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Hypothermia reduces ischemia- and stimulation-induced myocardial interstitial norepinephrine and acetylcholine releases

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Kawada T, Kitagawa H, Yamazaki T, Akiyama T, Kamiya A, Uemura K, Mori H, Sugimachi M. Hypothermia reduces ischemia- and stimulation-induced myocardial interstitial norepinephrine and acetylcholine releases. *J Appl Physiol* 102: 622–627, 2007. First published November 2, 2006; doi:10.1152/japplphysiol.00622.2006.—Although hypothermia is one of the most powerful modulators that can reduce ischemic injury, the effects of hypothermia on the function of the cardiac autonomic nerves in vivo are not well understood. We examined the effects of hypothermia on the myocardial interstitial norepinephrine (NE) and ACh releases in response to acute myocardial ischemia and to efferent sympathetic or vagal nerve stimulation in anesthetized cats. We induced acute myocardial ischemia by coronary artery occlusion. Compared with normothermia ($n = 8$), hypothermia at 33°C ($n = 6$) suppressed the ischemia-induced NE release [63 nM (SD 39) vs. 18 nM (SD 25), $P < 0.01$] and ACh release [11.6 nM (SD 7.6) vs. 2.4 nM (SD 1.3), $P < 0.01$] in the ischemic region. Under hypothermia, the coronary occlusion increased the ACh level from 0.67 nM (SD 0.44) to 6.0 nM (SD 6.0) ($P < 0.05$) and decreased the NE level from 0.63 nM (SD 0.19) to 0.40 nM (SD 0.25) ($P < 0.05$) in the nonischemic region. Hypothermia attenuated the nerve stimulation-induced NE release from 1.05 nM (SD 0.85) to 0.73 nM (SD 0.73) ($P < 0.05$, $n = 6$) and ACh release from 10.2 nM (SD 5.1) to 7.1 nM (SD 3.4) ($P < 0.05$, $n = 5$). In conclusion, hypothermia attenuated the ischemia-induced NE and ACh releases in the ischemic region. Moreover, hypothermia also attenuated the nerve stimulation-induced NE and ACh releases. The Bezold-Jarisch reflex evoked by the left anterior descending coronary artery occlusion, however, did not appear to be affected under hypothermia.

vagal nerve; sympathetic nerve; cardiac microdialysis; cats

HYPOTHERMIA IS ONE OF THE most powerful modulators that can reduce ischemic injury in the central nervous system, heart, and other organs. The general consensus is that hypothermia induces a hypometabolic state in tissues and balances energy supply and demand (25). With respect to the myocardial ischemia, the size of a myocardial infarction correlates with temperature (6), and mild hypothermia can protect the myocardium against acute ischemic injury (9). The effects of hypothermia on the function of the cardiac autonomic nerves in terms of neurotransmitter releases, however, are not fully understood. Because autonomic neurotransmitters such as norepinephrine (NE) and ACh directly impinge on the myocardium, they would be implicated in the cardioprotection by hypothermia.

In previous studies from our laboratory, Kitagawa et al. (16) demonstrated that hypothermia attenuated the nonexocytotic NE release induced pharmacologically by ouabain, tyramine, or cyanide. Kitagawa et al. (15) also demonstrated that hypothermia attenuated the exocytotic NE release in response to vena cava occlusion or to local administration of high K^+. The effects of hypothermia on the ischemia-induced myocardial interstitial NE release, however, were not examined in those studies. In addition, the effects of hypothermia on the ischemia-induced myocardial interstitial ACh release have never been examined. Because both sympathetic and parasympathetic nerves control the heart, simultaneous monitoring of the myocardial interstitial releases of NE and ACh (14, 31) would help integrative understanding of the autonomic nerve terminal function under hypothermia in conjunction with acute myocardial ischemia.

In the present study, the effects of hypothermia on the ischemia-induced and nerve stimulation-induced myocardial interstitial neurotransmitter releases were examined. We implanted a dialysis probe into the left ventricular free wall of anesthetized cats and measured dialysate NE and ACh levels as indexes of neurotransmitter outputs from the cardiac sympathetic and vagal nerve terminals, respectively. Based on our laboratory's previous results (15, 16), we hypothesized that hypothermia would attenuate the neurotransmitter releases in response to acute myocardial ischemia and to electrical nerve stimulation.

MATERIALS AND METHODS

Surgical Preparation and Protocols

Animals were cared for in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, approved by the Physiological Society of Japan. All protocols were reviewed and approved by the Animal Subjects Committee of National Cardiovascular Center. Adult cats were anesthetized via an intraperitoneal injection of pentobarbital sodium (30–35 mg/kg) and ventilated mechanically through an endotracheal tube with oxygen-enriched room air. The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 $mg \cdot kg^{-1} \cdot h^{-1}$) through a catheter inserted from the right femoral vein. Mean arterial pressure (MAP) was measured using a pressure transducer connected to a catheter inserted from the right femoral artery. Heart rate (HR) was determined from an electrocardiogram.

Protocol 1: acute myocardial ischemia. We examined the effects of hypothermia on the ischemia-induced myocardial interstitial releases of NE and ACh. The heart was exposed by partially removing the left fifth and/or sixth rib. A dialysis probe was implanted transversely into

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the anterolateral free wall of the left ventricle perfused by the left anterior descending coronary artery (LAD) to monitor myocardial interstitial NE and ACh levels in the ischemic region during occlusion of the LAD (13). Another dialysis probe was implanted transversely into the posterior free wall of the left ventricle perfused by the left circumflex coronary artery to monitor myocardial interstitial NE and ACh levels in a nonischemic region. Heparin sodium (100 U/kg) was administered intravenously to prevent blood coagulation. Animals were divided into a normothermic group ($n = 8$) and a hypothermic group ($n = 6$). In the hypothermic group, surface cooling with ice bags was performed until the esophageal temperature decreased to 33°C (15, 16). A stable hypothermic condition was obtained within ~2 h. In each group, we occluded the LAD for 60 min and examined changes in the myocardial interstitial NE and ACh levels in the ischemic region (i.e., the LAD region) and nonischemic region (i.e., the left circumflex coronary artery region). Fifteen-minute dialysate samples were obtained during the preocclusion baseline condition and during the periods of 0–15, 15–30, 30–45, and 45–60 min of the LAD occlusion.

Protocol 2: sympathetic stimulation. We examined the effects of hypothermia on the sympathetic nerve stimulation-induced myocardial interstitial NE release ($n = 6$). A dialysis probe was implanted transversely into the anterolateral free wall of the left ventricle. The bilateral cardiac sympathetic nerves originating from the stellate ganglia were exposed through a second intercostal space and sectioned. The cardiac end of each sectioned nerve was placed on a bipolar platinum electrode for sympathetic stimulation (5 Hz, 10 V, 1-ms pulse duration). The electrodes and nerves were covered with mineral oil to provide insulation and prevent desiccation. A 4-min dialysate sample was obtained during the sympathetic stimulation under the normothermic condition. Thereafter, hypothermia was introduced using the same cooling procedure as in *protocol 1*, and a second 4-min dialysate sample was obtained during the sympathetic stimulation.

Protocol 3: vagal stimulation. We examined the effects of hypothermia on the vagal nerve stimulation-induced ACh release ($n = 5$). A dialysis probe was implanted transversely into the anterolateral free wall of the left ventricle. The bilateral vagi were exposed through a midline cervical incision and sectioned at the neck. The cardiac end of each sectioned nerve was placed on a bipolar platinum electrode for vagal stimulation (20 Hz, 10 V, 1-ms pulse duration). To prevent severe bradycardia and cardiac arrest, which can be induced by the vagal stimulation, the heart was paced at 200 beats/min using pacing wires attached to the apex of the heart during the stimulation period. A 4-min dialysate sample was obtained during the vagal stimulation under the normothermic condition. Thereafter, hypothermia was introduced using the same cooling procedure as in *protocol 1*, and a second 4-min dialysate sample was obtained during the vagal stimulation.

Because of the relatively intense stimulation of the sympathetic or vagal nerve, the stimulation period in *protocols 2* and *3* was limited to 4 min to minimize gradual waning of the stimulation effects. At the end of the experiment, the animals were killed by increasing the depth of anesthesia with an overdose of pentobarbital sodium. We then confirmed that the dialysis probes had been threaded in the middle layer of the left ventricular myocardium.

Dialysis Technique

The dialysate NE and ACh concentrations were measured as indexes of myocardial interstitial NE and ACh levels, respectively. The materials and properties of the dialysis probe have been described previously (2, 3). Briefly, we designed a transverse dialysis probe. A dialysis fiber (13-mm length, 310-μm outer diameter, 200-μm inner diameter; PAN-1200, 50,000 molecular weight cutoff; Asahi Chemical) was connected at both ends to polyethylene tubes (25-cm length, 500-μm outer diameter, 200-μm inner diameter). The dialysis probe was perfused with Ringer solution containing a cholinesterase inhibitor eserine (10^{-4} M) at a rate of 2 μl/min. We started dialysate sampling from 2 h after the implantation of the dialysis probe(s), when the dialysate NE and ACh concentrations had reached steady states. The actual dialysate sampling was delayed by 5 min from the collection period to account for the dead space volume between the semipermeable membrane and the sample tube. Each sample was collected in a microtube containing 3 μl of HCl to prevent amine oxidation. The dialysate ACh concentration was measured directly by HPLC with electrochemical detection (Eicom). The in vitro recovery rate of ACh was ~70%. With the use of a criterion of signal-to-noise ratio of higher than three, the detection limit for ACh was 3 pg per injection. The dialysate NE concentration was measured by another HPLC-electrochemical detection system after the removal of interfering compounds by an alumina procedure. The in vitro recovery rate of NE was ~55%. With the use of a criterion of signal-to-noise ratio of higher than three, the detection limit for NE was 200 fg per injection.

Statistical Analysis

All data are presented as means and SD values. For *protocol 1*, we performed two-way repeated-measures ANOVA using hypothermia as one factor and the dialysate sampling periods (the effects of ischemia) as the other factor. For *protocols 2* and *3*, we compared stimulation-induced releases of NE and ACh before and during hypothermia using a paired *t*-test. For all of the statistics, the difference was considered significant when $P < 0.05$.

