

Fig. 4 Equilibrium diagrams between the arterial baroreflex neural and peripheral arcs. The *dashed* and *solid curves* represent the open-loop characteristics of the baroreflex neural arc under the control and ANG II-treated conditions, respectively. The *dashed* and *solid lines* represent the open-loop characteristics of the baroreflex peripheral arc under the control and ANG II-treated conditions, respectively. The *open circle* indicates the closed-loop operating point under the control condition. ANG II causes an upward shift in the peripheral arc. If ANG II does not affect the neural arc, the closed-loop operating point would be at the point depicted by the *open triangle*. In this case, the estimation of baroreflex control of SNA based on the closed-loop operating points (the *open circle* and *open triangle*) approximates the slope of the baroreflex neural arc (*dashed curve*). ANG II, however, causes a rightward shift in the neural arc. Thus, the estimation of the baroreflex control of SNA based on closed-loop operating points (the *open* and *filled circles*) does not match the slope of the neural arc under either the control (*dashed curve*) or ANG II-treated condition (*solid curve*)

II administration. ANG II does modify the arterial baroreflex in that it increases SNA at a given baroreceptor pressure level but does not appear to attenuate the range of arterial baroreflex control of SNA, HR or AP.

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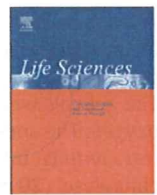
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Detection of endogenous acetylcholine release during brief ischemia in the rabbit ventricle: A possible trigger for ischemic preconditioning

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ABSTRACT

Aims: To examine endogenous acetylcholine (ACh) release in the rabbit left ventricle during acute ischemia, ischemic preconditioning and electrical vagal stimulation.

Main methods: We measured myocardial interstitial ACh levels in the rabbit left ventricle using a cardiac microdialysis technique. In Protocol 1 ($n=6$), the left circumflex coronary artery (LCX) was occluded for 30 min and reperused for 30 min. In Protocol 2 ($n=5$), the LCX was temporarily occluded for 5 min. Ten minutes later, the LCX was occluded for 30 min and reperused for 30 min. In Protocol 3 ($n=5$), bilateral efferent vagal nerves were stimulated at 20 Hz and 40 Hz (10 V, 1-ms pulse duration).

Key findings: In Protocol 1, a 30-min coronary occlusion increased the ACh level from 0.39 ± 0.15 to 7.0 ± 2.2 nM (mean \pm SE, $P < 0.01$). In Protocol 2, a 5-min coronary occlusion increased the ACh level from 0.33 ± 0.07 to 0.75 ± 0.11 nM ($P < 0.05$). The ACh level returned to 0.48 ± 0.10 nM during the interval. After that, a 30-min coronary occlusion increased the ACh level to 2.4 ± 0.49 nM ($P < 0.01$). In Protocol 3, vagal stimulation at 20 Hz and 40 Hz increased the ACh level from 0.29 ± 0.06 to 1.23 ± 0.48 ($P < 0.05$) and 2.44 ± 1.13 nM ($P < 0.01$), respectively.

Significance: Acute ischemia significantly increased the ACh levels in the rabbit left ventricle, which appeared to exceed the vagal stimulation-induced ACh release. Brief ischemia as short as 5 min can also increase the ACh level, suggesting that endogenous ACh release can be a trigger for ischemic preconditioning.

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Introduction

Although ventricular vagal innervation is sparser than that observed in the atrium, we have previously demonstrated that electrical vagal stimulation and acute myocardial ischemia significantly increased myocardial interstitial acetylcholine (ACh) levels in the feline left ventricle (Kawada et al. 2000, 2001, 2006a,b, 2007). Potential differences between species, however, suggest that data obtained from the feline left ventricle may not be directly extrapolated to ventricular vagal innervation in other species (Brown 1976; Kilbinger and Löffelholz 1976). Compared with the feline heart, the rabbit heart is more frequently analyzed in investigations of myocardial ischemia and ischemic preconditioning. For instance, Qin et al. (2003) used isolated rabbit hearts to demonstrate that ACh and adenosine induce ischemic preconditioning mimetic effects through different signaling pathways. In our previous study, vagal stimulation increased the level of tissue inhibitor of metalloproteinase-1 (TIMP-1)

and reduced the level of endogenous active matrix metalloproteinase-9 (MMP-9) during ischemia–reperfusion injury in the rabbit left ventricle (Uemura et al. 2007). Despite its potential cardioprotective effects against myocardial ischemia, the profile of endogenous ACh release in the rabbit left ventricle is poorly understood *in vivo* owing to the difficulty in detecting low levels of myocardial interstitial ACh. Quantification of endogenous ACh release during myocardial ischemia and electrical vagal stimulation would help understand the potential cardioprotective effects of vagal stimulation. In the present study, we examined the effects of acute myocardial ischemia, ischemic preconditioning, and electrical vagal stimulation on myocardial interstitial ACh levels in the rabbit left ventricle *in vivo* using an improved high-performance liquid chromatography (HPLC) system that allowed us to detect low concentrations of ACh (Shimizu et al. 2009).

