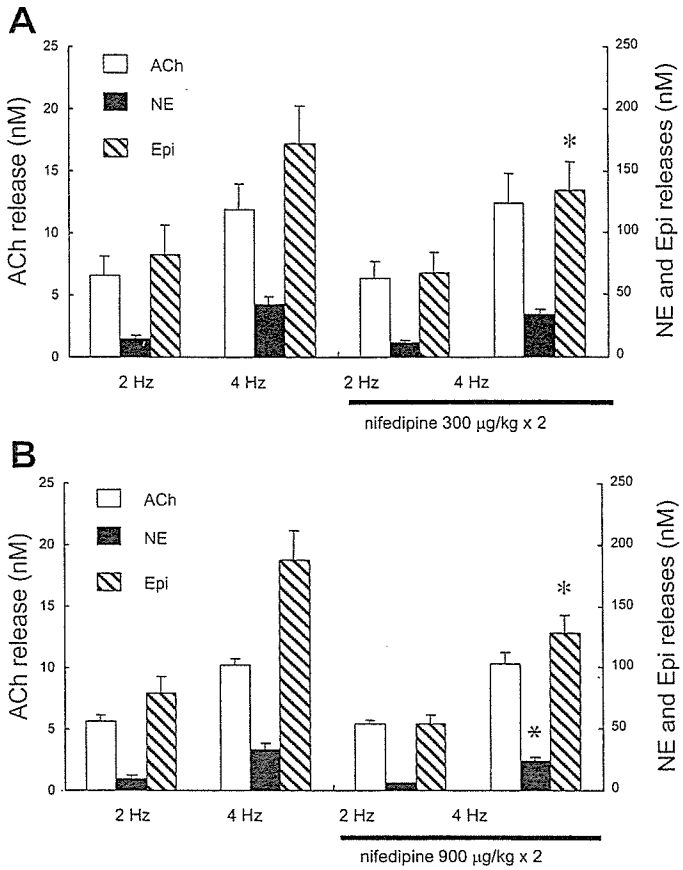

Fig. 4. Effects of nifedipine on ACh, NE, and Epi releases. Nifedipine (300 μg/kg) did not change ACh release at either frequency but inhibited Epi release at 4 Hz (*A*). Nifedipine (900 μg/kg) did not change ACh release at either frequency but inhibited NE and Epi releases at 4 Hz (*B*). Values are means ± SE from 6 rats. $*P < 0.05$ vs. ACh, NE, or Epi release at same frequency before administration.

endings or not play a major role in ACh release. This is the first direct study to demonstrate the type of Ca^{2+} channels controlling ACh release from splanchnic nerve endings.

In isolated rat adrenal glands, catecholamine release induced by field stimulation is sensitive to P/Q-type Ca^{2+} channel antagonist, whereas that induced by exogenous ACh is insensitive (27). This indirect study suggested the involvement of P/Q-type Ca^{2+} channels in ACh release but failed to show the involvement of N-type Ca^{2+} channels. In isolated bovine adrenal glands, a direct measurement study showed that a reduction of the extracellular Ca^{2+} concentration inhibits ^{3}H-labeled ACh release induced by field stimulation, but N- and L-type Ca^{2+} channel antagonists do not (28). Thus our findings are in part consistent with these direct and indirect studies but inconsistent as to the involvement of N-type Ca^{2+} channels.

This discrepancy might be ascribed to the experimental method. The contribution of Ca^{2+} channels may vary with the type of method used to evoke ACh release. In these studies, ACh release was evoked by electrical field stimulation of isolated adrenal glands, which is known to induce ACh release but not direct depolarization of chromaffin cells (34). In the present study, ACh release was evoked in the in vivo state by electrical stimulation of splanchnic nerves. The type of Ca^{2+} channels involved in ACh release may vary with the frequency, amplitude, or time period of stimulation. Actually, in the present study, we observed the involvement of P/Q-type Ca^{2+} channels at only high-frequency stimulation, while it has been reported in perfused rat adrenal glands that N-type Ca^{2+} channels are involved in the maintenance of catecholamine release in response to long splanchnic nerve stimulation (31). The time period of 2 min in the present study seems to be longer than those in earlier studies but could be within the physiological range. Moreover, the blocking action of ω-conotoxin GVIA is time dependent as well as dose dependent and irreversible (8, 11, 32, 36). The maximum functional effect of ω-conotoxin GVIA has been observed to be at least 15 min after administration. We evaluated the effect of ω-conotoxin GVIA 30 min after intravenous administration, when heart rate and mean arterial pressure had already been stabilized. The evaluation early after administration might lead to underestimation of the inhibitory effects of ω-conotoxin GVIA.