RESULTS

Figure 1*A* illustrates changes in myocardial interstitial NE levels in the ischemic region during LAD occlusion obtained from *protocol 1*. The *inset* shows the magnified ordinate for the

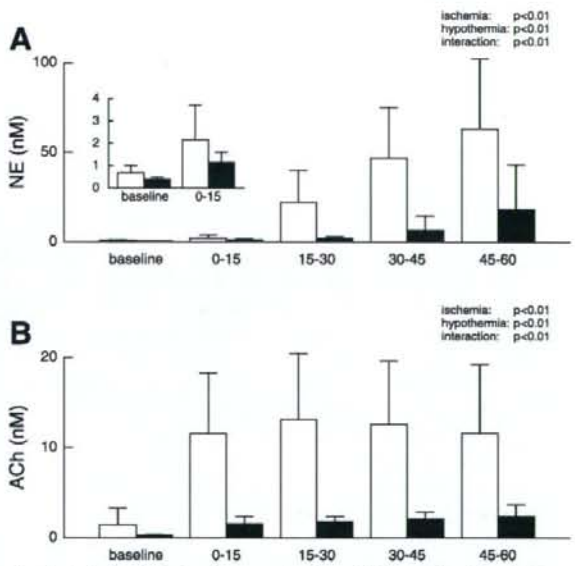

Fig. 1. *A*: ischemia-induced myocardial interstitial norepinephrine (NE) release in the ischemic region. Acute myocardial ischemia caused a progressive increase in the level of myocardial interstitial NE. Hypothermia attenuated the ischemia-induced NE release. *Inset*: magnified ordinate for the baseline and the 0- to 15-min period of ischemia. *B*: ischemia-induced myocardial interstitial ACh release in the ischemic region. Acute myocardial ischemia increased the myocardial interstitial ACh levels. Hypothermia attenuated the ischemia-induced ACh release. Open bars: normothermia; solid bars: hypothermia.

baseline and the 0- to 15-min period of ischemia. In the normothermic group (open bars), the LAD occlusion caused an ~94-fold increase in the NE level during the 45- to 60-min interval. In the hypothermic group (solid bars), the LAD occlusion caused an ~45-fold increase in the NE level during the 45- to 60-min interval. Compared with normothermia, hypothermia suppressed the baseline NE level to ~59% and the NE level during the 45- to 60-min period to ~29%. Statistical analysis indicated that the effects of both hypothermia and ischemia on the NE release were significant, and the interaction between hypothermia and ischemia was also significant.

Figure 1*B* illustrates changes in myocardial interstitial ACh levels in the ischemic region during the LAD occlusion. In both the normothermic (open bars) and hypothermic (solid bars) groups, the LAD occlusion caused an approximately eightfold increase in the ACh level during the 45- to 60-min interval. Compared with normothermia, however, hypothermia suppressed both the baseline ACh level and the ACh level during the 45- to 60-min period of ischemia to ~20%. Statistical analysis indicated that the effects of both hypothermia and ischemia on the ACh release were significant, and the interaction between hypothermia and ischemia was also significant.

Figure 2*A* illustrates changes in myocardial interstitial NE levels in the nonischemic region during the LAD occlusion. Note that scale of the ordinate is only one-hundredth of that in Fig. 1*A*. The LAD occlusion decreased the NE level in the normothermic group (open bars); the NE level during the 45- to 60-min interval was ~59% of the baseline level. The LAD occlusion also decreased the NE level in the hypothermic group (solid bars); the NE level during the 45- to 60-min interval was ~64% of the baseline level. Although the LAD occlusion resulted in a decrease in the NE level under both conditions, the NE level under hypothermia was nearly twice that measured under normothermia. The statistical analysis indicated that the effects of both hypothermia and ischemia on the NE release were significant, whereas the interaction between hypothermia and ischemia was not significant.

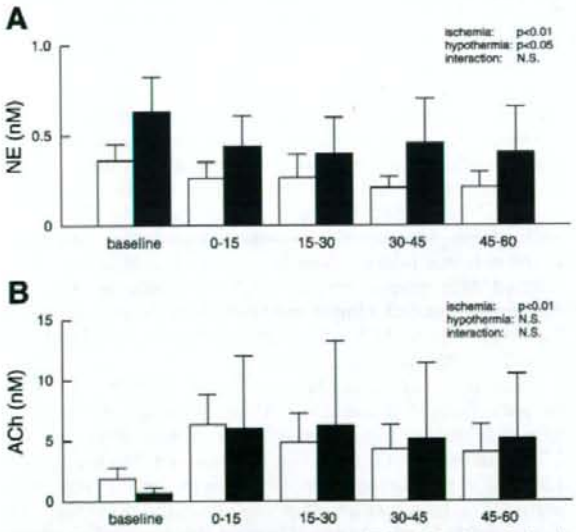

Fig. 2. *A*: changes in the myocardial interstitial NE levels in the nonischemic region. Acute myocardial ischemia decreased the level of myocardial interstitial NE from the baseline level. Hypothermia increased the myocardial interstitial NE levels in the nonischemic region. *B*: changes in the myocardial interstitial ACh levels in the nonischemic region. Acute myocardial ischemia increased the myocardial interstitial ACh level. Hypothermia did not attenuate the increasing response of ACh to the left anterior descending coronary artery occlusion. Open bars: normothermia; solid bars: hypothermia. NS, not significant.

Table 1. *Mean arterial pressure during acute myocardial ischemia obtained in protocol 1*

	Baseline	5 min	15 min	30 min	45 min	60 min
Normothermia	108 (23)	102 (28)	101 (24)	101 (20)	102 (21)	102 (21)
Hypothermia	108 (11)	80 (17)	87 (10)	85 (10)	86 (10)	91 (11)

Values are means (SD) (in mmHg) obtained during preocclusion baseline period and 5-, 15-, 30-, 45-, and 60-min periods of coronary artery occlusion. Ischemia: $P < 0.01$; hypothermia: not significant; interaction: $P < 0.01$.

Figure 2*B* illustrates changes in myocardial interstitial ACh levels in the nonischemic region during the LAD occlusion. The LAD occlusion caused an ~3.4-fold increase in the ACh level during the 0- to 15-min interval in the normothermic group (open bars). The LAD occlusion caused an approximately ninefold increase in the ACh level during the 0- to 15-min interval in the hypothermic group (solid bars). These effects of ischemia on the ACh release were statistically significant. Although hypothermia seemed to attenuate the baseline ACh level, the overall effects of hypothermia on the ACh level were insignificant.

Tables 1 and 2 summarize the MAP and HR data, respectively, obtained in *protocol 1*. Acute myocardial ischemia significantly reduced MAP ($P < 0.01$) and HR ($P < 0.01$). Hypothermia did not affect MAP but did decrease HR ($P < 0.01$). The interaction between ischemia and hypothermia was significant for MAP but not for HR by the two-way repeated-measures ANOVA.

For *protocol 2*, hypothermia significantly attenuated the sympathetic stimulation-induced NE release to ~70% of the level observed during normothermia (Fig. 3*A*). Under normothermia, the sympathetic stimulation increased MAP from 114 mmHg (SD 27) to 134 mmHg (SD 33) ($P < 0.01$) and HR from 147 beats/min (SD 9) to 207 beats/min (SD 5) ($P < 0.01$). Under hypothermia, the sympathetic stimulation increased MAP from 117 mmHg (SD 11) to 136 mmHg (SD 22) ($P < 0.05$) and HR from 125 beats/min (SD 16) to 164 beats/min (SD 10) ($P < 0.01$).

For *protocol 3*, hypothermia significantly attenuated the vagal stimulation-induced ACh release to ~70% of the level observed during normothermia (Fig. 3*B*). Hypothermia did not change MAP [117 mmHg (SD 18) vs. 118 mmHg (SD 27)] but

Table 2. *Heart rate during acute myocardial ischemia obtained in protocol 1*

	Baseline	5 min	15 min	30 min	45 min	60 min
Normothermia	183 (26)	160 (18)	163 (16)	163 (18)	166 (20)	165 (21)
Hypothermia	146 (25)	116 (19)	113 (19)	126 (39)	112 (20)	97 (31)

Values are means (SD) (in beats/min) obtained during preocclusion baseline period and 5-, 15-, 30-, 45-, and 60-min periods of coronary artery occlusion. Ischemia: $P < 0.01$; hypothermia: $P < 0.01$; interaction: not significant.

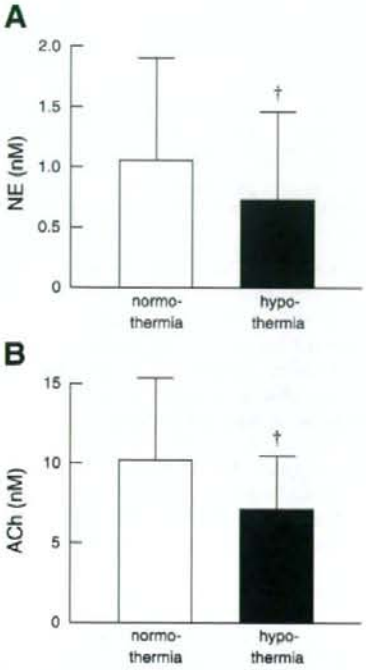

Fig. 3. *A*: efferent sympathetic nerve stimulation-induced release of myocardial interstitial NE before and during hypothermia. †Hypothermia significantly attenuated the stimulation-induced NE release. *B*: efferent vagal nerve stimulation-induced release of myocardial interstitial ACh before and during hypothermia. †Hypothermia significantly attenuated the stimulation-induced ACh release.

did decrease HR from 202 beats/min (SD 24) to 179 beats/min (SD 15) ($P < 0.05$) during the prestimulation, unpaced condition. MAP during the stimulation was 105 mmHg (SD 19) under normothermia and 93 mmHg (SD 33) under hypothermia.

DISCUSSION

A cardiac microdialysis is a powerful tool to estimate neurotransmitter levels in the myocardial interstitium in vivo (2, 3, 14, 19, 20, 31). The present study demonstrated that hypothermia significantly attenuated the myocardial interstitial releases of NE and ACh in the ischemic region during the LAD occlusion. In contrast, the increasing response in the ACh level from its baseline level and the decreasing response in the NE level from its baseline level observed in the nonischemic region were maintained under hypothermia. To our knowledge, this is the first report showing the effects of hypothermia on the myocardial interstitial releases of NE and ACh during acute myocardial ischemia in vivo. In addition, the present study showed that hypothermia significantly attenuated nerve stimulation-induced myocardial interstitial NE and ACh releases in vivo.

Effects of Hypothermia on Ischemia-induced NE and ACh Releases in the Ischemic Region

Acute myocardial ischemia causes energy depletion, which leads to myocardial interstitial NE release in the ischemic region (Fig. 1*A*). The NE release can be classified as exocytotic or nonexocytotic (18, 24). Exocytotic release indicates NE release from synaptic vesicles, which normally occurs in response to nerve discharge and subsequent Ca^{2+} influx through voltage-dependent Ca^{2+} channels. On the other hand, nonexocytotic release indicates NE release from the axoplasm, such as that mediated by a reverse transport through the NE transporter. A neuronal uptake blocker, desipramine, can suppress the ischemia-induced NE release (19, 24). Whereas exocytotic release contributes to the ischemia-induced NE release in the initial phase of ischemia (within ~20 min), carrier-mediated nonexocytotic release becomes predominant as the ischemic period is prolonged (1). Hypothermia significantly attenuated the ischemia-induced NE release (Fig. 1*A*). The NE level during the 45- to 60-min period of ischemia under hypothermia was ~20% of that obtained under normothermia. The NE uptake transporter is driven by the Na^+ gradient across the cell membrane (23). The loss of the Na^+ gradient due to ischemia causes NE to be transported out of the cell by reversing the action of the NE transporter. Hypothermia inhibits the action of the NE transporter and also suppresses the intracellular Na^+ accumulation (8), thereby reducing nonexocytotic NE release during ischemia. The present results are in line with an in vitro study that showed hypothermia suppressed nonexocytotic NE release induced by deprivation of oxygen and glucose (30). The present results are also consistent with a previous study from our laboratory that showed hypothermia attenuated the nonexocytotic NE release induced by ouabain, tyramine, or cyanide (16).