Materials and methods

Surgical preparation and protocols

Animal care was conducted in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, which has been approved by the Physiological Society of

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Japan. Japanese white rabbits weighing 2.5 kg to 3.1 kg (2.8 ± 0.1 kg, mean \pm SE) were anesthetized via intravenous administration of pentobarbital sodium (30–35 mg/kg) through a marginal ear vein. The animals were ventilated mechanically with room air mixed with oxygen. The anesthetic condition was maintained using a continuous intravenous infusion of urethane ($125 \text{ mg kg}^{-1} \text{ h}^{-1}$) and α -chloralose ($20 \text{ mg kg}^{-1} \text{ h}^{-1}$) through a catheter inserted in the right femoral vein. Mean arterial pressure (AP) was measured using a catheter inserted in the right femoral artery. Heart rate (HR) was measured from an electrocardiogram obtained using a cardiotelemetry. The animal was placed in a lateral position, and the left fourth and fifth ribs were partially resected to allow access to the heart. The heart was suspended in a pericardial cradle.

In Protocol 1 ($n=6$), which was designed to examine the effects of acute myocardial ischemia and reperfusion, a 3-0 silk suture was passed around a branch of the left circumflex coronary artery (LCX); both ends were passed through a polyethylene tube to make a snare to occlude the artery. A dialysis probe was implanted into the anterolateral free wall of the left ventricle perfused by the LCX. After collecting a baseline dialysate sample, the LCX was occluded for 30 min and reperused for 30 min. After the ischemia–reperfusion protocol was finished, the LCX was occluded again and a 5-ml bolus of 1% methylene blue was injected intravenously to confirm that the dialysis probe had been implanted within the area at risk for myocardial ischemia.

In Protocol 2 ($n=5$), which was designed to examine the effects of ischemic preconditioning (*i.e.*, a brief ischemic event preceding a major ischemic event), a 3-0 silk suture was passed around a branch of the LCX and both ends were passed through a polyethylene tube to make a snare. Two dialysis probes were implanted into the anterolateral free wall of the left ventricle perfused by the LCX; the probes were separated by at least 5 mm. Combining the dialysate samples obtained from the two dialysis probes increased the time resolution of the ACh measurement. After collecting a baseline dialysate sample, the LCX was temporarily occluded for 5 min which was followed by a 10-min interval. The LCX was then occluded for 30 min and reperused for 30 min. After the ischemia–reperfusion protocol was completed, the LCX was occluded again and a 5-ml bolus of 1% methylene blue was injected intravenously to confirm that the two dialysis probes had been implanted within the area at risk for myocardial ischemia.

In Protocol 3 ($n=5$), which was designed to examine the effects of electrical vagal stimulation, the vagus nerves were exposed and sectioned at the neck. Each sectioned vagus nerve was placed on a pair of bipolar platinum electrodes to stimulate the efferent vagus nerve. The nerve and the electrodes were fixed using silicone glue (Kwik-Sil, World Precision Instruments, Sarasota, FL, USA). Two dialysis probes were implanted into the anterolateral free wall of the left ventricle; the probes were separated by at least 5 mm. Dialysate samples obtained from the two dialysis probes were analyzed separately. After collecting baseline dialysate samples, the vagus nerves were stimulated at 20 Hz for 15 min and 40 Hz for 15 min. The stimulation amplitude was 10 V and the pulse duration was 1 ms. The 40-Hz stimulation often caused an initial cardiac arrest for a few seconds and was considered to be the most intensive stimulation in the present experimental settings. The 20-Hz stimulation was arbitrarily selected at a half of the maximum stimulation rate to observe the dependence of the ACh release on the stimulation rate.

At the end of each protocol, the experimental animals were sacrificed with an overdose of intravenous pentobarbital sodium. We performed a postmortem examination and confirmed that the dialysis probe(s) had been implanted within the left ventricular myocardium.

