(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

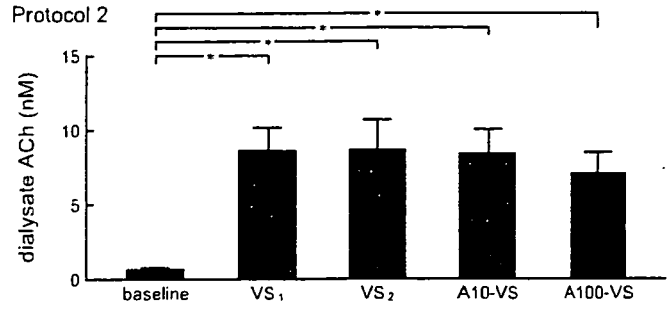

Fig. 3. Changes in dialysate ACh concentrations obtained from *protocol 2*. Vagal stimulation significantly increased the ACh levels. There was no significant difference in the ACh level among the 4 dialysate samples during vagal stimulation (VS_1, VS_2, A10-VS, and A100-VS). Values are presented as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

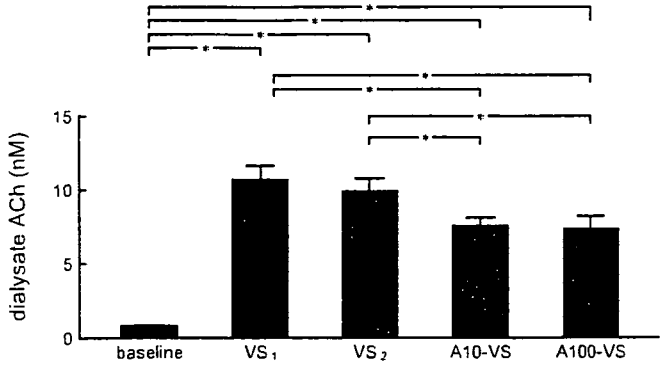

Fig. 2. Changes in dialysate ACh concentrations obtained from *protocol 1*. Vagal stimulation significantly increased the ACh levels. There was no significant difference in the ACh level between the 2 control trials (VS_1 and VS_2). The ACh level was significantly lower in A10-VS and A100-VS compared 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.

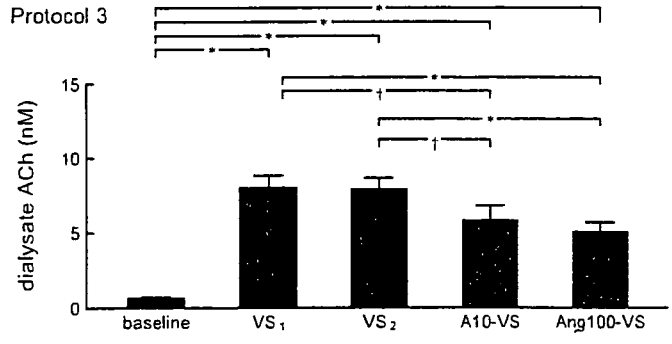

Fig. 4. Changes in dialysate ACh concentrations obtained from *protocol 3*. Vagal stimulation significantly increased the ACh levels. There was no significant difference in the ACh level between the 2 control trials (VS_1 and VS_2). The ACh level was significantly lower in A10-VS and A100-VS compared 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. †$P < 0.05$ 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).

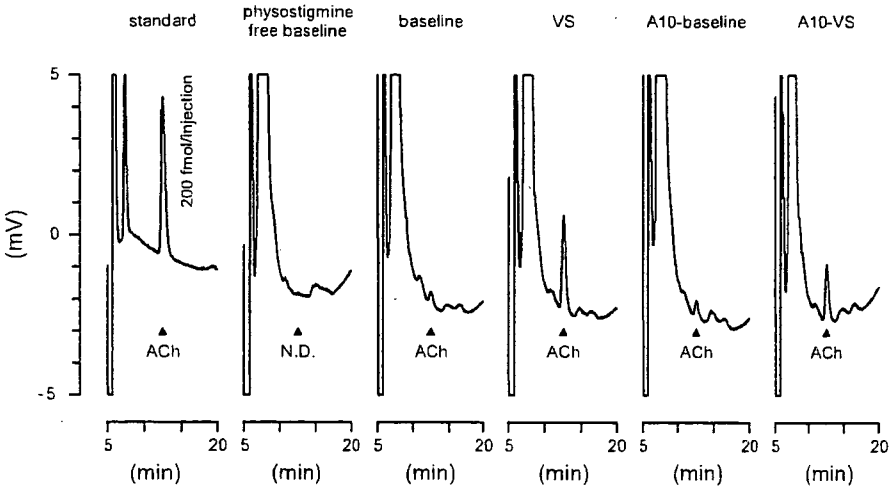

Fig. 5. Typical chromatograms for the ACh measurements obtained from *protocol 4*. ACh was less than the limit of determination when perfusate did not contain physostigmine (physostigmine-free baseline). The baseline ACh level was above the limit of determination when perfusate contained 100 μM physostigmine. (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.