Acute myocardial ischemia increases myocardial interstitial ACh level in the ischemic region, as reported previously (Fig. 1*B*) (13). The level of ischemia-induced ACh release during 0- to 15-, 15- to 30-, 30- to 45-, or 45- to 60-min period of ischemia is comparable to that evoked by 4-min electrical stimulation of the bilateral vagi (Fig. 3*B*). Compared with the normothermic condition, hypothermia significantly attenuated the ischemia-induced myocardial interstitial release of ACh in the ischemic region. Our laboratory's previous study indicated that intracellular Ca^{2+} mobilization is essential for the ischemia-induced release of ACh (13). Hypothermia may have prevented the Ca^{2+} overload, thereby reducing the ischemia-induced ACh release. Alternatively, hypothermia may reduce the extent of the ischemic injury, which in turn suppressed the ischemia-induced ACh release. Because ACh has protective effects on the cardiomyocytes against ischemia (11), the suppression of ischemia-induced ACh release during hypothermia itself may be unfavorable for cardioprotection.

There is considerable controversy regarding the cardioprotective effects of β-adrenergic blockade during severe ischemia, with studies demonstrating a reduction of infarct size (10, 17) or no effects (7, 27). The β-adrenergic blockade seems effective to protect the heart only when the heart is reperfused within a certain period after the coronary occlusion. The β-adrenergic blockade would reduce the myocardial oxygen consumption through the reduction of HR and ventricular contractility and delay the progression of ischemic injury. Hence the infarct size might be reduced when the heart is reperfused before the ischemic damage becomes irreversible. The ischemia-induced NE release reached nearly 100 times the baseline NE level under normothermia (Fig. 1*A*), which by far exceeded the NE level attained by electrical stimulation of the

bilateral stellate ganglia (Fig. 3*A*). Because high NE levels have cardiotoxic effects (22), ischemia-induced NE release might aggravate the ischemic injury. However, catecholamine depletion by a reserpine treatment fails to reduce the infarct size (26, 29), throwing a doubt on the involvement of catecholamine toxicity in the progression of myocardial damage during ischemia. It is, therefore, most likely that the hypothermia-induced reductions in NE and ACh are the result of reduced myocardial damage or a direct effect on nerve endings.

Van den Doel et al. (28) showed that hypothermia does not abolish necrosis, but rather delays necrosis during sustained ischemia, so that hypothermia protected against infarction produced by a 30-min occlusion but not against infarction produced by a 60-min occlusion in the rat heart. At the same time, they mentioned that hypothermia was able to reduce the infarct size after a 60-min coronary occlusion in the dog, possibly because of the significant collateral flow in the canine hearts. Because the feline hearts are similar to the canine hearts in that they have considerable collateral flow compared with the rat hearts (21), hypothermia should have protected the feline heart against the 60-min coronary occlusion in the present study.

Effects of Hypothermia on the NE and ACh Releases in the Nonischemic Region and on the Electrical Stimulation-induced NE and ACh Releases

The NE and ACh levels in the nonischemic region may reflect the sympathetic and parasympathetic drives to this region. As an example, myocardial interstitial ACh levels increase during activations of the arterial baroreflex and the Bezold-Jarisch reflex (14). In the present study, acute myocardial ischemia decreased the NE level from its baseline level, whereas it increased the ACh level from its baseline level (Fig. 2). Ischemia also decreased MAP and HR (Tables 1 and 2), suggesting that the Bezold-Jarisch reflex was induced by the LAD occlusion under both normothermia and hypothermia. Taking into account the fact that electrical stimulation-induced ACh release was attenuated to ~70% (Fig. 3), similar ACh levels during ischemia imply the enhancement of the parasympathetic outflow via the Bezold-Jarisch reflex under hypothermia. These results are in line with the study by Zheng et al. (32), where pulmonary chemoreflex-induced bradycardia was maintained under hypothermia. Hypothermia increased the NE level in the nonischemic region, suggesting that sympathetic drive to this region also increased. Hypothermic stress is known to cause sympathetic activation, accompanying increases in MAP, HR, plasma NE, and epinephrine levels (4). In the present study, because the effect of hypothermia on MAP was insignificant (Table 1) and HR decreased under hypothermia (Table 2), the sympathetic activation observed in the nonischemic region might have been regional and not systemic.

Hypothermia attenuated the releases of NE and ACh in response to respective nerve stimulation to ~70% of that observed under normothermia (Fig. 3). The suppression of the exocytotic NE release by hypothermia is consistent with a previous study from our laboratory, where hypothermia attenuated the myocardial interstitial NE release in response to vena cava occlusion or to a local high K^+ administration (15). The suppression of NE release by hypothermia is consistent with an in vitro study by Kao and Westhead (12) in which catecholamine secretion from adrenal chromaffin cells induced by elevated K^+ levels increased as the temperature increased from 4 to 37°C. On the other hand, because hypothermia inhibits the neuronal NE uptake, the NE concentration at the synaptic cleft is expected to be increased if the level of NE release remains unchanged. Actually, Vizi (30) demonstrated that hypothermia increased NE release in response to field stimulation in vitro. In the present study, however, the suppression of NE release might have canceled the potential accumulation of NE due to NE uptake inhibition. The present study also demonstrated that the ACh release was suppressed by hypothermia. In the rat striatum, hypothermia decreases the extracellular ACh concentration and increases the choline concentration (5). Hypothermia may inhibit a choline uptake transporter in the same manner as it inhibits a NE uptake transporter. The inhibition of the choline transporter by hypothermia may have hampered the replenishment of the available pool of ACh and thereby contributed to the suppression of the stimulation-induced ACh release.

Limitations

In *protocol 1*, because we did not measure the infarct size in the present study, the degree of myocardial protection by hypothermia was undetermined. Whether the reduction of ischemia-induced neurotransmitter release correlates with the reduction of infact size requires further investigations. In *protocols 2* and *3*, baseline NE and ACh levels were not measured. The reduction of stimulation-induced NE and ACh release by hypothermia might be partly due to the reduction of baseline NE and ACh levels. However, because transection of the stellate ganglia (31) or vagi (3) reduces the baseline NE and ACh levels, changes in the baseline NE and ACh levels by hypothermia in *protocols 2* and *3* could not be as large as those observed under innervated conditions in *protocol 1* (Figs. 1 and 2).

In conclusion, hypothermia attenuated the ischemia-induced releases of NE and ACh in the ischemic region to ~30 and 20% of those observed under normothermia, respectively. Hypothermia also attenuated the nerve stimulation-induced releases of NE and ACh to ~70% of those observed during normothermia. In contrast, hypothermia did not affect the decreasing response in the NE level and the increasing response in the ACh level in the nonischemic region, suggesting that the Bezold-Jarisch reflex evoked by the LAD occlusion was maintained.

GRANTS

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Angiotensin II attenuates myocardial interstitial acetylcholine release in response to vagal stimulation

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Kawada T, Yamazaki T, Akiyama T, Li M, Zheng C, Shishido T, Mori H, Sugimachi M. Angiotensin II attenuates myocardial interstitial acetylcholine release in response to vagal stimulation. *Am J Physiol Heart Circ Physiol* 293: H2516–H2522, 2007. First published July 20, 2007; doi:10.1152/ajpheart.00424.2007.—Although ANG II exerts a variety of effects on the cardiovascular system, its effects on the peripheral parasympathetic neurotransmission have only been evaluated by changes in heart rate (an effect on the sinus node). To elucidate the effect of ANG II on the parasympathetic neurotransmission in the left ventricle, we measured myocardial interstitial ACh release in response to vagal stimulation (1 ms, 10 V, 20 Hz) using cardiac microdialysis in anesthetized cats. In a control group ($n = 6$), vagal stimulation increased the ACh level from 0.85 ± 0.03 to 10.7 ± 1.0 (SE) nM. Intravenous administration of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ suppressed the stimulation-induced ACh release to 7.5 ± 0.6 nM ($P < 0.01$). In a group with pretreatment of intravenous ANG II receptor subtype 1 (AT_1 receptor) blocker losartan (10 mg/kg, $n = 6$), ANG II was unable to inhibit the stimulation-induced ACh release (8.6 ± 1.5 vs. 8.4 ± 1.7 nM). In contrast, in a group with local administration of losartan (10 mM, $n = 6$) through the dialysis probe, ANG II inhibited the stimulation-induced ACh release (8.0 ± 0.8 vs. 5.8 ± 1.0 nM, $P < 0.05$). In conclusion, intravenous ANG II significantly inhibited the parasympathetic neurotransmission through AT_1 receptors. The failure of local losartan administration to nullify the inhibitory effect of ANG II on the stimulation-induced ACh release indicates that the site of this inhibitory action is likely at parasympathetic ganglia rather than at postganglionic vagal nerve terminals.

cardiac microdialysis; cats; losartan

ANG II HAS a variety of effects on the cardiovascular system (22): it acts on the vascular beds to increase peripheral vascular resistance and also on the adrenal cortex to cause volume retention. These direct effects of ANG II contribute to the maintenance of arterial pressure (AP). Aside from these direct effects, ANG II has been shown to modulate the sympathetic nervous system both centrally (7, 9) and peripherally (10). With respect to the sympathetic regulation in the heart, however, exogenous ANG II does not facilitate stimulation- and ischemia-induced norepinephrine release in the porcine left ventricle (18). Compared with a number of reports on the sympathetic system, only a few reports are available as to the effects of ANG II on the parasympathetic system. In 1982, Potter (23) demonstrated that ANG II (5–10 μg iv, body wt not reported) inhibited bradycardia induced by vagal stimulation in dogs. In that study, administration of ACh reduced the heart rate to an identical degree in the presence or absence of ANG II, suggesting that the inhibition of bradycardia by ANG II was attributable to the inhibition of the ACh release from the vagal nerve terminals. In contrast, Andrews et al. (3) reported that ANG II (500 ng/kg iv) did not inhibit bradycardia induced by vagal stimulation in ferrets. In a rat heart failure model, ANG II receptor subtype 1 (AT_1 receptor) antagonist losartan enhanced the bradycardic response to vagal stimulation (5). In pithed rats, an angiotensin-converting enzyme (ACE) inhibitor captopril also enhanced the bradycardic response to vagal stimulation (25, 26). In all of these studies, changes in the heart rate were used as a functional measurement of peripheral vagal function because of the difficulty in measuring the ACh release in the in vivo heart. Accordingly, whether ANG II affects the vagal control over the ventricle remains unknown. The aim of the present study was to examine the effect of ANG II on the vagal stimulation-induced ACh release in the left ventricular myocardium by measuring the interstitial ACh levels directly using a cardiac microdialysis technique (1, 13–15). We also explored the possible sites of action for the effect of ANG II on the stimulation-induced ACh release by administering losartan systemically from the femoral vein or locally through the dialysis fiber. Because ACh has a protective effect on the ischemic myocardium (12, 24, 29), elucidating the effect of ANG II on the ACh release in the ventricle would be helpful to understand the mechanism of ACE inhibitor or AT_1 receptor antagonist for the treatment of heart diseases (16, 17).