Dialysis technique

We measured dialysate concentrations of ACh as indices of myocardial interstitial ACh levels. The materials and properties of the

dialysis probe have been described previously (Akiyama et al. 1994). Briefly, we designed a transverse dialysis probe. A dialysis fiber (length, 8 mm; outer diameter, 310 μm ; inner diameter, 200 μm ; PAN-1200, 50,000-Da molecular-weight cutoff, Asahi Chemical, Japan) was glued at both ends to polyethylene tubes (length, 25 cm; outer diameter, 500 μm ; inner diameter, 200 μm). The dialysis probe was perfused at a rate of 2 $\mu\text{l}/\text{min}$ with Ringer's solution containing a cholinesterase inhibitor eserine (100 μM). Dialysate sampling was started from 2 h after probe implantation. In Protocols 1 and 3, one sampling period was set at 15 min, which yielded a sample volume of 30 μl . The actual dialysate sampling lagged behind a given collection period by 5 min owing to the dead space volume between the dialysis membrane and collecting tube. In Protocol 2, one sampling period was set at 5 min to increase the time resolution during the ischemic preconditioning, and dialysate samples from the two dialysis probes were combined to yield a sample volume of 20 μl . The sampling period was changed to 10 min during the main ischemic event to reduce the total number of samples. The amount of ACh in the dialysate was measured using an HPLC system with electrochemical detection (Eicom, Japan) adjusted to measure low levels of ACh (Shimizu et al. 2009). The concentration of ACh was calculated taking the sample volume in account.

Statistical analysis

All data are presented as the mean and SE values. We performed repeated-measures analysis of variance, followed by a Tukey test for all pairwise, multiple comparisons to examine changes in the ACh levels (Glantz 2002). Because the variance of measured ACh levels increased with their mean, statistical analysis was performed after logarithmic conversion of the ACh data (Snedecor and Cochran 1989). The AP and HR data were examined using repeated-measures analysis of variance, followed by a Dunnett's test for multiple comparisons against a single control (Glantz 2002). In Protocols 1 and 3, the baseline value was treated as the single control. In Protocol 2, the value measured just before the main ischemic event was treated as the single control. In all of the statistical analyses, differences were considered significant when $P < 0.05$.

Results

In Protocol 1, the myocardial interstitial ACh levels significantly increased during ischemia compared with the baseline value (Fig. 1). Although the ACh levels declined during reperfusion, they were still significantly higher than the baseline value. Changes in AP and HR are summarized in Table 1. Although AP did not change significantly during ischemia, it decreased significantly throughout the reperfusion period. The HR increased significantly after 30 min of ischemia, and remained high during the reperfusion period with the exception of the last data point.

In Protocol 2, the LCX was occluded for 5 min (ischemic preconditioning) and released for 10 min before the major ischemic event. The brief 5-min occlusion significantly increased the myocardial interstitial ACh level compared with the baseline value (Fig. 2). The ACh levels during the interval between the brief occlusion and the major occlusion did not differ from the baseline value. The ACh levels increased significantly during the major ischemic event compared with the baseline value. Although the ACh levels declined during reperfusion, they were still significantly higher than the baseline value. Changes in AP and HR are summarized in Table 2. Neither AP nor HR changed significantly compared with the respective control values measured after the 10-min middle interval.

In Protocol 3, electrical vagal stimulation significantly increased the myocardial interstitial ACh levels (Fig. 3). The ACh levels returned close to the baseline value just after vagal stimulation was terminated. The AP and HR values were significantly reduced by vagal stimulation (Table 3).

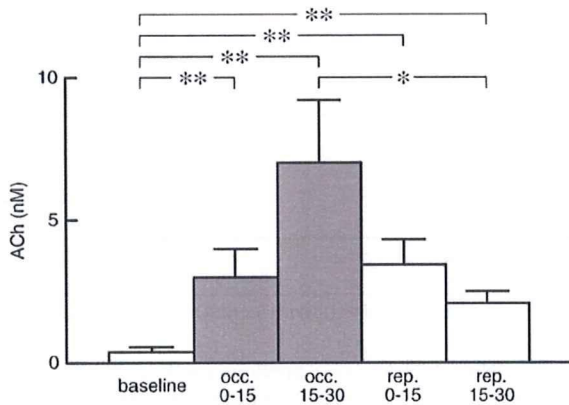


Fig. 1. Changes in the myocardial interstitial ACh levels in Protocol 1. The left circumflex coronary artery was occluded for 30 min and reperused for 30 min. occ: occlusion; rep: reperfusion. Data are shown as the mean + SE (n = 6). *P < 0.05 and **P < 0.01; Tukey test.