GRANTS

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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

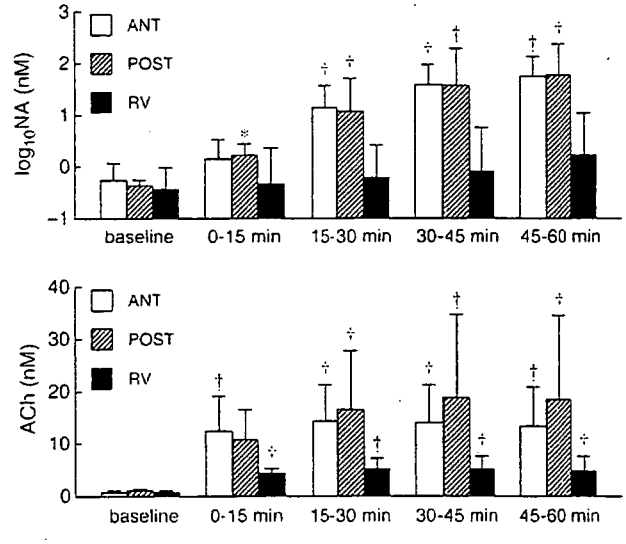

Fig. 1. Changes in myocardial interstitial noradrenaline (NA) and acetylcholine (ACh) levels during coronary occlusion. ANT: region of the anterior free wall of the left ventricle perfused by the left anterior descending coronary artery, POST: region of the posterior free wall of the left ventricle perfused 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.

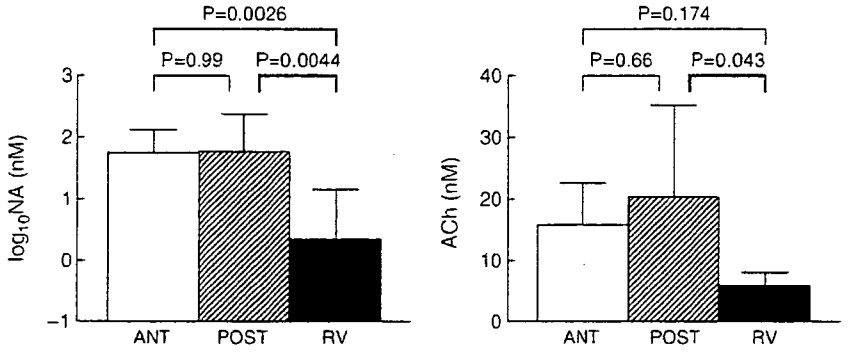

Fig. 2. Maximum levels of myocardial interstitial NA and ACh induced by coronary occlusions. The maximum NA level did not differ between the ANT and POST groups, but it was significantly lower in the RV group (left panel). The maximum ACh level did not differ between the ANT and POST groups, but it was 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.

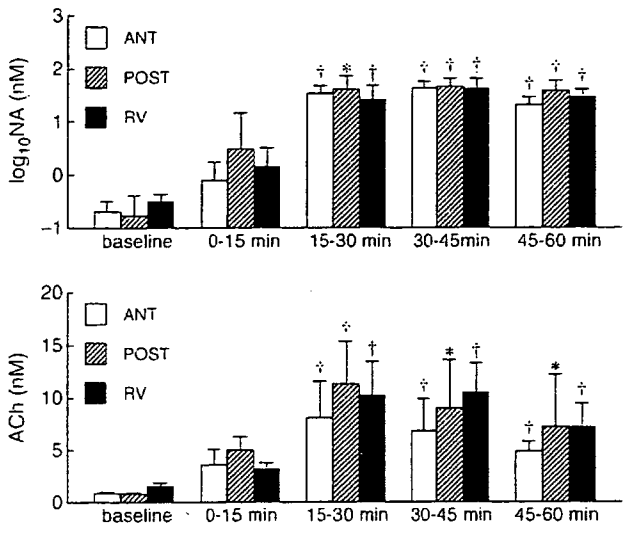

Fig. 3. Changes in myocardial interstitial NA and ACh levels induced by a local administration of ouabain through the dialysis probe. Ouabain significantly increased myocardial interstitial NA levels in all of the ANT, POST, and RV groups (top panel). The maximum NA level did not differ among the three 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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Cardiac epinephrine synthesis and ischemia-induced myocardial epinephrine release