MATERIALS AND METHODS

Surgical Preparation

Animal care was provided in strict accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences* approved by the Physiological Society of Japan. All protocols were approved by the Animal Subject Committee of the National Cardiovascular Center. Twenty eight adult cats weighing from 1.9 to 4.9 kg were anesthetized using an intraperitoneal injection of pentobarbital sodium (30–35 mg/kg) and were then ventilated mechanically with room air mixed with oxygen. The depth of anesthesia was maintained by a continuous intravenous infusion of pentobarbital sodium (1–2 $mg \cdot kg^{-1} \cdot h^{-1}$) through a catheter inserted in the right femoral vein. Systemic AP was monitored by a catheter inserted in the right femoral artery. Heart rate was determined from an

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electrocardiogram using a cardiotachometer. Esophageal temperature of the animal, measured using a thermometer (CTM-303; TERUMO), was maintained at ~37°C using a heating pad and a lamp. Both vagal nerves were exposed and sectioned bilaterally through a midline cervical incision. With the animal in the lateral position, we resected the left fifth and sixth ribs to approach the heart. After the incision of the pericardium, the heart was suspended in a pericardial cradle. Stainless steel wires were attached to the apex and the posterior wall of the left ventricle to pace the heart. Using a fine guiding needle, we implanted a dialysis probe transversely through the anterolateral free wall of the left ventricle. Next, we attached a pair of bipolar platinum electrodes to the cardiac end of each sectioned vagal nerve. The nerves and electrodes were covered in warmed mineral oil for insulation. We gave heparin sodium (100 U/kg) intravenously to prevent blood coagulation. At the end of the experiment, postmortem examination confirmed that the semipermeable membrane of the dialysis probe had been implanted in the left ventricular myocardium.

Dialysis Technique

The materials and properties of the dialysis probe have been described previously (1). Briefly, we designed a transverse dialysis probe in which a dialysis fiber of semipermeable membrane (13 mm length, 310 μm outer diameter, 200 μm inner diameter; PAN-1200, 50,000 mol wt cutoff; Asahi Chemical) was attached at both ends to polyethylene tubes (25 cm length, 500 μm outer diameter, 200 μm inner diameter). The dialysis probe was perfused at a rate of 2 μl/min with Ringer solution containing the cholinesterase inhibitor physostigmine (100 μM). Experimental protocols were started 2 h after implanting the dialysis probe when the ACh concentration in the dialysate reached a steady state. ACh concentrations in the dialysate were measured by an HPLC system with electrochemical detection (Eicom, Kyoto, Japan).

Figure 1 schematizes the three original protocols and two supplemental protocols utilized in the present study. The hatched rectangle indicates the baseline sampling, whereas the solid rectangles indicate the sampling during the 10-min vagal stimulation period (1 ms, 10 V, 20 Hz) in each protocol. The stimulus was set supramaximal to most easily delineate the possible effect of ANG II on myocardial interstitial ACh release. In all of the vagal stimulation periods, we paced the heart at 200 beats/min to avoid the difference in heart rate affecting the vagal stimulation-induced ACh release (14). For baseline sampling periods, we paced the heart at 200 beats/min when spontaneous heart rate was <200 beats/min.

Protocol 1 (n = 6). We examined the effects of intravenous administration of ANG II on vagal stimulation-induced myocardial ACh release. We collected a dialysate sample under baseline conditions. We then stimulated the vagal nerve and paced the heart for 10 min and collected a dialysate sample during the stimulation period

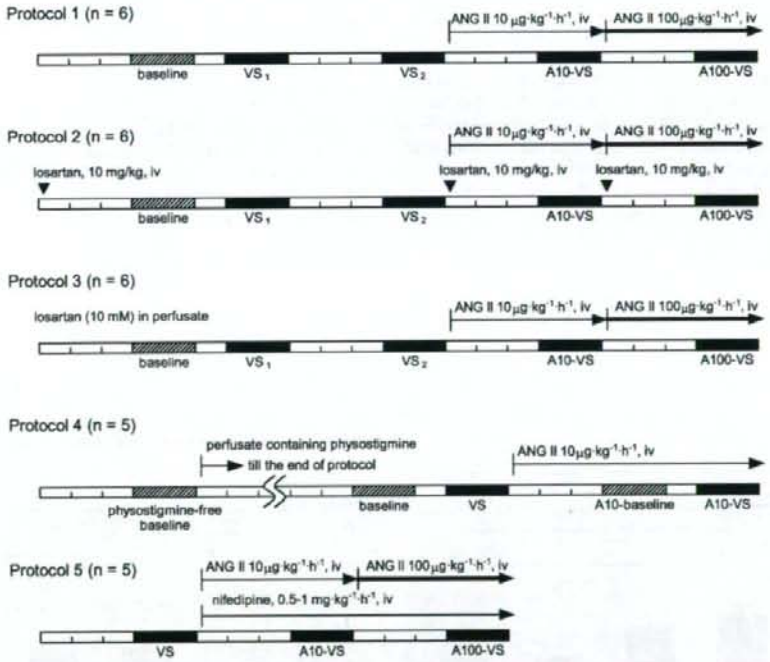

Fig. 1. Schematic representation of the protocols used in the present study. After implantation of the dialysis probe (2 h), we obtained a baseline dialysate sample (hatched rectangles) for 10 min. Thereafter, we obtained 4 dialysate samples during vagal stimulation with fixed-rate pacing for 10 min (filled rectangles) at intervening intervals of 15 min. In *protocols 1* through *3*, after obtaining 2 control trials (VS_1 and VS_2), we initiated intravenous administration of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ and waited for 15 min to obtain a dialysate sample during vagal stimulation with fixed-rate pacing (A10-VS). We then increased the dose of ANG II to 100 $\mu g \cdot kg^{-1} \cdot h^{-1}$ and waited for an additional 15 min before obtaining a dialysate sample during vagal stimulation with fixed-rate pacing (A100-VS). In *protocol 2*, the ANG II receptor subtype 1 blocker losartan was administered by bolus injection (10 mg/kg) before obtaining a baseline dialysate sample and also immediately before the beginning of each dose of ANG II administration (▼). In *protocol 3*, we administered losartan (10 mM) through the dialysis probe throughout the protocol. In *protocol 4*, we first collected a dialysate sample using perfusate free of physostigmine. We then replaced the perfusate with Ringer solution containing physostigmine and collected dialysate samples of baseline and vagal stimulation (VS). Approximately 15 min after the onset of iv ANG II administration at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$, we collected dialysate samples of baseline (A10-baseline) and vagal stimulation (A10-VS). In *protocol 5*, we collected dialysate samples during a control vagal stimulation (VS) and during the 2 doses of iv ANG II administration (A10-VS and A100-VS). The pressor effect of ANG II was counteracted by simultaneous iv infusion of the L-type Ca^{2+} channel blocker nifedipine.

(VS_1). After an intervening interval of 15 min, we repeated the 10-min vagal stimulation with fixed-rate pacing and collected another dialysate sample (VS_2). After performing these two control trials, we began intravenous administration of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$. Approximately 15 min after the onset of the ANG II administration, we collected a dialysate sample (A10-VS) during 10-min vagal stimulation with fixed-rate pacing. We then increased the dose of ANG II at 100 $\mu g \cdot kg^{-1} \cdot h^{-1}$. Approximately 15 min after the onset of the higher-dose ANG II administration, we collected a final dialysate sample (A100-VS) during 10-min vagal stimulation with fixed-rate pacing.

Protocol 2 (n = 6). We examined whether the intravenous AT_1 receptor antagonist losartan would block the effects of ANG II on the vagal stimulation-induced myocardial ACh release. We infused losartan potassium intravenously at 10 mg/kg and waited for ~15 min. We then collected baseline, VS_1, and VS_2 samples with an intervening interval of 15 min, as described in *protocol 1*. Next, after an additional bolus injection of losartan potassium at 10 mg/kg, we began intravenous infusion of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$. After ~15 min, we obtained a dialysate sample of A10-VS. Finally, after another bolus injection of losartan potassium at 10 mg/kg, we began intravenous infusion of ANG II at 100 $\mu g \cdot kg^{-1} \cdot h^{-1}$. After an additional 15 min, we obtained a dialysate sample of A100-VS.

Protocol 3 (n = 6). We examined whether local administration of losartan would block the effects of ANG II on the vagal stimulation-induced myocardial ACh release. We perfused the dialysis probe with Ringer solution containing 10 mM of losartan potassium. Taking into account the distribution across the semipermeable membrane, we administered losartan at a concentration >400 times higher than that for intravenous administration in *protocol 2*. Because local administrations of larger molecules such as ω-conotoxin GVIA (molecular weight 3037) and ω-conotoxin MVIIC (mol wt 2,749) were able to suppress vagal stimulation-induced ACh release in our previous study (15), it would be reasonable to assume that losartan potassium (mol wt 461) should have spread in the vicinity of the dialysis fiber, from which the dialysate was collected. Using the same procedures as described in *protocol 1*, we obtained dialysate samples for baseline, VS_1, VS_2, A10-VS, and A100-VS. A previous study indicated that ACh measured by cardiac microdialysis in the left ventricle mainly reflected ACh released from the postganglionic nerve terminals and not from the parasympathetic ganglia (1 and see DISCUSSION for details).

Protocol 4 (n = 5). To examine the effects of ANG II on the baseline ACh level, we performed an additional protocol where the baseline ACh level was measured during intravenous infusion of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (A10-baseline). In this protocol, we also obtained a dialysate sample using the perfusate without the cholinesterase inhibitor physostigmine before the usual dialysate sampling using the perfusate containing physostigmine.

Protocol 5 (n = 5). To avoid the pressor effect of ANG II, we administered an L-type Ca^{2+} channel blocker nifedipine (0.5–2.0 $mg \cdot kg^{-1} \cdot h^{-1}$) simultaneously with ANG II and obtained dialysate samples for VS, A10-VS, and A100-VS. In a previous study, intravenous administration of an L-type Ca^{2+} channel blocker alone did not affect the vagal stimulation-induced myocardial ACh release significantly (15).