There are many similarities between synaptic transmission from splanchnic nerves to chromaffin cells and sympathetic ganglionic transmission (17). In isolated guinea pig paravertebral ganglia, an electrophysiological study has shown that both N- and P-type Ca^{2+} channel antagonists reduce cholinergic synaptic conductance, whereas L-type Ca^{2+} channel antagonist does not (19). In isolated rat superior cervical ganglia, both N- and P-type Ca^{2+} channel antagonists inhibit the rise in Ca^{2+} concentration in the terminal boutons (22). Moreover, in isolated rat superior cervical ganglia, ^{3}H-labeled ACh release induced by high K^+ is inhibited by both N- and P-type Ca^{2+} channel antagonists but unaffected by L-type Ca^{2+} channel antagonist (15). Our findings are similar to these findings obtained from isolated sympathetic preganglionic nerves.

The inhibition by ω-conotoxin GVIA (50 μg/kg) was almost the same as that by ω-conotoxin GVIA (10 μg/kg). Moreover, the inhibition by combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) was almost algebraically the sum of the individual inhibition by ω-conotoxin GVIA (10 μg/kg) and MVIIC (50 μg/kg). These results suggest that fivefold higher doses of ω-conotoxin GVIA and MVIIC are sufficient to cause inhibition of Ca^{2+} channels. However, ~30% of ACh release was resistant to combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg). Other types of Ca^{2+} channels except for N- and P/Q-types may be involved in ACh release from splanchnic nerve endings. Further examination could be needed.

Effects of Ca^{2+} channel antagonists on catecholamine release from chromaffin cells. In the present study, nifedipine (300 μg/kg) did not change ACh release at 2 and 4 Hz but inhibited Epi release at 4 Hz by ~20%. A threefold higher dose of nifedipine (900 μg/kg) did not change ACh release at 2 and 4 Hz but inhibited NE and Epi releases at 4 Hz by ~30%. Adrenal chromaffin cells are divided into two populations: NE- and Epi-storing cells (10). L-type Ca^{2+} channels could be present on the surface of both NE- and Epi-storing cells and play a role in NE and Epi releases.

Approximately 70% of catecholamine release was resistant to nifedipine (900 μg/kg). This result suggests that other types of Ca^{2+} channels except for L-type are present on chromaffin cells and involved in NE and Epi releases, although we cannot exclude the possibility of incomplete inhibition of L-type Ca^{2+} channels. Species differences in the types of Ca^{2+} channels controlling Ca^{2+} influx and catecholamine release have been

shown with rat, cat, and bovine chromaffin cells (5, 6, 13, 24). In patch-clamp studies of isolated rat chromaffin cells, Ca^{2+} inward current elicited by depolarization is sensitive to both L- and N-type Ca^{2+} channel antagonists (16, 21). The study measuring Ba^{2+} current by patch-clamp technique has shown the existence of L-, N-, and P/Q-type Ca^{2+} channels on rat chromaffin cells and the following distribution of Ca^{2+} channels in decreasing order: L-type > N-type > P/Q-type (13). In the present study, ω-conotoxin GVIA (10 and 50 μg/kg) inhibited NE release at 4 Hz and Epi release at 2 and 4 Hz by approximately 45–50%. ω-Conotoxin MVIIC (50 μg/kg) inhibited Epi release at 4 Hz by ~30%. Combined ω-conotoxin GVIA (50 μg/kg) and MVIIC (250 μg/kg) inhibited NE and Epi releases at 2 and 4 Hz by approximately 75–85%. However, these Ca^{2+} channel antagonists simultaneously inhibited ACh release to almost the same extent. It is difficult to determine how much Ca^{2+} antagonists are acting on chromaffin cells when Ca^{2+} channel antagonists inhibit ACh release. Thus, although much of these inhibitions of catecholamine release may be considered to be consequences of the inhibition of ACh release, we cannot exclude the possibility that N- or P/Q-type Ca^{2+} channels may be involved in the in vivo catecholamine release on chromaffin cells.

The inhibition of NE release at 2 Hz by ω-conotoxin GVIA (10 and 50 μg/kg) and the inhibition of NE release at 4 Hz by ω-conotoxin MVIIC (50 μg/kg) were not statistically significant despite significant inhibitions of ACh and Epi releases. In the same preparation, we have shown that cholinergic antagonists almost inhibited NE and Epi releases induced by nerve stimulation (1, 2). However, the correlation between ACh and NE releases was poorer than that between ACh and Epi releases when stimulation frequency was raised stepwise (2). Insignificant inhibitions of NE release may be ascribed to this poor correlation.

In the present study, Ca^{2+} channel antagonists did not affect basal dialysate NE and Epi levels. In our previous study of the same preparation, these basal levels were not affected by neostigmine, hexamethonium, or atropine (1). We then concluded that these basal dialysate NE and Epi levels reflect noncholinergic catecholamine release. N-, P/Q-, and L-type Ca^{2+} channels may not play a major role in basal noncholinergic catecholamine release from adrenal medulla.

Methodological considerations. We administered neostigmine locally to adrenal medulla through a dialysis probe. Cholinesterase inhibitor was necessary to monitor endogenous ACh even during splanchnic nerve stimulation because released ACh is rapidly degraded by acetylcholinesterase before reaching the dialysis fiber. In the same preparation, local administration of neostigmine enhanced the dialysate catecholamine response to nerve stimulation by approximately threefold, but dialysate ACh and catecholamine responses are correlated with the stimulation frequency of splanchnic nerves in the presence of neostigmine (2). Thus dialysate ACh and catecholamine responses are likely to be correlated with the amount of Ca^{2+} influx from voltage-dependent Ca^{2+} channels even in the presence of neostigmine.