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Abstract

Objective: Phenylethanolamine-*N*-methyltransferase (PNMT), the enzyme that synthesizes epinephrine (EPI) from norepinephrine (NE) in the adrenal gland, is present in extra-adrenal tissues including heart. Ischemia evokes an excessive NE accumulation in the myocardial interstitial spaces. Therefore, cardiac PNMT activity with high NE levels may contribute to cardiac EPI synthesis and release evoked by ischemia.
Methods: We measured dialysate EPI levels in the left ventricle of anesthetized rabbits using a cardiac microdialysis technique. The dialysate EPI level served as an index of the myocardial interstitial EPI level. Locally administered NE-induced dialysate EPI responses were measured. The left circumflex coronary artery was occluded for 60 min and the dialysate EPI and NE levels in the ischemic region were measured. Coronary occlusion-induced EPI responses were compared with and without administration of a PNMT inhibitor (SKF29661) in the presence and absence of desipramine (catecholamine transport blocker).
Results: Local administration of NE (250, 2500 ng/ml) increased the EPI levels to 734±125 and 2088±367 pg/ml respectively. These increases in dialysate EPI were suppressed by the PNMT inhibitor. Acute myocardial ischemia significantly increased the EPI levels to 3607±1069 pg/ml in the ischemic region, and these were suppressed by the PNMT inhibitor (1417±581 pg/ml). The pretreatment with desipramine suppressed ischemia-induced EPI release, which did not differ with (725±155 pg/ml) and without administration of a PNMT inhibitor (743±172 pg/ml).
Conclusion: The cardiac PNMT in the left ventricle is capable of synthesizing EPI with markedly elevated NE levels in the myocardial interstitial space.

Keywords: Autonomic nervous system; Interstitial space; Ischemia; Reperfusion; Neurotransmitters

1. Introduction

It is generally accepted that myocardial ischemia evokes an excessive catecholamine accumulation in the myocardial interstitial space [1,2]. This high catecholamine level in the myocardial interstitium is thought to aggravate the progression of myocardial cell injury and incidence of malignant arrhythmia [3,4]. From in vitro and in vivo studies, several mechanisms are presently suggested to induce release of norepinephrine (NE) from the nerve endings [1,5,6]. The outward NE transport through uptake$_1$ carrier has been proposed as an important mechanism responsible for the ischemia-induced NE release. With respect to epinephrine (EPI), however, it is uncertain whether the mechanism of release differs between EPI and NE.

EPI is synthesized mainly from NE in the adrenal medulla by phenylethanolamine *N*-methyltransferase (PNMT) [7] and released into the bloodstream [8]. Myocardial ischemia evokes an excessive NE and EPI accumulation in the myocardial interstitial space although the blood supply is blocked. Therefore, regional release mechanism has been suggested to induce release of EPI in the ischemic region. Early studies reported that PNMT activity was measured in

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homogenates from the heart [9,10]. Excessive NE level and cardiac PNMT activity may provide the prerequisite for cardiac EPI synthesis evoked by ischemia. We speculate that ischemia may promote EPI synthesis and release by high NE accumulation via cardiac PNMT activity.

We have demonstrated the usefulness of dialysis technique for the in vivo monitoring of regional myocardial interstitial catecholamine kinetics [11,12]. In the present study, we extend this approach to assessment of PNMT activity using NE as a substrate of EPI synthesis. We examined the role of PNMT activity in the EPI release evoked by myocardial ischemia. Furthermore, we examined the contribution of neuronal catecholamine transport to EPI release evoked by ischemia.

2. Materials and methods

2.1. Animal preparation

Animal care proceeded in strict accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Adult Japanese white rabbits (2.5–3.3 kg) were anesthetized with pentobarbital sodium (30–35 mg/kg i.v.). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). The rabbits were intubated and ventilated with room air mixed with oxygen. Heart rate, arterial pressure, and electrocardiogram were simultaneously monitored with a data recorder. The fifth or sixth rib on the left side was partially removed to expose the heart. A snare was placed around the main branch of the left circumflex coronary artery (LCX) to act as the occluder for later coronary occlusion. With a fine guiding needle, a dialysis probe was implanted in the region perfused by LCX of the left ventricular wall. Judging from changes in the color of the ventricular wall during a brief coronary occlusion, the dialysis probe was located in the midst of the ischemic region. Heparin sodium (100 IU/kg) was administered intravenously to prevent blood coagulation.

2.2. Dialysis technique and epinephrine measurements

Materials suitable for cardiac dialysis probes have been described in detail elsewhere [13]. Briefly, we designed a handmade long transverse dialysis probe. A dialysis fiber (8 mm length, 0.31 mm o.d., and 0.20 mm i.d.; PAN-1200 50,000 molecular weight cutoff, Asahi Chemical Japan) was glued at both ends of a polyethylene tube. The dialysis probe was perfused with Ringer's solution or Ringer's solution containing pharmacological agents at a perfusion speed of 2 μl/min using a microinjection pump (Carnegie Medicine CMA/100). One sampling period was 15 min (1 sampling volume = 30 μl). Each sample was collected in a microtube containing 3 μl of 0.1 N HCl to prevent amine oxidation. Dialysate EPI and NE level were measured by high-performance liquid chromatography with electrochemical detection (ECD-300, Eicom, Kyoto, Japan) as previously described [14,15]. Dialysate EPI level served as an index of myocardial interstitial EPI level. We commenced the protocol followed by a stabilization period of 2 h. Taking into consideration the dead space between the dialysis fiber and sample tube, we sampled the dialysate.