Statistical Analysis

All data are presented as mean ± SE values. In *protocols 1* through *3*, myocardial interstitial ACh levels were compared among baseline, VS_1, VS_2, A10-VS, and A100-VS samples using a repeated-measures ANOVA (8). When there was a significant difference, Tukey's test for all-pairwise comparisons was applied to identify the differences between any two of the samples. Differences were considered significant at $P < 0.05$. The mean AP value in the last 1 min of the 10-min vagal stimulation period was treated as the AP value during vagal stimulation. The AP data were compared using a repeated-measures ANOVA among baseline, during the two control stimulations (VS_1 and VS_2), and before and during vagal stimulation under the two different doses of intravenous ANG II administrations. When there was a significant difference, Dunnett's test for comparison against a single control was applied to identify differences from the baseline value. Differences were considered significant at $P < 0.05$. In

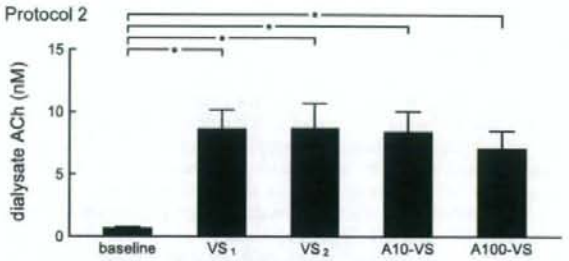

Fig. 3. Changes in dialysate ACh concentrations obtained from *protocol 2*. Vagal stimulation significantly increased the ACh levels. There was no significant difference in the ACh level among the 4 dialysate samples during vagal stimulation (VS_1, VS_2, A10-VS, and A100-VS). Values are presented as means and SE. $*P < 0.01$ by Tukey's test.

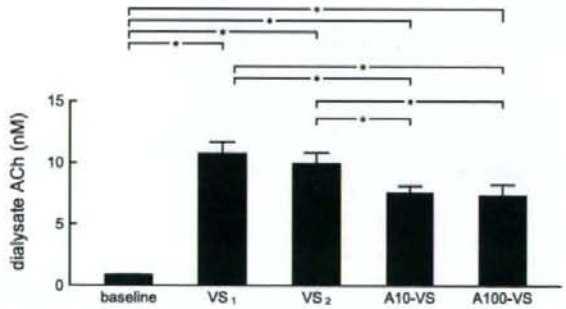

Fig. 2. Changes in dialysate ACh concentrations obtained from *protocol 1*. Vagal stimulation significantly increased the ACh levels. There was no significant difference in the ACh level between the 2 control trials (VS_1 and VS_2). The ACh level was significantly lower in A10-VS and A100-VS compared with that measured in VS_1 and VS_2. There was no significant difference in the ACh level between A10-VS and A100-VS. Values are presented as mean and SE. $*P < 0.01$ by Tukey's test.

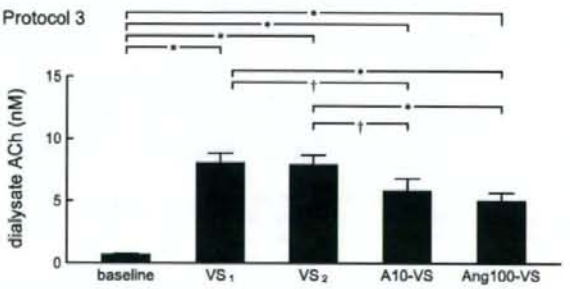

Fig. 4. Changes in dialysate ACh concentrations obtained from *protocol 3*. Vagal stimulation significantly increased the ACh levels. There was no significant difference in the ACh level between the 2 control trials (VS_1 and VS_2). The ACh level was significantly lower in A10-VS and A100-VS compared with that measured in VS_1 and VS_2. There was no significant difference in the ACh level between A10-VS and A100-VS. Values are presented as means and SE. $\dagger P < 0.05$ and $*P < 0.01$ by Tukey's test.

Table 1. *Mean arterial pressure values before vagal stimulation and during the last 1 min of stimulation*

	Baseline	VS_1	VS_2	A10	A10-VS	A100	A100-VS
Protocol 1	102±11	93±17	91±17	132±9†	105±19	129±13†	105±21
Protocol 2	102±17	71±16*	69±16*	80±15	68±17*	86±19	72±18*
Protocol 3	102±13	100±17	92±17	139±11*	120±19	147±11*	122±21

Data are means ± SE obtained from baseline, two control trials (VS_1 and VS_2), before (A10) and during (A10-VS) vagal stimulation under iv administration of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$, and before (A100) and during (A100-VS) vagal stimulation under iv administration of ANG II at 100 $\mu g \cdot kg^{-1} \cdot h^{-1}$. The heart was paced at 200 beats/min whenever vagal stimulation was applied. †$P < 0.05$ and *$P < 0.01$ from the respective baseline values by Dunnett's test.

protocol 4, the baseline ACh levels were compared before and during the ANG II administration using a paired *t*-test. The ACh levels during vagal stimulation were also compared before and during ANG II administration using a paired *t*-test. In *protocol 5*, the ACh levels and the mean AP values were compared among VS, A10-VS, and A100-VS using a repeated-measures ANOVA followed by Tukey's test.

RESULTS

In *protocol 1*, vagal stimulation significantly increased myocardial interstitial ACh levels (Fig. 2). There was no significant difference between two control trials with an intervening interval of 15 min [VS_1: 10.7 ± 1.0 (SE) nM and VS_2: 9.9 ± 0.9 (SE) nM]. Intravenous administration of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ significantly attenuated the vagal stimulation-induced ACh release (A10-VS: 7.5 ± 0.6 nM) to ~71% of VS_1. Although the intravenous administration of ANG II at 100 $\mu g \cdot kg^{-1} \cdot h^{-1}$ also significantly attenuated the vagal stimulation-induced ACh release (A100-VS: 7.3 ± 0.9 nM) to ~68% of VS_1, the ACh levels were not different from those of A10-VS.

In *protocol 2*, vagal stimulation significantly increased myocardial interstitial ACh levels under control stimulations (VS_1: 8.6 ± 1.5 nM and VS_2: 8.7 ± 2.0 nM; Fig. 3). With a pretreatment of intravenous losartan, intravenous ANG II was unable to suppress the vagal stimulation-induced ACh release (A10-VS: 8.4 ± 1.7 nM and A100-VS: 7.1 ± 1.4 nM). Although the mean level of ACh tended to be lower in A100-VS compared with VS_1 or VS_2, the differences were not statistically significant.

In *protocol 3*, vagal stimulation significantly increased myocardial interstitial ACh levels under control stimulations (VS_1: 8.0 ± 0.8 nM and VS_2: 7.9 ± 0.8 nM; Fig. 4). Intravenous ANG II at either 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ or 100 $\mu g \cdot kg^{-1} \cdot h^{-1}$ significantly suppressed the vagal stimulation-induced ACh release to ~72% (A10-VS: 5.8 ± 1.0 nM) and 62% (A100-VS: 5.0 ± 0.7 nM of that seen in VS_1), respectively.

In *protocol 1*, the AP values before the vagal stimulation during the intravenous ANG II administrations (A10 and A100) were significantly higher than the baseline AP value (Table 1). The AP values during vagal stimulation (VS_1, VS_2, A10-VS, and A100-VS) were not different from the baseline AP value. In *protocol 2*, the AP value before the first administration of losartan was 126 ± 14 mmHg. The AP values before the vagal stimulation during the intravenous ANG II administrations (A10 and A100) were not significantly different from the baseline AP value. The AP values during vagal stimulation (VS_1, VS_2, A10-VS, and A100-VS) were significantly lower than the baseline AP value. In *protocol 3*, the AP values before vagal stimulation during the intravenous ANG II administrations (A10 and A100) were significantly higher than the baseline AP value. The AP values during vagal stimulation (VS_1, VS_2, A10-VS, and A100-VS) did not differ statistically from the baseline AP value.

Figure 5 illustrates typical chromatograms obtained from one animal in *protocol 4*. The baseline ACh level was below the limit of determination (0.5 nM) when the perfusate did not contain physostigmine. Approximately 1 h after replacing the perfusate with Ringer solution containing physostigmine, the baseline ACh level was above the limit of determination. As shown in Table 2, vagal stimulation significantly increased the ACh level (VS). The intravenous administration of ANG II did not affect the baseline ACh level (A10-basline) but significantly attenuated the ACh level during vagal stimulation (A10-VS).

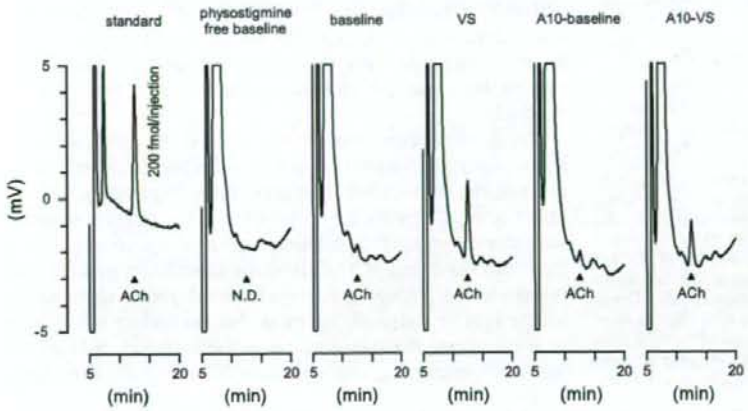

Fig. 5. Typical chromatograms for the ACh measurements obtained from *protocol 4*. ACh was less than the limit of determination when perfusate did not contain physostigmine (physostigmine-free baseline). The baseline ACh level was above the limit of determination when perfusate contained 100 μM physostigmine. (This perfusate was usually used for the ACh measurements.) Vagal stimulation increased the ACh level (VS). The administration of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ did not affect baseline ACh level (A10-baseline) but significantly attenuated the vagal stimulation-induced ACh release (A10-VS). See Table 2 for pooled data of ACh levels. ND, not detected.

Table 2. *Mean arterial pressure values and ACh concentrations obtained in protocol 4*

	Physostigmine-free Baseline	Baseline	VS	A10-Baseline	A10-VS
ACh, nM	Not detected	1.6±0.4	10.6±2.4	1.7±0.5	7.8±2.1*
Mean arterial pressure, mmHg	111±11	109±12	103±6	148±3*	118±6

Data are means ± SE obtained from physostigmine-free baseline, baseline, control vagal stimulation (VS), and baseline (A10-baseline) and vagal stimulation (A10-VS) under iv administration of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$. There was no significant difference in the ACh level between baseline and A10-baseline by a paired-*t*-test. The ACh level was significantly lower in A10-VS than in VS by a paired-*t*-test. Mean arterial pressure was significantly higher in A10-baseline compared with the physostigmine-free baseline value by Dunnett's test. *$P < 0.01$.

In *protocol 5*, the pressor effect of ANG II was counteracted by the simultaneous intravenous infusion of nifedipine (Table 3). Under this condition, the intravenous administration of ANG II significantly attenuated the stimulation-induced ACh level to ~83% (A10-VS) and 72% (A100-VS) of that seen in VS.

DISCUSSION

The present study demonstrated that intravenous ANG II significantly inhibited the vagal stimulation-induced myocardial interstitial ACh release in the left ventricle in anesthetized cats. Intravenous administration of losartan abolished the inhibitory effect of ANG II on the stimulation-induced ACh release, suggesting that the inhibitory action of ANG II was mediated by AT_1 receptors.