Intravenous administration of Ca^{2+} channel antagonists induced changes in heart rate or mean arterial pressure. These changes might affect ACh and catecholamine releases through a baroreflex mechanism. Moreover, these hemodynamic changes might decrease the spillover of ACh or catecholamine from adrenal medulla and affect the dialysate ACh or catecholamine concentrations (20). In our preparation, however, splanchnic nerves had been transected before control sampling, and basal dialysate catecholamine concentrations did not change before or after administration. Thus effects of these hemodynamic changes could be negligible when we considered the effects of Ca^{2+} channel antagonists on nerve stimulation-induced dialysate responses.

In conclusion, we applied dialysis technique to the adrenal medulla of anesthetized rats and investigated the effects of Ca^{2+} channel antagonists on ACh and catecholamine releases induced by electrical stimulation of splanchnic nerves. Both N- and P/Q-type Ca^{2+} channels control ACh release on preganglionic splanchnic nerve endings while L-type Ca^{2+} channels do not. L-type Ca^{2+} channels are involved in norepinephrine and epinephrine releases on chromaffin cells.

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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±2.8% versus 40.9±4.2% in controls), and this effect was completely reversed by the nonselective adenosine receptor antagonist 8-sulfophenyltheophylline (45.2±5.4%) or by an inhibitor of ecto-5′-nucleotidase (44.4±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 · 100 g^{-1} · 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 resistance[3] 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 membrane enzymes, pumps, and proteins, such as Na^+/K^+-ATPase,[9] Ca^{2+} 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% O_2 at flow rate of 1.0 to 1.5 L/min). The arterial blood pH, Po_2, and Pco_2 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 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ 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 lactate[14] 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 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 4.8 mg/mL, n=5), or 8-sulfophenyltheophylline (8-SPT) at 30 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ 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 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ 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 $\mu g \cdot kg^{-1} \cdot min^{-1}$, whereas the dose of carvedilol (1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was the minimum level that caused maximal coronary vasodilation.

In addition, propranolol was infused at 30 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ 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 $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 1.5 mg/mL, n=5) or human recombinant superoxide dismutase (SOD) (5340 IU/mg, >99% purity) at 25 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ 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 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ 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 $F_{2\alpha}$ level was measured in coronary arterial and venous blood, and the arteriovenous difference of 8-iso-prostaglandin $F_{2\alpha}$ [VAD(8-Iso-$F_{2\alpha}$)] 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 $\cdot kg^{-1} \cdot min^{-1}$, 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 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ 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 $\mu g \cdot kg^{-1} \cdot min^{-1}$ or AMP-CP at 80 $\mu g \cdot kg^{-1} \cdot min^{-1}$. 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 levels[13] have been reported previously.

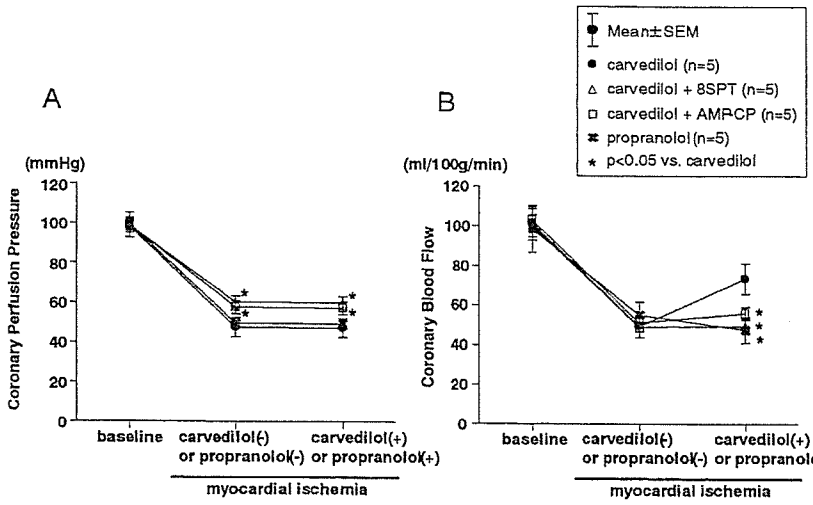

Figure 1. Effects of carvedilol on coronary hemodynamics in ischemic myocardium. A and B show CPP and CBF, respectively. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