2.3. Experimental protocols

2.3.1. Dialysate EPI levels during local administration of NE

First, to elucidate cardiac PNMT activity, we locally administered NE. The concentration of NE was chosen to be in the same range as in the myocardial ischemic region based on the results of our previous study [6]. After control sampling, we locally administered NE (250 or 2500 ng/ml) through a dialysis probe for 60 min, with dialysate collected during the last 15 min. The same protocol was followed after administration of a PNMT inhibitor (SKF29661) [16] in separate rabbits. SKF29661 (50 mg/kg) was administered intraperitoneally 60 min before control sampling. To confirm whether PNMT activity was located in sympathetic nerve endings or myocardium, we performed chemical sympathetic denervation with hydroxydopamine (6-OHDA) and examined the dialysate EPI response to local NE infusion. Five days previously, rabbits were given 60 mg/kg 6-OHDA intravenously [17]. Dialysate EPI response to NE infusion was measured. Furthermore, we examined the effect of NE uptake$_2$ inhibition on the EPI response to local NE infusion. We added corticosterone (1 mM) on the perfusate and measured the dialysate EPI response to local NE infusion.

2.3.2. Time course of dialysate EPI levels during the myocardial ischemia in the presence and absence of SKF29661

After control sampling, we occluded the main branch of LCX for 60 min and then released the occluder. We observed the time course of dialysate EPI levels in the ischemic region in six rabbits. We collected five consecutive 15-minute dialysate samples during coronary occlusion and reperfusion (vehicle group). To examine the involvement of PNMT activity on the EPI level, we intraperitoneally administered a PNMT inhibitor (SKF29661) 60 min before control sampling in separate rabbits (SKF group). We performed LCX occlusion and collected dialysate samples as described in vehicle group. We compared EPI responses to LCX occlusion between vehicle and SKF groups.

2.3.3. Influence of desipramine on dialysate EPI levels during myocardial ischemia with and without SKF29661

EPI can be released via non-exocytotic release at the sympathetic nerve terminals [18]. To elucidate the involvement of catecholamine transporter on EPI levels, we locally administered an inhibitor of NE uptake$_1$ carrier desipramine (100 μM) through a dialysis probe. One hour thereafter, we performed LCX occlusion and collected dialysate samples in

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

	Control	Coronary occlusion (min)				Reperfusion (min)
		15	30	45	60	15
Heart rate (bpm)						
Vehicle group ($n=6$)*	297±10	289±8	283±9	280±9	281±7	269±6
SKF29661 group ($n=6$)	319±11	312±12	311±13	313±13	311±14	306±13
Desipramine group ($n=6$)	312±10	315±11	317±10	318±12	314±13	309±12
Desipramine+SKF29661 group ($n=6$)	316±12	310±10	314±8	315±7	317±8	311±8
Mean arterial pressure (mm Hg)						
Vehicle group ($n=6$)	96±8	87±7	84±5	83±4	81±5	81±3 †
SKF29661 group ($n=6$)	97±3	93±4	93±4	92±4	92±4	91±4 †
Desipramine group ($n=6$)	98±6	94±6	91±5	89±5	88±4	87±4 †
Desipramine+SKF29661 group ($n=6$)	101±2	88±6	90±4	88±4	88±3	83±3 †

†Values are means±SE. *$P<0.05$ vs. values of pretreatment groups.

the above-described protocol in separate rabbits (desipramine group). Furthermore, we tried to determine the influence of desipramine on PNMT induced EPI release during myocardial ischemia. We co-administered intraperitoneally SKF29661 (50 mg/kg) and desipramine locally through a dialysis probe continuously 60 min before control sampling (desipramine+SKF group). We performed LCX occlusion and collected dialysate samples as described in vehicle group. We compared EPI responses to LCX occlusion between desipramine and desipramine+SKF groups.

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

2.4. Statistical methods

The effects of myocardial ischemia (NE infusion) and pretreatment were examined using two-way analysis of variance. When statistical significance was detected between two groups, the dialysate EPI levels with and without a PNMT inhibitor were compared by unpaired t-test. The dialysate NE levels were compared among four groups using one-way analysis variance followed by Newman–Keuls test for the multiple comparisons against each other. The data of heart rate and mean arterial pressure were compared among four groups using two-way analysis variance. When statistical significance was detected, the Newman–Keuls test was applied. Statistical significance was defined as $P<0.05$. Values are presented as means±SE.