Inhibitory Effect of ANG II on Myocardial Interstitial ACh Release

Only a few reports have focused on the modulatory effects of ANG II on the parasympathetic nervous system (3, 5, 25, 26), all of which have used the heart rate reduction in response to vagal stimulation as a functional measurement to assess the peripheral vagal function. Although ANG II has been shown to inhibit the ACh release in the rat entorhinal cortex in vitro (4), the direct evidence for the inhibitory effect of ANG II on the ACh release in the peripheral vagal neurotransmission in vivo has been lacking. The present study demonstrated that intravenous ANG II inhibited the vagal nerve stimulation-induced ACh release in the left ventricle in vivo (Fig. 2). As for the sympathetic system in the heart, Lameris et al. (18) have previously demonstrated that ANG II does not affect the sympathetic nerve stimulation-induced norepinephrine release. The insignificant effect of ANG II on the sympathetic neurotransmission and the inhibitory effect of ANG II on the parasympathetic neurotransmission may provide the basis for a study by Takata et al. (26) in which ACE inhibitor enhanced cardiac vagal but not sympathetic neurotransmission.

Table 3. *Mean arterial pressure values and ACh concentrations obtained in protocol 5*

	VS	A10-VS	A100-VS
ACh, nM	12.7±1.1	10.6±1.1†	9.2±1.5*
Mean arterial pressure, mmHg	83.4±12.2	68.4±6.3	70.4±9.5

Data are means ± SE from a control vagal stimulation trial (VS), during vagal stimulation under iv administration of ANG II at 10 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (A10-VS) and during vagal stimulation under iv administration of ANG II at 100 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (A100-VS). The heart was paced at 200 beats/min during vagal stimulation. In this protocol, the pressor effect of ANG II was counteracted by simultaneous iv administration of the L-type Ca^{2+} channel blocker nifedipine (0.5–2 $mg \cdot kg^{-1} \cdot h^{-1}$). †$P < 0.05$ and *$P < 0.01$ from the VS group by Tukey's test. There was no significant difference between A10-VS and A100-VS in the ACh level. There were no significant differences in mean arterial pressure among the three trials.

An increased activity of the renin-angiotensin system is common in chronic heart failure and has been considered to be a stimulus for aggravation of the disease. Inhibition of the renin-angiotensin system by ACE inhibitors or by AT_1 receptor blockers can prevent the ventricular remodeling and improve the survival rate (16, 17), suggesting that ANG II is indeed involved in the aggravation of heart failure. ACh, on the other hand, can exert a cardioprotective effect against myocardial ischemia in several experimental settings (12, 24, 29). If ANG II inhibits the peripheral vagal neurotransmission, blockade of ANG II would increase the vagal effect on the heart. Actually, Du et al. (5) demonstrated that losartan enhanced bradycardia induced by vagal stimulation in rats with chronic myocardial infarction. In that study, however, the ventricular effect of vagal stimulation was not assessed. The results of the present study indicate that ANG II inhibited the vagal neurotransmission in the ventricle. Blockade of ANG II is therefore expected to increase the vagal effect on the ventricular myocardium when the vagal outflow from the central nervous system is unchanged. Although no literatures appear to be available as to the chronic effect of ACh on the prognosis of heart failure, electrical vagal stimulation was able to improve the survival rate of chronic heart failure in rats (19). In that study, the magnitude of the vagal stimulation was such that the heart rate decreased only by 20–30 beats/min in rats, suggesting that a modest increase in vagal tone would be sufficient to produce a cardioprotective effect. It is plausible that blockade of ANG II yields beneficial effects on chronic heart failure not only by antagonizing the sympathetic effects but also by enhancing the vagal effects on the ventricle.

Vagal stimulation was able to reduce the left ventricular contractility as assessed by end-systolic elastance only when sympathetic stimulation coexisted (20), suggesting that the effect of vagal stimulation on ventricular contractility would be secondary to sympathoinhibition. Accordingly, contribution of the inhibitory effect of ANG II on the stimulation-induced ACh release to the physiological regulation of ventricular contractility might be marginal. We think that the finding is important as a peripheral mechanism of vagal withdrawal in heart diseases accompanying the activation of the renin-angiotensin system.

Possible Site of the Inhibitory Action of ANG II on ACh Release

In *protocol 3*, we examined whether local administration of losartan was able to nullify the inhibitory effect of ANG II on the vagal stimulation-induced ACh release. The utility of local administration of pharmacological agents through the dialysis fiber has been confirmed previously. As an example, local administration the Na^+ channel inhibitor tetrodotoxin through the dialysis fiber completely blocked the nerve stimulation-induced ACh release (14). With respect to the source for ACh, intravenous administration of the nicotinic antagonist hexamethonium bromide completely blocked the stimulation-induced ACh release, whereas local administration of hexamethonium bromide did not, suggesting the lack of parasympathetic ganglia in the vicinity of dialysis fiber (1). In support of our interpretation, a neuroanatomic finding indicates that three ganglia, away from the left anterior free wall targeted by the dialysis probe, provide the major source of left ventricular postganglionic innervation in cats (11). Therefore, the myocardial interstitial ACh measured by cardiac microdialysis in the left ventricle mainly reflects the ACh release from the postganglionic vagal nerve terminals. The results of *protocol 3* indicate that losartan spread around the postganglionic vagal nerve terminals failed to abolish the inhibitory effect of ANG II on the stimulation-induced ACh release. Because intravenous administration of losartan was able to abolish the inhibitory effect of ANG II on the stimulation-induced ACh release (*protocol 2*), the site of this inhibitory action is likely at parasympathetic ganglia rather than at postganglionic vagal nerve terminals. The fact that AT_1 receptors are rich in parasympathetic ganglia (2) would support our interpretation.

ANG II has a direct vasoconstrictive effect on the coronary artery (30). At the same time, however, the intravenous administration of ANG II tended to increase mean AP during vagal stimulation by ~15 mmHg in *protocol 1* (Table 1). Although it was statistically insignificant, if this increase in mean AP increased cardiac oxygen demand, the coronary blood flow might have been increased (27), resulting in an increased rate of washout in the myocardial tissue. The possibility cannot be ruled out that such a washout mechanism contributed to the reduction of stimulation-induced ACh release during ANG II administration. However, the baseline ACh level was not decreased by ANG II in *protocol 4*, suggesting that the washout rate did not increase significantly. In addition, even when the pressor effect of ANG II was counteracted by nifedipine, ANG II was still able to inhibit the vagal stimulation-induced ACh release in *protocol 5*. Therefore, we think that the change in washout rate was not a principal mechanism for the reduction of stimulation-induced ACh release by ANG II.

The mechanisms for the baseline ACh release under the vagotomized condition were not identified in the present study. In the motor nerve terminals, a so-called nonquantal release of ACh is documented, which is independent of nerve activity (6). Incorporation of the vesicular transport system in the membrane of the nerve terminals during an exocytosis process is considered to be responsible for the mechanism of nonquantal ACh release. A similar mechanism might contribute to the baseline ACh release in the vagal nerve terminals.

Several limitations need to be addressed. First, the dose of ANG II might have increased the plasma ANG II concentration beyond the physiological range. In this regard, the observed effect might be rather pharmacological or pathological than physiological. Nevertheless, because there are local synthesis and degradation of ANG II in the heart (21, 28), the inhibition of ACh release by ANG II could operate locally in the heart. Second, whether ANG II inhibited the ACh release from the preganglionic nerve terminals or it suppressed the excitability of the postganglionic nerve fibers to ACh was not identified in the present study. Third, the involvement of ANG II receptor subtype 2 (AT_2 receptor) in the modulation of peripheral parasympathetic neurotransmission was not examined in the present study because intravenous losartan was able to abolish the inhibitory effect of ANG II on the stimulation-induced ACh release. However, if coactivation of AT_1 and AT_2 receptors is required for the inhibitory effect of ANG II, blockade of AT_2 receptors would also abolish the inhibitory effect. Forth, we tested just one level of vagal stimulation. Whether the effect of ANG II on the stimulation-induced ACh release depends on the vagal stimulation intensity remains to be resolved.

In conclusion, intravenous ANG II reduced the vagal nerve stimulation-induced ACh release in the left ventricle. Intravenous losartan abolished the inhibitory effect of ANG II on the stimulation-induced ACh release, suggesting that this inhibition was mediated by AT_1 receptors. Because local administration of losartan via dialysis fiber was unable to nullify the inhibitory effect of ANG II on the stimulation-induced ACh release, the site of this inhibitory action is likely parasympathetic ganglia. The present results imply that the beneficial effects of ACE inhibitors and AT_1 receptor antagonists in the treatment of heart diseases may include not only the suppression of sympathetic activity but also the enhancement of vagal activity to the ventricle.

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Regional difference in ischaemia-induced myocardial interstitial noradrenaline and acetylcholine releases

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Abstract

Knowledge of the regional differences in myocardial interstitial noradrenaline (NA) and acetylcholine (ACh) levels during ischaemia would be important to understand the abnormality of neuronal environment surrounding the ischaemic heart. Using a cardiac microdialysis technique, we compared ischaemia-induced changes in the myocardial interstitial NA and ACh levels among three groups of anesthetized cats: the anterior free wall of the left ventricle (ANT group, $n=7$; the left anterior descending coronary artery was occluded), the posterior free wall of the left ventricle (POST group, $n=6$; the left circumflex coronary artery was occluded), and the right ventricle (RV group, $n=6$; the right coronary artery was occluded). The maximum NA level was not different between the ANT and POST groups but was significantly lower in the RV group ($P<0.01$) [70 nM (SD 37), 106 nM (SD 99), and 7 nM (SD 10), respectively]. The maximum ACh level was not different between the ANT and POST groups but was significantly lower in the RV group ($P<0.05$) [16 nM (SD 7), 20 nM (SD 15), and 6 nM (SD 2), respectively]. In contrast, there were no significant differences in NA or ACh release in response to a local administration of ouabain (10 mM) among the ANT, POST, and RV groups ($n=6$ each). In conclusion, the regional difference of the ischaemic effects, rather than the regional difference in the functional distributions of sympathetic and vagal efferent nerve terminals, might contribute to the lower levels of ischaemia-induced NA and ACh releases in the RV group.

Keywords: Cardiac microdialysis; Coronary artery occlusion; Ouabain; Cats

The heart is under an incessant control by the autonomic nervous system. The principal molecule that affects the myocardium is noradrenaline (NA) for the sympathetic nerve and acetylcholine (ACh) for the parasympathetic nerve. Acute myocardial ischaemia causes abnormality of the neural regulation via mechanisms such as pathological cardio-cardiac reflexes and disruption of the nerves traversing the ischaemic region (Zipes 1990; Hainsworth, 1991; Elvan and Zipes, 1998; Armour, 1999; Kawada et al., 2002). Although occlusion of the left anterior descending coronary artery (LAD) has been shown to increase myocardial interstitial NA and ACh levels in the ischaemic region (Shindo et al., 1996; Kawada et al., 2000; Lameris et al., 2000), whether the effects of ischaemia on the myocardial interstitial NA and ACh levels are homogeneous within the left ventricle and between the left and right ventricles remains unknown. The cardio-depressor reflex similar to that induced by veratridine (the Bezold–Jarisch reflex) is frequently observed in inferoposterior but not in anterior myocardial infarction, suggesting the regional difference in the vagal afferent fibre distribution within the left ventricle (Thames et al., 1978; Walker et al., 1978). Regional differences are also reported in the distributions of sympathetic and vagal efferent nerves within the left ventricle and between the left and right ventricles

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(Pierpont et al., 1984; Schmid et al., 1978). We hypothesized that the effects of ischaemia on the myocardial interstitial NA and ACh levels would also show regional differences.