Measurement of Infarct Size and Collateral Blood Flow

In protocol 4, the area of myocardial necrosis and the area at risk[16] 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: time flow=(tissue count)×(reference flow)/(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 mL · 100 g^{-1} · min^{-1}, (2) a heart rate >170 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±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±3.2 mm Hg and heart rate, 130.2±3.7 versus 128.0±3.3 bpm) nor FS (20.1±1.0% versus 21.5±1.0%) changed during the infusion of carvedilol. In contrast, CBF was increased (98.4±8.5 versus 112.6±9.6 mL · 100 g^{-1} · min^{-1}, $P<0.05$), as was VAD(Ado) (40.9±4.0 versus 68.6±5.5 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±5.8 mm Hg before and after 8-SPT and 99.0±3.0 versus 102.0±3.2 mm Hg before and after AMP-CP; heart rate, 132.2±6.9 versus 132.4±6.1 min^{-1} before and after 8-SPT and 131.8±4.6 versus 132.8±3.4 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

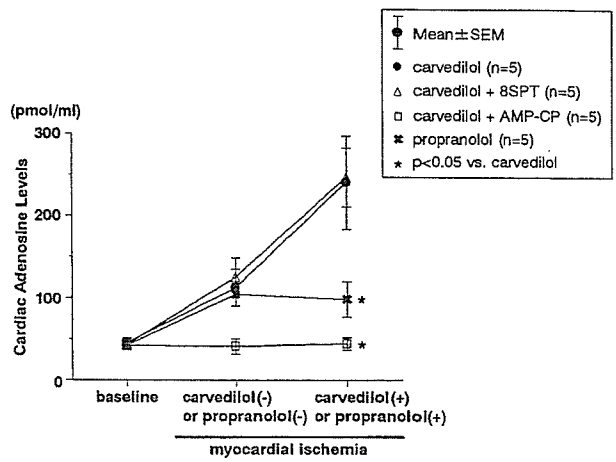

Figure 2. Changes of difference in adenosine levels between coronary venous and arterial blood [VAD(Ado)] in ischemic myocardium. Carvedilol increased VAD(Ado), which was attenuated by an ecto-5'-nucleotidase inhibitor. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

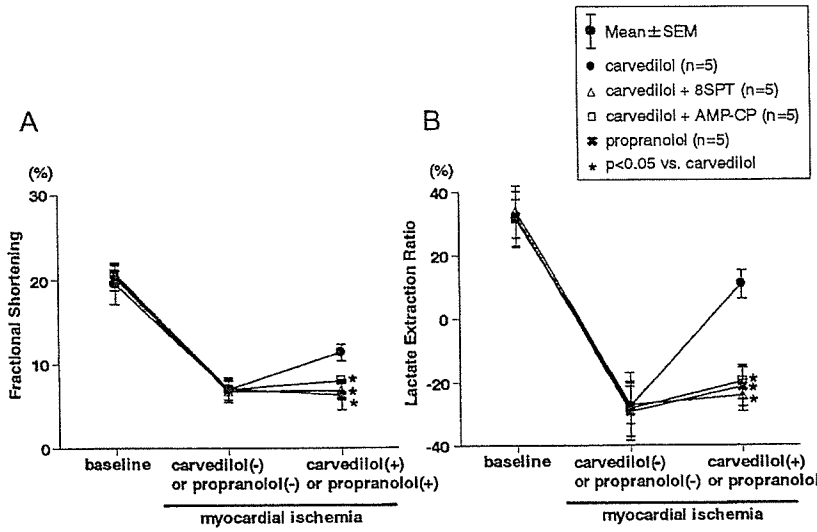

Figure 3. Changes of FS (A) and LER (B) in ischemic myocardium. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

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-$F_{2\alpha}$), 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-$F_{2\alpha}$) 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-$F_{2\alpha}$) did not increase (Figure 4C). Addition of carvedilol did not further attenuate VAD(8-Iso-$F_{2\alpha}$) 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

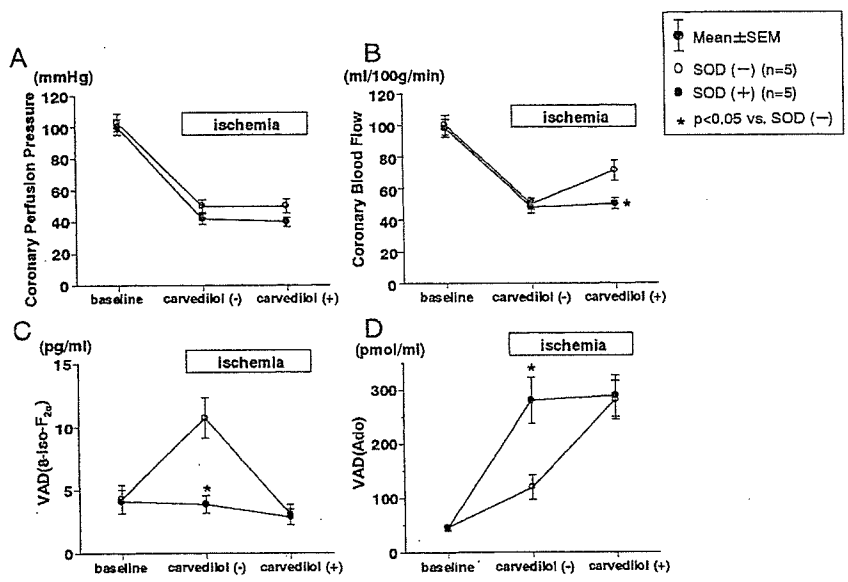

Figure 4. Changes of CPP (A), CBF (B), VAD(8-Iso-$F_{2\alpha}$) (C), and VAD(Ado) (D) in ischemic myocardium. Statistical significance was tested by ANOVA followed by Bonferroni's test.