3. Results

3.1. Time course of heart rate and mean arterial pressure

Local administration of NE through a dialysis probe did not alter heart rate (HR) or mean arterial pressure (MAP) in vehicle or SKF groups. The time course of HR and MAP during myocardial ischemia and reperfusion is shown in Table 1. Coronary occlusion tended to cause a fall in HR and MAP, but no statistically significant alterations in HR or MAP were obtained against a baseline value within each group. Basal HR in vehicle group was lower than that in the other three groups.

3.2. Dialysate EPI response during local NE administration through a dialysis probe

Fig. 1 shows data obtained from local administration of exogenous NE through a dialysis probe. Dialysate EPI levels increased significantly with increases in the rate of NE infusion. Dialysate EPI levels reached 734.5±125, and 2081±367 pg/ml ($n=6$) at 250 and 2500 ng/ml of NE infusion, respectively. In the presence of SKF29661, dialysate EPI levels were significantly suppressed compared to those of the vehicle group. Dialysate EPI levels were 68±25, 282±70 pg/ml ($n=6$) at 250 and 2500 ng/ml of NE infusion, respectively. SKF29661 attenuated EPI responses to 10% of vehicle group. In sympathetic denervation with 6-OHDA, the dialysate EPI response to NE infusion (250 ng) was preserved ($n=4$, 760±193 pg/ml). With the perfusate containing the NE uptake$_2$ inhibitor, corticosterone (1 mM), the dialysate EPI response to NE infusion (250 ng) was suppressed ($n=6$, 167±27 pg/ml).

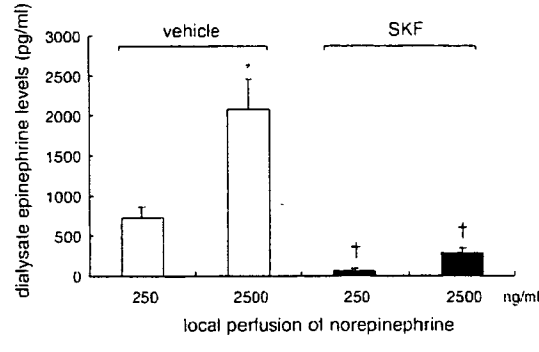

Fig. 1. Dialysate epinephrine levels evoked by norepinephrine perfusion through dialysis probe. SKF = SKF29661, *$P<0.05$ vs. value at 0–15 min of norepinephrine perfusion, †$P<0.05$ vs. concurrent value of vehicle group.

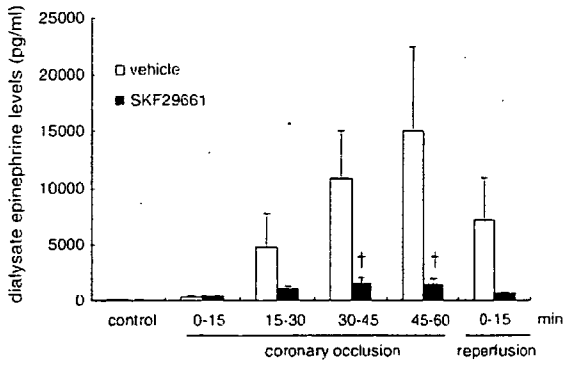

Fig. 2. Time course of dialysate epinephrine levels during coronary occlusion. Values are means±SE. †$P<0.05$ vs. concurrent value of vehicle group.

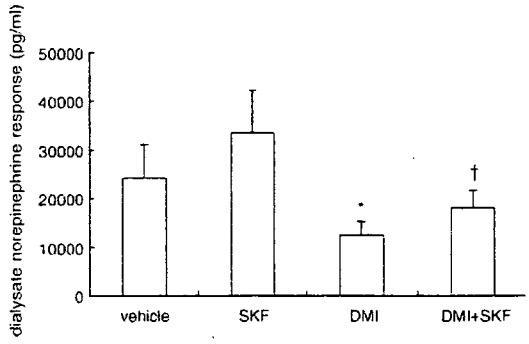

Fig. 4. Dialysate norepinephrine responses to 60 min coronary occlusion. SKF = SKF29661, DMI = desipramine, DMI+SKF = desipramine+SKF29661. Values are means±SE. *$P<0.05$ vs. value of vehicle group. †$P<0.05$ vs. value of SKF group.

3.3. Dialysate EPI levels in the ischemic region

Coronary occlusion significantly increased dialysate EPI levels (Fig. 2). In the vehicle group, dialysate EPI levels were 59.6±39.8 pg/ml in the control and increased after coronary occlusion. During 60 min coronary occlusion, dialysate EPI levels markedly increased and reached 15030±7418 pg/ml ($n=6$) at 45–60 min of occlusion. After reperfusion, dialysate EPI levels decreased to 7193±3722 pg/ml, although their levels were higher than those in the control. In the presence of SKF29661, dialysate EPI levels also increased and reached 1493±196 pg/ml ($n=6$) at 45–60 min of occlusion. These increases in dialysate EPI levels after 30 min of coronary occlusion were significantly attenuated by SKF29661.