Discussion

Effects of acute ischemia on myocardial interstitial ACh levels

Acute myocardial ischemia significantly increased myocardial interstitial ACh levels in the ischemic region (Fig. 1). To our knowledge, this is the first report demonstrating ischemia-induced ACh release in the rabbit left ventricle *in vivo*. Because electrical vagal stimulation increased the myocardial interstitial ACh levels (Fig. 3), centrally mediated activation of the efferent vagus nerve could contribute to these effects. LCX occlusion, however, did not decrease the HR significantly (Table 1), suggesting that centrally mediated vagal activation did not have a marked role in the present study. In a previous study, acute myocardial ischemia increased myocardial interstitial ACh levels in vagotomized cats, suggesting an important role of a local release mechanism that is independent of efferent vagal activity (Kawada et al. 2000). Intracellular Ca²⁺ mobilization related to cation-selective stretch-activated channels is thought to be involved in this local release mechanism (Kawada et al. 2000, 2006b). A similar local mechanism may be responsible for ischemia-induced ACh release in the rabbit left ventricle.

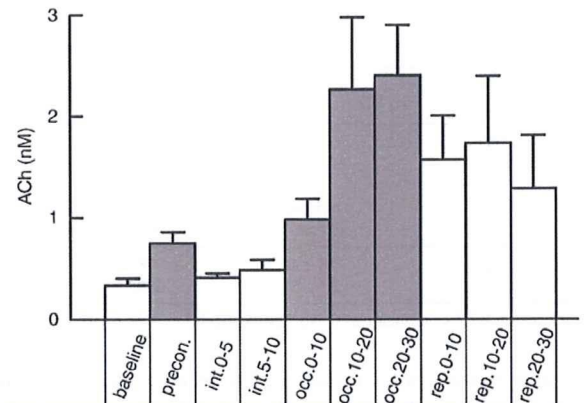
In our previous study, topical perfusion of ACh through a dialysis probe increased TIMP-1 levels in the rabbit left ventricle (Uemura et al. 2007). The production of TIMP-1 reduces endogenous levels of active MMP-9, which can limit ventricular remodeling following myocardial ischemia and reperfusion. Whether ischemia-induced ACh release can induce such an anti-remodeling effect remains unanswered, however, because reperfusion reduced the myocardial interstitial ACh levels toward the baseline value. Whether prolonged ischemia for more than 30 min induces sustained elevations of ACh levels is an interesting topic for future studies.

The ACh levels were decreased toward the baseline value upon reperfusion, probably by the washout of ACh from the interstitial fluid. In the case of myocardial interstitial myoglobin levels, the reperfusion further increases the myoglobin levels, suggesting an occurrence of reperfusion injury to the myocardium (Kitagawa et al. 2005).

Table 1
Mean arterial pressure (AP) and heart rate (HR) obtained during Protocol 1 (n = 6).

	Baseline	Occlusion 5 min	Occlusion 15 min	Occlusion 30 min	Reperfusion 5 min	Reperfusion 15 min	Reperfusion 30 min
AP(mm Hg)	82 ± 4	77 ± 4	72 ± 5	75 ± 5	72 ± 5*	70 ± 4*	70 ± 2**
HR (beats/min)	247 ± 16	264 ± 14	265 ± 13	280 ± 10**	278 ± 9*	277 ± 8*	274 ± 9

Data are shown as the mean ± SE. *P < 0.05 and **P < 0.01 vs. baseline using Dunnett's test.



baseline										
preconditioning	*									
interval 0-5										
interval 5-10										
occlusion 0-10	**		*							
occlusion 10-20	**	**	**	**						
occlusion 20-30	**	**	**	**	*					*
reperfusion 0-10	**	**	**	**						
reperfusion 10-20	**		**	**						
reperfusion 20-30	**		**	*			*			

Fig. 2. Changes in the myocardial interstitial ACh levels in Protocol 2. The left circumflex coronary artery was occluded for 5 min. Ten minutes later, the left circumflex coronary artery was occluded for 30 min and reperused for 30 min. precon: preconditioning; int: interval; occ: occlusion; rep: reperfusion. Data are shown as the mean + SE (n = 5). *P < 0.05 and **P < 0.01; Tukey test.

Reoxygenation upon reperfusion rapidly restores the ATP synthesis, which can cause hypercontracture of myofibrils and undesired cytoskeletal lesions (Piper et al. 2004). Because the vagal nerve endings do not have contractile elements, the hypercontracture-induced cell injury does not occur, and the further release of ACh may have been prevented.