We used a cardiac microdialysis technique and measured dialysate NA and ACh concentrations as indices of myocardial interstitial NA and ACh levels in anesthetized cats (Akiyama et al., 1991, 1994; Yamazaki et al., 1997; Kawada et al., 2001). We compared ischaemia-induced changes in the myocardial interstitial NA and ACh levels among the following regions: the anterior free wall of the left ventricle (ANT group) perfused by the LAD, the posterior free wall of the left ventricle (POST group) perfused by the left circumflex coronary artery (LCX), and the right ventricle (RV group) perfused by the right coronary artery (RCA). In addition, we compared changes in the myocardial interstitial NA and ACh levels in response to a local administration of ouabain through the dialysis probe (Yamazaki et al., 1999; Kawada et al., 2001). The advantage of the local administration of ouabain might be that we can assess the transmitter releasing function of the sympathetic and vagal efferent nerve terminals in the working heart without significant effects on the systemic haemodynamics.

1. Materials and methods

1.1. Surgical preparation

Animal care was conducted in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences* approved by the Physiological Society of Japan. All protocols were approved by the Animal Subjects Committee of the National Cardiovascular Center. Adult cats weighing 2.2 to 5.0 kg were anaesthetized via an intraperitoneal injection of pentobarbital sodium (30–35 mg/kg) and ventilated mechanically with room air mixed with oxygen. The depth of anaesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium ($1–2$ mg kg^{-1} h^{-1}) through a catheter inserted into the right femoral vein. Mean systemic arterial pressure was measured from a catheter inserted into the right femoral artery. The heart rate was determined using an electrocardiogram.

We performed an ischaemia protocol and a local ouabain protocol in different animals. In each protocol, the experimental animals were divided into ANT, POST, and RV groups. In the ANT and POST groups, the left fifth and/or sixth ribs were resected to allow access to the heart. In the ANT group, a 3-0 silk suture was passed around the LAD just distal to the first diagonal branch for later occlusion. Using a fine guiding needle, a dialysis probe was implanted transversely into the anterior free wall of the left ventricle perfused by the LAD. In the POST group, a 3-0 silk suture was passed around the LCX for later occlusion, and a dialysis probe was implanted transversely into the posterior free wall of the left ventricle perfused by the LCX. In the RV group, the right fifth and/or sixth ribs were resected to expose the heart. A 3-0 silk suture was passed around the RCA for later occlusion. The right ventricular wall was picked up with a pair of forceps, and a dialysis probe was threaded transversely through the myocardium using a fine guiding needle. Heparin sodium (100 U/kg) was administered intravenously to prevent blood coagulation. A postmortem examination confirmed that the dialysis probe did not penetrate into the ventricular cavity. In the local ouabain protocol, similar experimental settings without a coronary snare were prepared for the three groups of animals.

1.2. Dialysis technique

The materials and properties of the dialysis probe have been described previously (Akiyama et al., 1991, 1994). Briefly, a dialysis fibre (13 mm length, 310 μm O.D., 200 μm I.D.; PAN-1200, 50,000 molecular weight cutoff, Asahi Chemical, Osaka, Japan) was glued at both ends to polyethylene tubes (25 cm length, 500 μm O.D., 200 μm I.D.). The dialysis probe was perfused at a rate of 2 μl/min with Ringer solution containing a cholinesterase inhibitor eserine (10^{-4} M). Two hours elapsed before the dialysate sampling was started to allow dialysate NA and ACh concentrations reached steady states. One sampling period was set at 15 min, which yielded a sample volume of 30 μl. The actual dialysate sampling lagged by 5 min behind a given collection period taking into account the dead space volume between the dialysis membrane and the sample tube. Each sample was collected in a microtube containing 3 μl of phosphate buffer

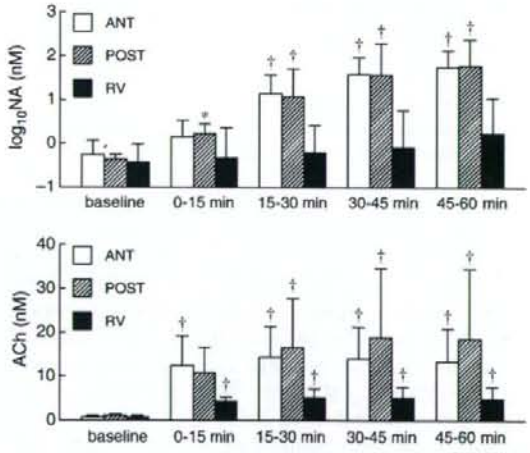

Fig. 1. Changes in myocardial interstitial noradrenaline (NA) and acetylcholine (ACh) levels during coronary occlusion. ANT: region of the anterior free wall of the left ventricle perfused by the left anterior descending coronary artery, POST: region of the posterior free wall of the left ventricle perfused by the left circumflex coronary artery, RV: region of the right ventricle perfused by the right coronary artery. The coronary occlusion significantly increased myocardial interstitial NA levels in the ANT and POST groups (top panel). Changes in the NA levels were not statistically significant in the RV group. The coronary occlusion significantly increased myocardial interstitial ACh levels in all of the ANT, POST, and RV groups (bottom panel). Data are mean and SD values. $^{*}P<0.05$ and $^{\dagger}P<0.01$ from the corresponding baseline value by Dunnett's test.

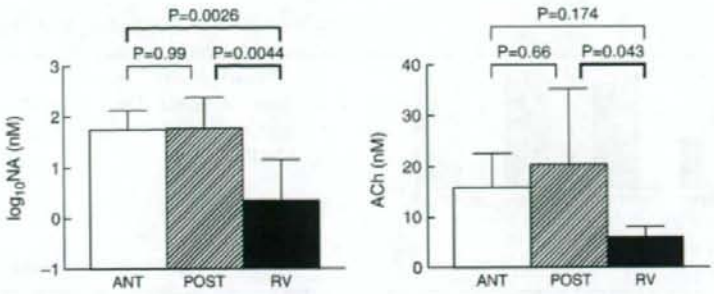

Fig. 2. Maximum levels of myocardial interstitial NA and ACh induced by coronary occlusions. The maximum NA level did not differ between the ANT and POST groups, but it was significantly lower in the RV group (left panel). The maximum ACh level did not differ between the ANT and POST groups, but it was significantly lower in the RV group than in the POST group (right panel). Data are mean and SD values. Exact P values determined by Tukey test are supplied.

(0.1 M, pH 3.5) to prevent amine oxidation. The ACh concentration in the dialysate was measured directly by high performance liquid chromatography with electrochemical detection (HPLC-ECD) system (Eicom, Kyoto, Japan). The NA concentration in the dialysate was measured by another HPLC-ECD system after removing interfering compounds with an alumina procedure.

1.3. Protocols

1.3.1. Ischaemia protocol

In each animal in the ANT ($n=7$), POST ($n=6$), and RV ($n=6$) groups, a 15-min dialysate sample was collected under baseline conditions. Thereafter, each corresponding coronary artery was occluded for 60 min, and four consecutive 15-min dialysate samples were obtained during the occlusion period.

1.3.2. Local ouabain protocol

In each animal in the ANT ($n=6$), POST ($n=6$), and RV ($n=6$) groups, a 15-min dialysate sample was collected under baseline conditions. The perfusate for the dialysis probe was then replaced with Ringer solution containing 10 mM of ouabain. The local administration of ouabain has been shown to evoke myocardial interstitial NA and ACh releases without significant effects on the systemic haemodynamics (Yamazaki et al., 1999; Kawada et al., 2001). Four consecutive 15-min dialysate samples were obtained during the local administration of ouabain.

1.4. Statistical analysis

All data are presented as mean (SD) values. Because the increase in NA reached more than 100 times the baseline value in the ANT and POST groups, NA data were compared after logarithmic transformation. To examine the effects of ischaemia or the local administration of ouabain on the NA or ACh level in each group, we used one-way repeated-measures analysis of variance (Glantz, 2002). When there was a significant difference in measured values among the collection periods, Dunnett's test was applied to identify the difference from the baseline value. To compare the maximum NA or ACh response among the ANT, POST, and RV groups, we used one-way analysis of variance. When there was a significant difference among the three groups, Tukey test was applied for simultaneous all pairwise comparisons. The differences were considered significant at $P<0.05$.

2. Results

Coronary artery occlusion increased the NA levels to more than 100 times the respective baseline levels in the ANT and POST groups (Fig. 1, top). In the RV group, although the mean NA level increased to nearly 10 times the baseline level, the change was not statistically significant, possibly due to the large variance of the NA responses across the animals. The coronary occlusion increased the ACh levels to approximately 15 times the respective baseline levels in the ANT and POST groups whereas it increased the

Table 1
Changes in mean arterial pressure by occlusions of the anterior descending coronary artery (ANT), the left circumflex coronary artery (POST), and the right coronary artery (RV)

	Baseline	5	15	30	45	60
ANT	116 (31)	114 (27)	108 (24)	108 (25)	108 (23)	110 (25)
POST	139 (31)	119 (31)†	125 (31)†	130 (31)*	132 (28)	135 (33)
RV	101 (20)	101 (26)	99 (22)	101 (23)	103 (25)	103 (23)

Baseline and values after 5, 15, 30, 45, and 60 min of the occlusion are presented (in mm Hg). Data are mean (SD) values. *$P<0.05$ and †$P<0.01$ from the corresponding baseline value.

Table 2
Changes in the heart rate by occlusions of the anterior descending coronary artery (ANT), the left circumflex coronary artery (POST), and the right coronary artery (RV)

	Baseline	5	15	30	45	60
ANT	193 (18)	166 (13)†	174 (15)†	175 (18)†	173 (15)†	171 (15)†
POST	183 (25)	159 (21)†	167 (20)†	167 (25)†	170 (25)†	169 (27)†
RV	188 (34)	175 (35)	176 (33)	179 (35)	181 (36)	183 (38)

Baseline and values after 5, 15, 30, 45, and 60 min of the occlusion are presented (in beats min^{-1}). Data are mean (SD) values. †$P<0.01$ from the corresponding baseline value.

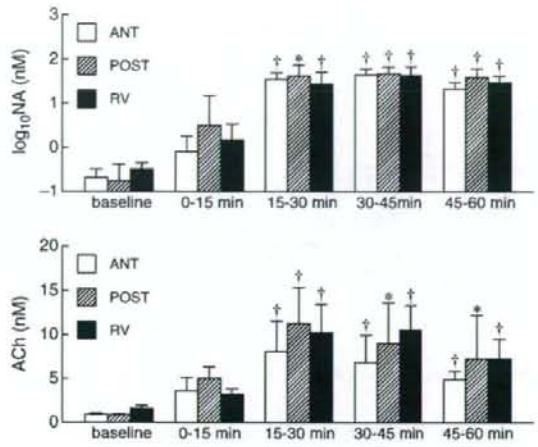

Fig. 3. Changes in myocardial interstitial NA and ACh levels induced by a local administration of ouabain through the dialysis probe. Ouabain significantly increased myocardial interstitial NA levels in all of the ANT, POST, and RV groups (top panel). The maximum NA level did not differ among the three groups. Ouabain significantly increased myocardial interstitial ACh levels in all of the ANT, POST, and RV groups (bottom panel). The maximum ACh level did not differ among the three groups. Data are mean and SD values. $^{*}P<0.05$ and $^{\dagger}P<0.01$ from the corresponding baseline value by Dunnett's test.