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

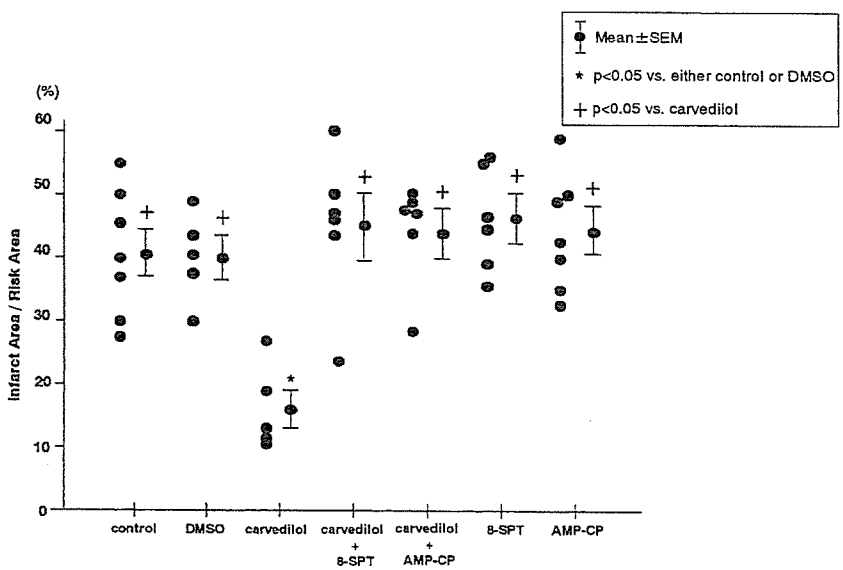

Figure 5. Infarct size as a percentage of area at risk. Infarct size was decreased in carvedilol group compared with control group, and this improvement was blocked by either 8-SPT or AMP-CP. Statistical significance was tested by ANOVA followed by Bonferroni's test.

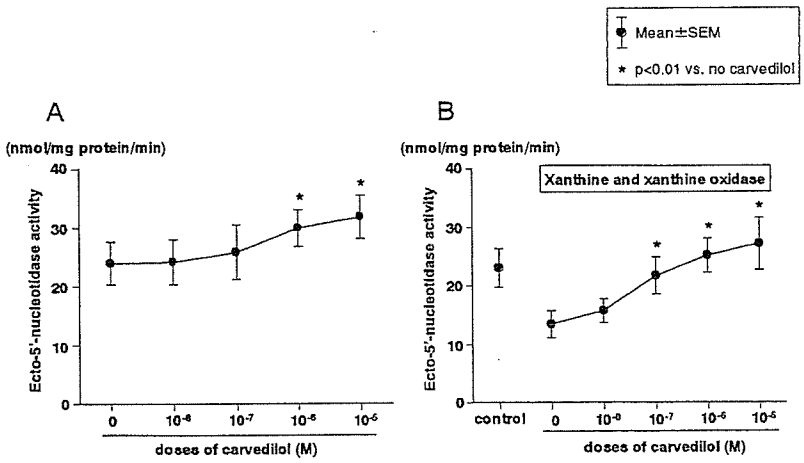

Figure 6. Ecto-5′-nucleotidase activity of HUVECs in presence or absence of carvedilol or xanthine and xanthine oxidase. Statistical significance was tested by ANOVA followed by Bonferroni's test.

increases of CBF in nonischemic and ischemic myocardium were 14.4±1.1% and 50.6±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 upregulated 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 μg/mL, and the calculated cardiac concentration of carvedilol in the present study is ≈1 μg/mL. In dogs, carvedilol at 1 and 4 μg/mL decreased blood pressure by 9% and 32%, respectively (data not shown), suggesting that the concentration of 1 μg/mL of carvedilol in canine heats 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 potently ameliorates heart failure, especially ischemic heart failure, may be related to adenosine.[12] Carvedilol may have the ability to both antagonize β-adrenoceptors 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-pteridinyl) methyl)-3,4-dihydro-2H-1,4-benzothiazine-7- carbonyl]-N-2- aminoadipic 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 ± 5.3%, $P < 0.01$). This effect was completely blunted by either the adenosine receptor antagonist 8-sulfophenyltheophylline (8-SPT) (45.0 ± 4.6% and 46.8 ± 5.8% in the 8-SPT and MX-68 + 8-SPT groups, respectively) or by the ecto-5′-nucleotidase inhibitor α,β-methylenadenosine 5′-diphosphate (AMP-CP) (44.0 ± 4.5% and 46.7 ± 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.