Effects of ischemic preconditioning on myocardial interstitial ACh levels

Ischemic preconditioning is a phenomenon in which a brief ischemic event makes the heart resistant to a subsequent ischemic insult (Murry et al. 1986). Acetylcholine, bradykinin, and adenosine are endogenous substances that can induce ischemic preconditioning mimetic effects in the rabbit heart (Liu et al. 1991; Qin et al. 2003; Krieg et al. 2004). In a previous study, we showed that a 5-min ischemic event increased myocardial interstitial ACh levels in the feline ventricle (Kawada et al. 2002). Ischemic preconditioning, however, is not frequently examined in the feline ventricle, making interpretation of these results difficult. In the present study, a 5-min ischemic event caused a significant increase in the ACh level in the rabbit left ventricle (Fig. 2), suggesting that brief ischemia-induced ACh release may serve as a trigger for the ischemic preconditioning. Krieg et al. (2004) demonstrated that ACh triggers preconditioning by sequentially activating Akt and nitric oxide synthase to produce reactive oxygen species. An acetylcholine-induced preconditioning mimetic effect has also been observed in canine (Yao and Gross 1993; Przyklenk and Kloner 1995) and rat (Richard et al. 1995) models.

Table 2
Mean arterial pressure (AP) and heart rate (HR) obtained during Protocol 2 (n = 5).

	Baseline	Preconditioning 5 min	Interval 5 min	Interval 10 min	Occlusion 5 min	Occlusion 10 min
AP(mm Hg)	83 ± 5	77 ± 5	78 ± 4	80 ± 4	78 ± 5	78 ± 5
HR(beats/min)	277 ± 7	282 ± 8	282 ± 7	284 ± 5	285 ± 5	286 ± 6
	Occlusion 20 min	Occlusion 30 min	Reperfusion 5 min	Reperfusion 10 min	Reperfusion 20 min	Reperfusion 30 min
AP(mm Hg)	77 ± 4	78 ± 5	77 ± 5	78 ± 5	77 ± 3	79 ± 3
HR(beats/min)	287 ± 5	289 ± 6	290 ± 5	289 ± 5	290 ± 6	293 ± 5

Data are shown as the mean ± SE. No significant differences relative to control values (the value 10 min after the preconditioning) were observed based on Dunnett's test.

In a previous study examining the feline ventricle (Kawada et al. 2002), brief ischemia significantly decreased the HR, highlighting the presence of a significant vagal reflex from the heart. Vagotomy abolished the ACh release induced by brief ischemia in that study, suggesting an important role of centrally mediated vagal activation. The vagal reflex from the heart, however, shows regional differences and varies among species (Thames et al. 1978; Kawada et al. 2007). In the present study, brief ischemia did not decrease the HR significantly (Table 2), suggesting that centrally mediated vagal activation was not a major factor for the brief ischemia-induced ACh release in the rabbit heart.

Rabbits exhibit marked effects from ischemic preconditioning, including reduced infarct size (Cohen et al. 1991; Cason et al. 1997). Although whether the ACh release induced by the brief ischemic event exerted cardioprotective effects was not examined in the present study, there was a notable difference in the changes in AP observed with Protocol 1 and Protocol 2. Although AP decreased significantly upon reperfusion in Protocol 1 (Table 1), it did not change significantly during the major ischemic event in Protocol 2 (Table 2), possibly reflecting preserved cardiac function as a result of the ischemic preconditioning.

Effects of electrical vagal stimulation on myocardial interstitial ACh levels

In the feline left ventricle, electrical vagal stimulation at 20 Hz (10 V, 1-ms pulse duration) increases myocardial interstitial ACh levels to approximately 20 nM as measured with a dialysis fiber 13 mm in length (Kawada et al. 2000). In contrast, electrical vagal stimulation at 20 Hz in the rabbit left ventricle (10 V, 1-ms pulse duration) increased the ACh levels to approximately 1.2 nM as measured with a dialysis fiber 8 mm long (Fig. 3). The small increase in the ACh level detected during electrical vagal stimulation may indicate that vagal innervation is much sparser in the rabbit ventricle

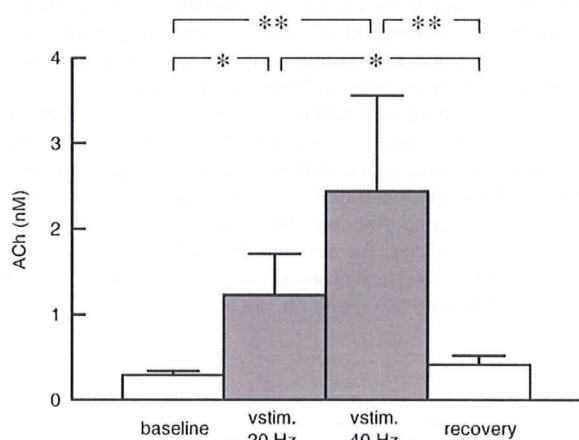


Fig. 3. Changes in the myocardial interstitial ACh levels in Protocol 3. The bilateral efferent vagus nerves were stimulated at 20 Hz for 15 min and 40 Hz for 15 min. Data are shown as the mean ± SE (n = 10, 2 samples from each of the 5 animals). *P < 0.05 and **P < 0.01; Tukey test.