3.4. Dialysate EPI levels in the ischemic region during local desipramine administration

Although coronary occlusion increased dialysate EPI levels, these levels were suppressed during local desipramine administration compared to the vehicle group (Fig. 3). During 60 min coronary occlusion, dialysate EPI levels increased and reached 743±171 pg/ml ($n=6$) at 45–60 min of occlusion. After reperfusion, dialysate EPI levels decreased to 400±181 pg/ml, although their levels were higher than those in the control. In the presence of SKF29661, dialysate EPI levels also increased and reached 725±154 pg/ml ($n=6$) at 45–60 min of occlusion. These increases in dialysate EPI levels were not attenuated by SKF29661.

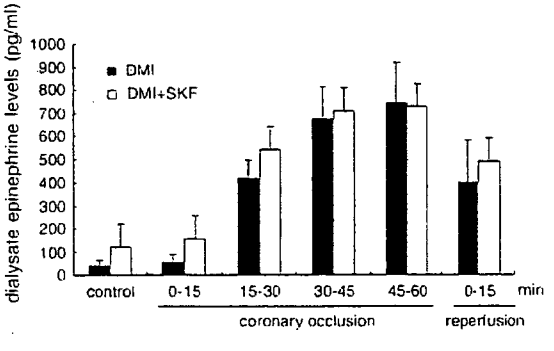

Fig. 3. Influence of desipramine on dialysate EPI levels during myocardial ischemia with and without SKF29661. DMI = desipramine, DMI+SKF = desipramine+SKF29661. Values are means±SE.

3.5. Comparison of peak dialysate NE levels of the 4 groups

EPI is synthesized from NE, and so myocardial interstitial NE levels might affect myocardial interstitial EPI levels. We compared with myocardial interstitial NE levels at 45–60 min of coronary occlusion in the vehicle group, the SKF29661 group, the desipramine group and the desipramine and SKF29661 group (Fig. 4) ($n=6$-6-6-6). In the latter two groups (desipramine, desipramine+SKF29661), ischemia-induced peak dialysate NE levels were significantly suppressed in comparison with that of the vehicle or SKF29661 group. Calculated ratios of interstitial EPI/NE were 1.5±0.4 and 12±4% at NE infusion (250 ng) and 45–60 min of occlusion, respectively.

4. Discussion

4.1. Changes in myocardial interstitial EPI levels during local administration of NE through a dialysis probe

Local administration of NE dose dependently increased dialysate EPI levels. The pretreatment with PNMT inhibitor SKF29661 significantly suppressed these increases. Therefore, the EPI levels in dialysate could serve as an index of PNMT activity during local administration of NE. To our knowledge, this is the first direct assessment of cardiac PNMT activity in in vivo heart. These results indicate that EPI can be regionally synthesized from NE with PNMT activity in the heart. Regionally administered NE in myocardial interstitium was taken up by cardiac sympathetic nerve endings via the uptake$_1$ carrier or by extraneuronal cells via uptake$_2$ carrier [19–21]. Several studies demonstrated the existence of PNMT in the myocardium rather than

sympathetic nerve endings [17,22]. In sympathetic denervation with 6-OHDA, the dialysate EPI response to NE infusion was preserved. The dialysate EPI response was suppressed by pretreatment with corticosterone (an NE uptake$_2$ inhibitor). Our data were also consistent with those studies. NE might be taken up by myocardial cell via uptake$_2$ carrier and converted to EPI with PNMT. Recent study has shown that gene expression of the PNMT is localized not in cardiac ganglion, but in cardiomyocytes [23]. Therefore, our data suggest that high NE level in myocardial interstitium yields EPI synthesis by regional PNMT activity. NE that was taken up by extraneuronal cells was metabolized mainly to normetanephrine (NMN) or 3-methoxy-4-hydroxyphenylglycol (MHPG) by catechol *O*-methyltransferase (COMT) [19], but high NE was partly available for EPI synthesis with PNMT. These elevated NE levels were similar to the levels of myocardial ischemic regions in our previous studies [6,24]. Therefore, EPI synthesis with PNMT may gain relevance during myocardial ischemia.

4.2. Myocardial interstitial EPI levels during coronary artery occlusion

Coronary occlusion-induced progressive increases in dialysate EPI levels. These increases corresponded to increases in dialysate NE levels. Our data suggest that the high NE level evoked by myocardial ischemia yields EPI synthesis by regional PNMT activity. During myocardial ischemia, calculated ratio of interstitial EPI/NE was eight-times higher than that of NE infusion. In the ischemic heart, normal transport of NE is impaired because of a reduced sodium gradient [5], whereas another uptake system is operative by extraneuronal cells via the uptake$_2$ carrier which is independent of the sodium gradient [25]. Actually myocardial ischemia evoked increases in myocardial interstitial NMN or MHPG via the uptake$_2$ carrier [26]. The time course of dialysate EPI levels corresponded to increases in dialysate NE and NMN levels. Therefore, we consider that released NE is taken up by extraneuronal cells and PNMT activity for EPI synthesis is operative at high concentration of NE.