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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 size[9] 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-

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-aminoadipic acid[20] (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, PO_2, and PCO_2 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-sulfophenyltheophylline (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 × 10^6 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-triphenyltetrazolium 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 significant 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

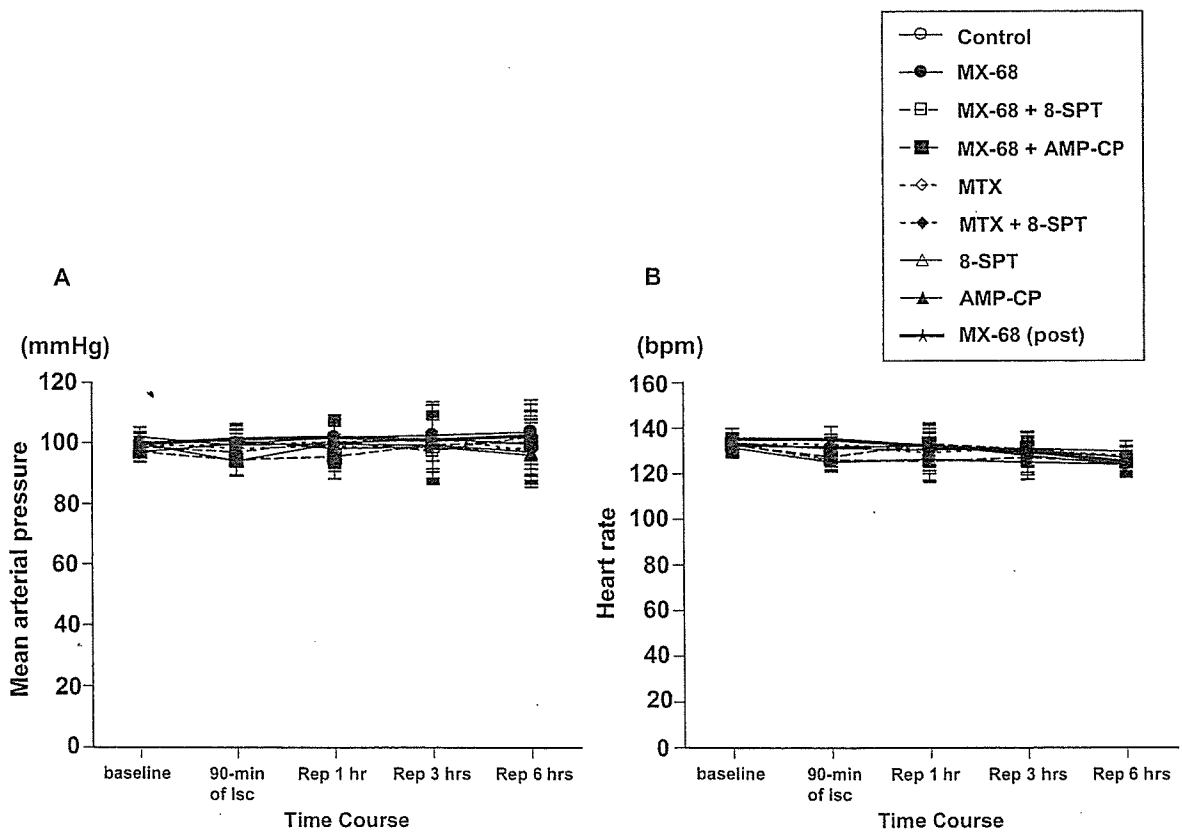

FIGURE 1. Systemic hemodynamic parameters (mean arterial pressure (A) and heart rate (B)) throughout the study. There were no significant changes of these parameters in all the 9 groups. Statistical significance was tested by ANOVA.