than in the feline ventricle. In a previous study that used a dialysis fiber 4 mm in length, right vagal stimulation at 20 Hz increased the dialysate ACh concentration from 0.4 ± 0.2 nM to 0.9 ± 0.3 nM, whereas left vagal stimulation at 20 Hz increased it from 0.3 ± 0.1 nM to 1.0 ± 0.4 nM in the rabbit right ventricle (Shimizu et al. 2009). Considering the bilateral stimulation and fiber length of 8 mm in the present study, the vagal innervation of the left ventricle may be comparable to or slightly sparser than that of the right ventricle.

The dialysis fiber differed in length among studies due to anatomical restrictions related to the fiber implantation procedure (i.e., size of the heart etc.). If we consider diffusive processes alone, the relative recovery (RR) can be expressed as:

$$RR = \frac{C_{inside}}{C_{outside}} = 1 - \exp\left(-k\frac{A}{F}\right) = 1 - \exp\left(-k\frac{mL}{F}\right)$$

where C_{inside} and C_{outside} are the ACh concentrations inside and outside the dialysis fiber; A is the surface area of the dialysis membrane, which can be proportional to the fiber length L with a coefficient m; F is a perfusion flow rate; and k is the mass transfer coefficient (Stähle 1991). The *in vitro* RR for ACh is approximately 70% with F = 2 μl/min and L = 13 mm (Akiyama et al. 1994), which yields km = 0.1852. Using this value, the *in vitro* RR would be approximately 52% for L = 8 mm and 31% for L = 4 mm. Although these values provide some clues to speculate the effects of fiber length on the detected ACh concentrations, they cannot be directly extrapolated to the present results, because k should be different in *in vivo* conditions.

The physiological significance of vagal innervation of the left ventricle is controversial, because fixed-rate atrial pacing abolishes vagally induced inhibition of left ventricular contractility in an experimental setting without significant background sympathetic tone (Matsuura et al. 1997). On the other hand, when the cardiac sympathetic nerve is activated, vagal stimulation can reduce ventricular contractility even under fixed-rate atrial pacing by antagonizing the sympathetic effect (Nakayama et al. 2001). In addition, vagal stimulation suppresses myocardial interstitial myoglobin release during acute myocardial ischemia in anesthetized cats (Kawada et al. 2008). Chronic vagal stimulation improves the survival rate of rat models of chronic heart failure after myocardial infarction (Li et al. 2004). These lines of evidence suggest that vagal innervation of the left ventricle may be of therapeutic significance.

An unresolved question regarding the cardioprotective effects of vagal stimulation is that a large quantity of ACh is released in the ischemic region without vagal stimulation (Fig. 1). In the present

Table 3
Mean arterial pressure (AP) and heart rate (HR) obtained during Protocol 3 (n = 5).

	Baseline	Vagal stimulation 20 Hz	Vagal stimulation 40 Hz	Recovery
AP (mm Hg)	100 ± 3	59 ± 9**	54 ± 9**	86 ± 5
HR (beats/min)	322 ± 14	126 ± 5**	100 ± 8**	311 ± 8

Data are shown as the mean ± SE. **P < 0.01 vs. baseline based on Dunnett's test.

study, vagal stimulation at 20-Hz lowered the HR by approximately 200 beats/min (to less than 40% of the control value) but the stimulation-induced ACh release did not exceed the ischemia-induced ACh release (Figs. 1 and 3). On the other hand, vagal stimulation that reduced the HR by only 10% produces a significant increase in the survival rate of chronic heart failure rats (Li et al. 2004). Therefore, vagal stimulation probably exerts its beneficial effects not only within the ischemic region but also outside of this region. For instance, vagal stimulation in dogs with a healed myocardial infarction is known to prevent lethal arrhythmia induced by exercise (Vanoli et al. 1991). Afferent vagal activation may also contribute to the cardioprotective effects. Further studies are clearly needed to identify the mechanisms underlying the vagally induced cardioprotective effects against myocardial infarction and chronic heart failure.