To confirm EPI synthesis via PNMT activity, we examined ischemia-induced dialysate EPI levels in the presence and absence of a PNMT inhibitor. PNMT inhibition suppressed the increase in dialysate EPI (synthesis by PNMT) and augmented the increase in dialysate NE (substrate of PNMT) levels. Thus, in the ischemic period as well as local administration of NE, PNMT activity plays an important physiological role in NE gradation and EPI synthesis. The PNMT activity in the ischemic left ventricle augmented EPI production by excess of substrate. The increased PNMT activity might reflect compensatory or adaptation processes secondary to impairments of the catecholamine uptake system and its degradation via monoamine oxidase [6].

At the myocardial interstitial space, local ω-conotoxin GVIA treatment attenuated the EPI release in response to cardiac sympathetic nerve stimulation [18]. Furthermore, local tyramine administration caused an increase in dialysate EPI level via a non-exocytotic mechanism. The previous study demonstrated that EPI is released from vesicle and axoplasma via exocytosis and carrier-mediated transport in the cardiac sympathetic nerve endings. In the resting state, myocardial interstitial EPI is extracted mainly from circulating EPI and taken up via catecholamine transporter to nerve endings. Therefore, in the sympathetic nerve endings containing EPI, the non-exocytotic release via outward transport would be involved in EPI release evoked by myocardial ischemia.

Myocardial ischemia-induced increases in dialysate EPI levels were suppressed by the pretreatment with desipramine. Desipramine suppressed peak EPI levels by 5% of vehicle group. Marked suppression of EPI release can be explained by two possible mechanisms. First, desipramine inhibits both directions of NE transport through uptake$_1$ carrier [5,27]. Ischemia-induced outward NE transport through uptake$_1$ carrier is inhibited by desipramine, and so myocardial interstitial NE levels are also attenuated [6]. In this way, desipramine reduced the substrate of EPI via PNMT and EPI synthesis in extraneuronal cells. Alternatively, EPI is released via carrier-mediated outward transport of EPI from sympathetic nerve endings. The present study could not clarify whether EPI specific transporter or NE transporter is involved in carrier-mediated outward transport of EPI. But desipramine inhibits transports of both EPI and NE. Thus, both actions of desipramine on NE and EPI caused a marked decrease in the EPI release evoked by myocardial ischemia. Although desipramine markedly suppressed the EPI release evoked by myocardial ischemia, it is uncertain which factor is more responsible for EPI release.

Finally, to elucidate which of these two mechanisms is mainly involved in the EPI release evoked by myocardial ischemia, we compared ischemia-induced EPI release between desipramine alone and the combination of desipramine and SKF29661 pretreatments. Myocardial ischemia-induced EPI release did not differ between the two groups. This result indicates that the EPI synthesis by PNMT might not be involved in the EPI release evoked by myocardial ischemia. In the presence of desipramine, myocardial interstitial NE levels were markedly suppressed. These NE levels might not be operative as the substrate of EPI whereas only the markedly higher NE level in vehicle group might be operative as the substrate of EPI and yield EPI synthesis via PNMT activity. Thus, PNMT in the left ventricle is capable of synthesizing EPI with markedly elevated NE in the myocardial interstitial space. As well as COMT system [28], cardiac PNMT plays an important physiological role in NE degradation during high concentrations of myocardial interstitial NE. Since EPI preferentially interacts with beta$_2$-adrenergic receptors in heart [29]. Regional EPI might promote exocytotic NE release by activating presynaptic beta$_2$-adrenergic receptors. Future work should concentrate on these aspects of cardiac PNMT.

4.3. Methodological considerations

In general, EPI is released from the adrenal medulla and carried to the heart via the bloodstream [9]. In the present study, we administered a PNMT inhibitor SKF29661 intraperitoneally to block EPI synthesis. SKF29661 may inhibit EPI synthesis at the adrenal grand and reduce blood EPI levels. In this way, administration of SKF29661 might affect EPI uptake and the content of EPI at the cardiac sympathetic nerve endings. There was no significant difference in the control dialysate EPI level between vehicle and SKF29661 group. Therefore, we believe that extraction of EPI from plasma EPI does not change the quantitative results obtained from the cardiac dialysis.

Animal studies demonstrated that two enzymes are involved in EPI synthesis: PNMT and nonspecific *N*-methyltransferase [30]. Nonspecific *N*-methyltransferase is less inhibited by the PNMT inhibitor SKF29661. This nonspecific *N*-methyltransferase was reported to be present in the heart, but the predominant cardiac enzyme is apparently PNMT. Actually pretreatment with SKF29661 suppressed NE-induced EPI release by 10% of vehicle group. Therefore, it is thought that nonspecific *N*-methyltransferase exerts little effect on the EPI release evoked by NE administration or myocardial ischemia.