ACh levels to approximately 8 times the baseline level in the RV group (Fig. 1, bottom).

The maximum NA level observed during ischaemia was not different between the ANT and POST groups, but it was significantly lower in the RV group than in the ANT and POST groups (Fig. 2, left). The maximum ACh level observed during ischaemia was not different between the ANT and POST groups, but it was significantly lower in the RV group than in the POST group (Fig. 2, right).

Changes in mean arterial pressure obtained from the ischaemia protocol are summarized in Table 1. Occlusion of the LAD did not change mean arterial pressure significantly (the ANT group), whereas occlusion of the LCX significantly decreased mean arterial pressure at 5, 15, and 30 min of the ischaemic period (the POST group). Occlusion of the RCA did not change mean arterial pressure (the RV group).

Changes in the heart rate obtained from the ischaemia protocol are summarized in Table 2. Occlusion of the LAD significantly decreased the heart rate throughout the occlusion period (the ANT group). Occlusion of the LCX also significantly decreased the heart rate throughout the occlusion period (the POST group). In contrast, occlusion of the RCA did not change the heart rate significantly (the RV group).

A local administration of ouabain increased the NA levels in all of the ANT, POST, and RV groups (Fig. 3, top). There were no significant differences in the maximum NA levels among the three groups ($P=0.40$ by ANOVA). The local administration of ouabain also increased the ACh levels in all of the ANT, POST, and RV groups (Fig. 3, bottom). The ACh levels reached their maximum levels during 15–30 min of the administration period, then slightly decreased from the maximum levels during 30–45 and 45–60 min of the administration period but remained higher than the respective baseline levels. There were no significant differences in the maximum ACh levels among the three groups ($P=0.35$ by ANOVA).

3. Discussion

The level of ischaemia-induced NA release was significantly lower in the RV group than in the ANT and POST groups (Fig. 2, left). The level of ischaemia-induced ACh release was significantly lower in the RV group than in the POST group (Fig. 2, right). To our knowledge, this is the first report showing the differential effects of ischaemia on the myocardial interstitial NA and ACh levels among the main coronary arteries.

3.1. Regional difference in the ischaemia-induced myocardial interstitial NA release

Mechanisms responsible for the ischaemia-induced NA release have been extensively studied (Schömig A et al., 1984, 1988). An exocytotic release mechanism participates in the NA release in the early phase of ischaemia (within approximately 20 min from the onset of ischaemia) (Akiyama and Yamazaki, 1999). Energy depletion in the ischaemic region impairs the Na^+–K^+ ATPase activity and induces axoplasmic Na^+ accumulation. Because the NA uptake carrier is driven by the Na^+ gradient across the plasma membrane (Schwartz, 2000), the accumulation of intracellular Na^+ causes the reverse transport of axoplasmic NA to extracellular space. This nonexocytotic release mechanism becomes predominant as the ischaemic period is prolonged (Akiyama and Yamazaki, 1999; Lameris et al., 2000). In the present study, there was no significant difference in the levels of ischaemia-induced NA release between the ANT and POST groups (Fig. 2, left), suggesting that the extent of energy depletion caused by the coronary artery occlusion might be similar between the anterior and posterior regions of the left ventricle. In contrast, the level of ischaemia-induced NA release was much lower in the RV group (Fig. 2, left). Much lower oxygen consumption in the right ventricle than in the left ventricle (Weiss et al., 1978; Kusachi et al. 1982) may have delayed the progression of ischaemia and/or mitigated the severity of ischaemia.

To examine whether the observed difference in the ischaemia-induced NA release was attributable to the regional difference in the functional distribution of sympathetic efferent nerve terminals, we measured the amount of myocardial interstitial NA release in response to a local administration of ouabain (Yamazaki et al., 1999). Because locally administered ouabain spreads in the vicinity of the semipermeable membrane of the dialysis fibre and evokes the NA release, the NA concentration in the dialysate thus measured is considered to reflect the density of sympathetic

nerve terminals around the dialysis fibre. There were no significant differences in the maximum NA levels in response to the ouabain administration among the ANT, POST, and RV groups (Fig. 3, top), suggesting that the functional distribution of sympathetic efferent nerve terminals did not account for the lower level of ischaemia-induced NA release in the RV group.

The results of local administration of ouabain, showing no significant regional differences in the functional distribution of sympathetic efferent nerve terminals, are comparable to histological studies. In the human heart (Kawano et al., 2003), the number of tyrosine hydroxylase (TH)-positive nerves is similar between the left and right ventricles, and the number of TH-positive nerves in the anterior wall is 1.2 times greater than that in the posterior wall of the left ventricle. Although Pierpont et al. (Pierpont et al., 1984) reported a regional difference in the NA content in the canine left ventricle, the major difference observed in their study is an increasing gradient of NA from the apex to the base of the ventricle. In addition, the NA content was similar between the left and right ventricles in their study [495 ng/g (SD 267) vs. 503 ng/g (SD 123)].

3.2. Regional difference in the ischaemia-induced myocardial interstitial ACh release

In previous studies, we have demonstrated that acute myocardial ischaemia causes myocardial interstitial ACh release in the ischaemic region (Kawada et al., 2000; 2006b). An exocytotic release mechanism may be involved in the ischaemia-induced ACh release within approximately 15 min from the onset of ischaemia (Kawada et al., 2000, 2006a). Thereafter, a local ACh release mechanism independent of vagal nerve discharge may play a dominant role in the ischaemia-induced ACh release. In the present study, there was no significant difference in the levels of ischaemia-induced ACh release between the ANT and POST groups (Fig. 2, right). In contrast, the level of ischaemia-induced ACh release was much lower in the RV group than in the POST group. These results suggest that the energy depletion during ischaemia might be less severe or delayed in the right ventricle compared to that in the left ventricle.

Similar to the ouabain-induced NA release, there were no significant differences in the levels of ouabain-induced ACh release among the ANT, POST, and RV groups (Fig. 3, bottom). In a histological study of the human heart (Kawano et al., 2003), the number of acetylcholine esterase (AChE)-positive nerves in the right ventricle is 1.2 times greater than that in the left ventricle, and the number of AChE-positive nerves in the posterior wall is 1.4 times greater than that in the anterior wall of the left ventricle. In the guinea pig heart, the level of choline acetyltransferase activity was approximately two times higher in the right ventricle than in the left ventricle (Schmid et al., 1978). Notwithstanding the discrepancies among reports, these histochemical studies indicate that the number of the vagal nerve terminals in the right ventricle is not less than that in the left ventricle. In other words, the regional difference in the vagal efferent nerve distribution may not account for the lower level of ischaemia-induced ACh release in the RV group as compared with the POST group.

3.3. Pathological significance

The pathological significance of the NA and ACh releases in the ischaemic region is still to be explored. Although high levels of NA reveal cardiotoxicity (Rona, 1985), depletion of catecholamine in reserpinized animals fails to reduce the myocardial infarct size (Toombs et al., 1993; Vander Heide et al., 1995). However, in the reserpinized animals, not only the ischaemic area but also the non-ischaemic area is subjected to catecholamine depletion, making the interpretation of the results difficult. Acetylcholine, when administered prior to coronary occlusion, induces an ischaemic preconditioning mimetic effect (Qin et al., 2002). Acetylcholine also exerts protection on myocytes against hypoxia (Kakinuma et al., 2005). Generally speaking, the excess NA might be harmful whereas the presence of ACh might be beneficial to the myocardium.

One possible feature of the cardiac microdialysis may be that it can monitor the time course of changes in myocardial interstitial NA and ACh levels (Shindo et al., 1996; Kawada et al., 2000; Lameris et al., 2000). Although myocardial ischaemia evokes both the NA and ACh releases, the ACh release is more prompt compared to the NA release. When calculating the percentage against the respective maximum level, the mean NA levels were only 3 and 2% whereas the mean ACh levels reached 79 and 53% in the ANT and POST groups, respectively, during the 0–15 min of the ischaemic period (Fig. 1, note the logarithmic scaling in the top panel). In the RV group, the mean NA level was 28% whereas the mean ACh level reached 72% during the 0–15 min of the ischaemic period. It seems that the ACh release is a protective mechanism against a forthcoming excess of NA in the ischaemic region. Further studies are required to elucidate the significance of a local neuronal environment in modifying the severity of myocardial ischaemia.

3.4. Regional difference in the reflex effects

In the ischaemia protocol, the reflexes from the heart and the arterial baroreflex might have modified the efferent nerve activities. Both the ANT and POST groups showed a significant decrease in the heart rate during ischaemia (Table 2), suggesting an increase in the vagal tone and/or a decrease in the sympathetic tone. Because mean arterial pressure was either unchanged (the ANT group) or decreased (the POST group) (Table 1), the baroreflex cannot account for the decrease in the heart rate during ischaemia. The decreased mean arterial pressure in the POST group compared to the unchanged mean arterial pressure in the ANT group suggests that the cardiodepressor reflex was stronger in the POST than

the ANT group. These differences, however, did not cause the difference in the maximum levels of ischaemia-induced NA and ACh releases between the ANT and POST groups (Fig. 2). Because local release mechanisms became predominant as the ischaemic period is prolonged (Akiyama and Yamazaki, 1999; Kawada et al., 2000, 2006a; Lameris et al., 2000), the difference in the efferent nerve activities might not have affected the maximum levels of NA and ACh releases significantly. In contrast to the ANT and POST groups, the RV group did not show significant changes in mean arterial pressure or the heart rate, suggesting that the effect of RCA occlusion on the systemic haemodynamics was minimal in the present study. Despite the absence of significant bradycardia, the myocardial interstitial ACh level was significantly increased in the RV group during ischaemia, suggesting the involvement of a local release mechanism.

There are limitations to the present study. First, we could not examine regional differences in the NA and ACh releases along the transmural axis from the epicardial layer to the endocardial layer, because we could not control the exact depth of the dialysis fibre implanted transversely in the ventricular wall. Second, species differences should be taken into account when interpreting the present data. As an example, we usually observed a bradycardic response not only during LCX occlusion but also during LAD occlusion in cats (Table 2). In contrast, LAD occlusion in dogs more frequently evokes tachycardia and hypertension (Thames et al., 1978; Zipes, 1990).

4. Conclusion

The maximum levels of ischaemia-induced NA and ACh releases did not differ between the ANT and POST groups but were significantly lower in the RV group. In contrast, myocardial interstitial NA and ACh releases in response to a local administration of ouabain did not show regional differences among the ANT, POST, and RV groups. The regional difference in the ischaemic effects, rather than the regional difference in the functional distributions of sympathetic and vagal efferent nerve terminals, might contribute to the lower levels of ischaemia-induced NA and ACh releases in the RV group.

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