Conclusion

The present study demonstrated the presence of vagal innervation in the rabbit left ventricle. Acute myocardial ischemia significantly increased the myocardial interstitial ACh levels. In addition, a brief ischemic event (5 min) caused detectable increases in ACh levels, indicating that endogenous ACh release may provide a trigger for ischemic preconditioning.

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Effect of the cholinesterase inhibitor donepezil on cardiac remodeling and autonomic balance in rats with heart failure

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Abstract

We have previously shown the beneficial effect of direct vagal electrical stimulation on cardiac remodeling and survival. In this study, we tried to reproduce the effect of vagal enhancement by administration of an acetylcholinesterase inhibitor, donepezil. A rat model of heart failure following extensive healed myocardial infarction was used. In rats given donepezil (5 mg/kg/day) in drinking water, biventricular weight was smaller (3.40 ± 0.13 vs. 3.02 ± 0.21 g/kg body weight, $p < 0.05$), and maximal rate of rise (3256 ± 955 vs. 3822 ± 389 mmHg/s, $p < 0.05$) and enddiastolic value (30.1 ± 5.6 vs. 23.2 ± 5.7 mmHg, $p < 0.05$) of left ventricular pressure were improved. Neurohumoral factors were suppressed (norepinephrine, 1885 ± 1423 vs. 316 ± 248 pg/ml, $p < 0.01$; BNP, 457 ± 68 vs. 362 ± 80 ng/ml, $p < 0.05$). High frequency component of heart rate variability showed a nocturnal increase. These findings indicated that donepezil reproduced the anti-remodeling effect of electrical vagal stimulation. Further studies are warranted to evaluate the clinical usefulness of donepezil in heart failure.

(149 words)

Keywords

Myocardial infarction, Vagal stimulation, Heart rate variability, Neurohumoral activation

Introduction

Profound imbalance of the autonomic nervous system has been considered to be an important factor that aggravates heart failure [1]. The imbalance includes not only overactive sympathetic activity but also diminished vagal activity [2]. Various therapeutic agents including beta-blockers [3, 4], angiotensin converting enzyme inhibitors [5, 6], and angiotensin receptor antagonists [7, 8] have proven useful at least partly by correcting the abnormally augmented sympathetic activity. However, few endeavors have been made to actively remedy the reduced vagal activity as a treatment for heart failure. As the first attempt of this therapeutic strategy, our group has shown that in rats with aggravating chronic heart failure after experimentally-induced healed myocardial infarction, electrical stimulation of the vagus nerve markedly improved survival through prevention of cardiac remodeling [9].

Since the efferent vagal nerve activity is transmitted by acetylcholine, drugs that increase the acetylcholine concentration at the neuro-effector junction are expected to have the similar effect as electrical stimulation. In fact, clinical trials of the acetylcholinesterase inhibitor pyridostigmine have been conducted in patients with chronic heart failure [10, 11], resulting in decreased ventricular arrhythmia, enhanced heart rate variability at rest, increased heart rate reserve and oxygen pulse during exercise, as well as improved heart rate recovery after exercise. However, these studies examined the effect of short-term administration (one to two days), and the long-term effect of pyridostigmine has not been investigated. Clinical trials have also been conducted on scopolamine that stimulates vagus nerve centrally at low doses [12, 13]. Transdermal administration of a small dose of scopolamine in patients with heart failure following myocardial infarction increased heart rate variability and enhanced baroreflex sensitivity. These studies have not shown, however, anti-remodeling effect, more direct evidence against the progression of heart failure.

We hypothesized that donepezil, a novel acetylcholinesterase inhibitor, would show

various clinically-relevant beneficial effects through its preferential effects on neural true cholinesterase (rather than hepatic pseudocholinesterase) [14]. Therefore, in the present study, we investigated the effect of donepezil on hemodynamics, neurohumoral activation, and cardiac remodeling in rats with chronic heart failure. In addition, we analyzed high-frequency component of the heart rate variability to assess changes in vagal tone [15, 16]. The results indicated that donepezil reproduced anti-remodeling effect of electrical stimulation of the vagus nerve, and increased vagal tone.

Materials and Methods

The protocol of this study was performed in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, and was approved by the Experimental Animal Committee of the National Cardiovascular Center.

Chronic heart failure model

Male Sprague-Dawley rats (8 weeks of age) were used. Under halothane anesthesia, a thoracotomy was performed and the main branch of the left coronary artery was ligated with nylon to produce myocardial infarction. The ligation resulted in myocardial infarction of 45 to 55%. The rats recovered from extensive myocardial infarction and progressed to the chronic state of heart failure (see Results). For ventricular fibrillation that occurred within one hour of ligation, defibrillation was conducted actively by cardiac massage in order to salvage as far as possible the rats with extensive myocardial infarction.