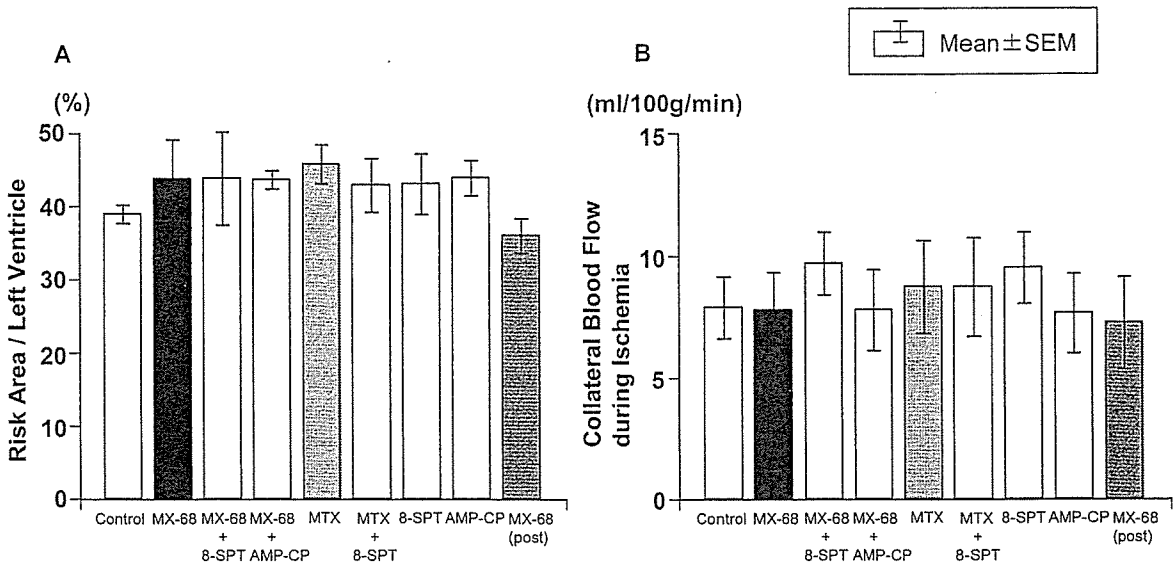

FIGURE 2. Area at risk (A) and collateral blood flow during ischemia (B) in the control group, MTX group, MX-68 group, MTX+8-SPT group, MX-68+8-SPT group, MX-68+AMP-CP group, MX-68(post) group, 8-SPT group, and AMP-CP group. There were no differences of the area at risk and collateral flow during ischemia between these groups. Statistical significance was tested by ANOVA.

blunted by infusion of either 8-SPT or AMP-CP. MTX also reduced infarct size in a similar manner to MX-68, and its protective effect was blunted by 8-SPT. Even when MX-68 was administered after the start of reperfusion, reduction of infarct size was observed to a level between that in the control group and that when MX-68 was administered before ischemia. Regression plots of infarct size versus collateral blood flow are shown in Figure 4, which indicate that the infarct size-limiting effect of either MX-68 or MTX was attributable to an adenosine-dependent mechanism.

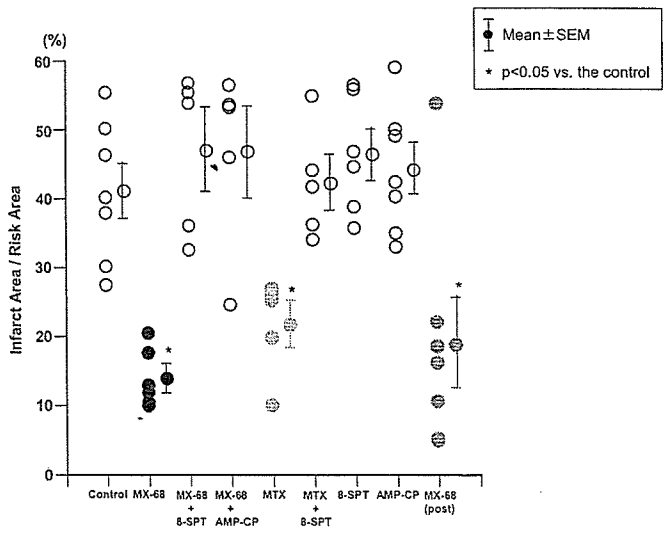

FIGURE 3. Infarct size expressed as a percentage of the area at risk. Infarct size was markedly decreased in the MTX and MX-68 groups compared with the control group, and this improvement was completely reversed by 8-SPT or AMP-CP. Statistical significance was tested by ANOVA.

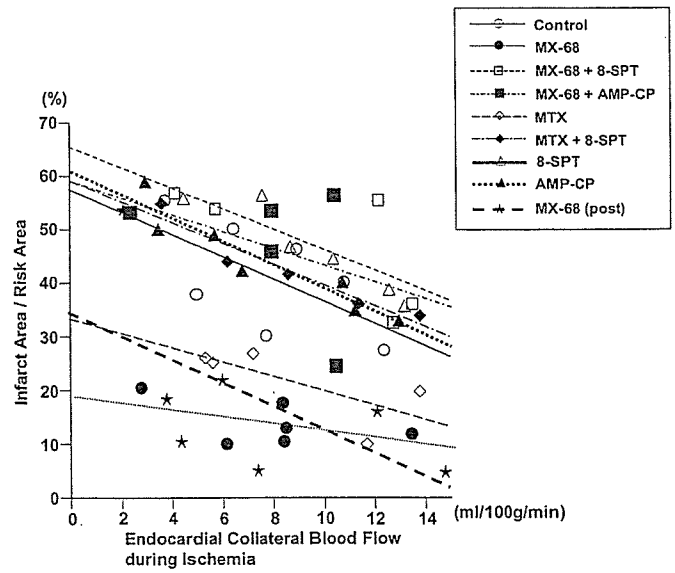

FIGURE 4. Infarct size after 90 minutes of ischemia versus regional collateral flow during ischemia. Infarct size is expressed as a percentage of the area at risk. Infarct size was markedly decreased in the MTX and MX-68 groups compared with the control group. This improvement was completely reversed by 8-SPT or AMP-CP. Statistical significance was tested by ANCOVA.

DISCUSSION

The present study demonstrated that either MTX or MX-68 could markedly reduce infarct size and that the cardioprotective effects of these agents were attributable to ecto-5′-nucleotidase- and adenosine-dependent mechanisms.