In conclusion, there is a PNMT activity in the heart. Under local administration of NE or ischemic conditions, PNMT in the left ventricle is capable of synthesizing EPI with markedly elevated NE in the myocardial interstitial space. We consider two mechanisms to be involved in the increment of EPI during myocardial ischemia, namely EPI synthesis by cardiac PNMT in extraneuronal cells and the non-exocytotic release from the sympathetic nerve endings.

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ORIGINAL RESEARCH

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Visualization of Intracerebral Arteries by Synchrotron Radiation Microangiography

BACKGROUND AND PURPOSE: Small cerebral vessels are a major site for vascular pathology leading to cerebral infarction and hemorrhage. However, such small cerebral vessels are difficult to visualize by using conventional methods. The goal of our study was the development of methodology allowing visualization of small cerebral arteries in rodents, suitable for experimental models.

MATERIALS AND METHODS: Using barium sulfate as a contrast material, we obtained microangiographic images of physiologic and pathologic changes consequent to cerebral infarction in mouse brain by monochromatic synchrotron radiation (SR). To achieve high-resolution and high-contrast images, we used a new x-ray camera with a pixel size of 4.5 μm, and we set the energy level at 37.5 keV, just above the K absorption of barium.

RESULTS: Small intracerebral arteries (~30 μm in diameter) were clearly visualized, as well as the cortical branches (50–70 μm in diameter) at the brain surface. The limit of detection appeared to be vessels ~10 μm in diameter. Compared with the noninfarcted side, the number of intracerebral arteries was dramatically decreased in the middle cerebral artery area affected by stroke.

CONCLUSIONS: These results indicate the potential of SR for evaluating pathologic changes in small cerebral arteries and for monitoring the impact of pro- and antiangiogenic therapeutic strategies.

Cerebrovascular disease is one of the major causes of death and disability in developed countries. To evaluate cerebral vasculature, conventional angiography and MR angiography are commonly used in clinical practice. The development of these imaging methods has allowed analysis of the pathologic features of cerebrovascular lesions and has guided therapeutic strategies. However, small cerebral vessels, including those known to harbor causative lesions in cerebral infarction and hemorrhage (due to lipohyalinotic changes and/or microaneurysm formation),[1] such as intracerebral arteries and perforators, are below the detection limit of conventional imaging techniques. An important step in developing therapeutic strategies effective against disease in small cerebral vessels is enhanced visualization of this vasculature, especially in experimental models.

Recently, ex vivo and in vivo microangiography using monochromatic synchrotron radiation (SR) has been suggested as a tool capable of visualizing pathophysiologic changes in small arteries. Using this system has made possible the detection of microcirculation in the dermis,[2] tumors,[3] and collateral microvessels in ischemic hind limbs.[4] Although fluorescence microscopy has also been used to image small arteries,[5-7] SR imaging has the advantage of visualizing microvessels, even after they enter the parenchyma of an organ. In contrast, fluorescence techniques do not allow adequate visualization of small arteries once a vessel is deep within brain or other parenchymal tissue. On the basis of these observations, we have developed a microangiographic system using SR and have investigated physiologic and pathologic features of rodent cerebral microvasculature.

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Materials and Methods

All procedures were performed in accordance with the National Cardiovascular Center Animal Care and Use Committee.

Preparation of Contrast Medium

For high-contrast images of the microcirculation, contrast agents included microspheres (Techpolymer I-2, Sekisui Plastics, Shiga, Japan) and barium sulfate (BarytgenSol, Fushimi, Tokushima, Japan). However, because the diameter of microspheres was 15 μm, whereas that of barium sulfate particles varied from 1–100 μm, the microcirculation of cerebral arteries could not be visualized by using these contrast media (not shown). To perfuse such microvessels (diameter <10 μm), we filtered barium sulfate (pore size 5 μm; Millex-SV, Millipore, Bedford, Mass) and obtained particles <5 μm in diameter. Filtered barium sulfate particles were then centrifuged (3000 G, 60 minutes) and concentrated to 50% by weight following removal of the supernatant.

Injection of Contrast Medium

Male severe combined immunodeficient (SCID) mice (6 weeks old; weight, 25–30 g; Oriental Yeast, Tokyo, Japan) were anesthetized by using inhaled diethyl ether and were perfused systemically with phosphate-buffered saline (PBS) containing heparin (40 U/mL) via the left ventricle of the heart with a peristaltic pump (Iwaki, Asahi Techno Glass, Chiba, Japan). Filtered barium sulfate particles (<5 μm in diameter, prepared as described previously; 50% by weight) were infused (0.7 mL), followed by isolation of the brain and fixation in formalin.