Adenosine and the Cardioprotective Effect of MTX or MX-68

Anti-inflammatory drugs such as steroids were thought to have an infarct size-limiting effect[7,8] because the pathophysiology of myocardial infarction resembles tissue inflammation and such drugs can potently ameliorate the tissue inflammatory process. However, steroids and related hormones have been variously reported to decrease infarct size, have no effect, or even increase infarct size.[4,9] On the other hand, other anti-inflammatory agents seem to be effective against ischemia/reperfusion injury. For example, 17β-estradiol is known to have an anti-inflammatory effect[10] and it markedly reduces infarct size.[11] Statins are known to improve vascular inflammation and atherosclerosis, and these drugs also reduce infarct size markedly.[12] Therefore, we cannot necessarily conclude that all anti-inflammatory drugs will be effective for ischemia/reperfusion injury, but we can suggest that these drugs have the possibility of mediating cardioprotection.

MTX and its analog MX-68 are disease-modifying antirheumatic drugs,[20] and their mechanism of action on immune cells was recently reported to be mediated via adenosine.[13] If this is the case in myocardial cells, either MTX or MX-68 would limit infarct size because adenosine markedly reduces the size of infarcts and triggers/mediates the cardioprotective effect of ischemic preconditioning.[24,25] Indeed, the present study revealed that MTX and its analog (MX-68) can ameliorate ischemia/reperfusion injury. We also showed that this action is adenosine-dependent, because the effect of either MTX or MX-68 was blunted by 8-SPT, an adenosine receptor antagonist. Accordingly, both MTX and MX-68 ameliorate ischemia/reperfusion injury via adenosine-related mechanisms.

In immune system cells, the adenosine-related action of MTX was reported to be attributable to ecto-5′-nucleotidase,[13] and this also seems to be the case in the myocardium because the effect of MX-68 and MTX was blunted by an ecto-5′-nucleotidase inhibitor or an adenosine receptor antagonist. Ecto-5′-nucleotidase produces adenosine, and adenosine inhibits norepinephrine release from presynaptic vesicles and attenuates Ca^{2+} influx into myocytes by acting on A_1 receptors and inhibitory G protein.[26,27] Adenosine also increases CBF, inhibits platelet aggregation, and inhibits leukocyte activation via A_2 receptors and stimulatory G protein.[14,16] Since factors such as an increase of norepinephrine, Ca^{2+} overload, decreased CBF, and activation of platelets and leukocytes are deleterious to the heart, control of these factors by adenosine may help to minimize ischemia/reperfusion injury. Several studies have shown that adenosine administration markedly attenuates ischemia/reperfusion injury.[3,15,17]

Role of Adenosine in the Effect of MTX or MX-68

How does MTX or MX-68 act on ecto-5′-nucleotidase? Several possibilities can be suggested. First, activation of ecto-5′-nucleotidase may occur after phosphorylation, as seen with ischemic preconditioning or treatment with phorbol ester, where activation of protein kinase C possibly leads to the phosphorylation and activation of ecto-5′-nucleotidase.[28,29] However, the in vitro activity of myocardial ecto-5′-nucleotidase was not increased by brief exposure to MTX (data not shown), whereas methoxamine and phorbol ester, which phosphorylate and activate ecto-5′-nucleotidase, both activated myocardial ecto-5′-nucleotidase in vitro.[28,29] These results suggest that MTX does not activate ecto-5′-nucleotidase via the process of phosphorylation, so a direct interaction between MTX and the active site of ecto-5′- nucleotidase may be responsible instead.

Second, MTX is reported to increase the tissue level of AICA riboside by inhibition of AICA riboside deaminase,[13] and we have previously shown that AICA riboside increases the activity of ecto-5′-nucleotidase.[19] Therefore, ecto-5′-nucleotidase may be activated when the myocardial AICA riboside is increased during administration of MTX in vivo. However, it has not been clarified how AICA riboside activates ecto-5′-nucleotidase in the heart. Since AICA riboside activates AMP deaminase and inactivates adenosine deaminase, it may also modulate the enzymes related to adenosine metabolism.[30] Accordingly, AICA riboside could increase adenosine production via activation of ecto-5′-nucleotidase, and maintain a high adenosine level by inhibiting enzymes involved in the metabolism of adenosine. In this context, there are many reports that AICA riboside is cardioprotective against ischemia/reperfusion injury via adenosine-dependent mechanisms.[16,30,31]

Clinical Relevance and Limitations

In this study, we demonstrated that both MX-68 and MTX can limit infarct size via adenosine-dependent mechanisms. It would be of interest to test the cardioprotective effect of MTX or MX-68 in the clinical setting of acute myocardial infarction with coronary revascularization, since infusion of adenosine during reperfusion has been shown to limit infarct size.[17] Furthermore, since administration of adenosine can precondition the myocardium prior to sustained ischemia,[24] treatment with MTX or MX-68 may be useful in patients who have coronary artery disease to precondition the myocardium and improve resistance to acute myocardial infarction. However, further studies are necessary to develop either MTX or MX-68 as a drug to treat acute ischemic heart disease.

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