Experimental protocol

One week after induction of myocardial infarction, the surviving rats underwent another operation under halothane anesthesia. An electrocardiogram (ECG) telemetry device was implanted in each rat to monitor ECG and heart rate continuously (Figure 1A).

Rats that survived another week were divided into a nontreated group and a donepezil group. The donepezil group was administered the acetylcholinesterase inhibitor donepezil (Aricept®, Eisai, Tokyo, Japan) dissolved in drinking water at a concentration of 50 mg/dl. The dose estimated from the volume of water consumed was 5 mg/kg/day on average. The selection of donepezil rested on the fact that it more inhibits the (true) acetylcholinesterase at synapses and effectors but less inhibits pseudoacetylcholinesterase (butyrylcholinesterase) in liver than other drugs [14].

At week 6 after treatment was started (week 8 after infarction), 13 rats in the nontreated group and 14 rats in the donepezil group were subjected to hemodynamic study under halothane anesthesia. After hemodynamic study and blood collection, the rats were euthanized by overdose of halothane, and histological examination was conducted.

In other 11 rats with similar healed myocardial infarction, heart rate variability was calculated from the continuous ECG recordings between weeks 12 to 20 after myocardial infarction. In 11 rats, 5 served as the nontreated group (weeks 12-20 after infarction) and 6 received the donepezil treatment (weeks 17 to 19 after infarction). Preliminary analysis indicated no differences in heart rate variability at 8 weeks after infarction.

Hemodynamic measurement

At week 6 of the treatment period, hemodynamic study was conducted in rats under halothane anesthesia. A Millar catheter (SPC-320, Millar Instruments, Houston, TX) was inserted from the carotid artery into the left ventricle to measure left ventricular pressure with high fidelity. From the left ventricular pressures, the maximal first derivative of left ventricular pressure over time (dP/dt_{max}) and left ventricular enddiastolic pressure was calculated. The right atrial pressure was measured by an external transducer via a catheter filled with physiological saline.

Neurohumoral factor measurements

Three ml of blood was collected and the neurohumoral factors in blood were assayed. As indices of sympathetic activity, norepinephrine (NE) and epinephrine (Epi) were measured using by high-performance liquid chromatography with electrochemical detection. Plasma level of brain (or B-type) natriuretic peptide (BNP) was measured by ELISA assay (BNP-32 Enzyme Immunoassay Kit, Peninsula Lab, San Carlos, CA). We included BNP for its importance as a strong predictor of prognosis [17, 18]. BNP has been useful in detecting new patients with heart failure and in predicting the mortality and cardiac events in patients as well as in asymptomatic subjects. BNP may also be useful with heart failure with preserved systolic function.

Heart tissue examination

The left and right ventricles were excised and the total weight was measured. Next, both ventricles were sectioned into 3 mm-thick three slices, starting from the apex towards the base of the heart. Myocardial infarction size was assessed from the proportion of the length of infarct to the left ventricular perimeter measured on each section.

Power spectral analysis of heart rate variability

The ECG telemetric data were processed as follows. Signals from the transmitter model TA11CTA-F40 (Data Sciences International, St. Paul, MN) were recorded on a recording software HEM (Notocord, Newark, NJ). From the data of the continuous recording (1 kHz sampling), an analysis software HRT10a1 (Notocord, Newark, NJ) was used to extract the RR intervals. All the RR intervals were extracted from 24-hour continuous recording data for the nontreated and the donepezil groups. The text data of 2-hour intervals were stored in files to be analyzed later using the heart rate variability analysis software that we developed. Due to the frequent occurrence of extrasystoles in

chronic heart failure, it was necessary to develop an original algorithm to process the data as explained below.

Heart rate variability analysis software

The following procedures were conducted.

(1) Data preparation

The 2-hour data were combined to obtain 24-hour data. The time of R wave detection and the RR interval were saved as combined data.

(2) Removal of extrasystole

A 20-point median filter was applied to all the RR interval data to produce a sequence. Heart beats with RR intervals differing from the median value by 15 msec (threshold) or above were recognized and recorded as extrasystole or post-extrasystole. These data were excluded from analysis.

(3) Resampling of valid interval data

The 24-hour data were divided into 6-minute data (with 50% overlap). After excluding the RR intervals associated with extrasystole, the valid RR interval data were resampled at intervals of 1/10 seconds using linear interpolation.

(4) Power spectral analysis

In the power spectral analysis, 1024 points of 1/10-second data were grouped into a segment (segment length = 100.24 seconds) for fast Fourier transformation (FFT). The power spectra obtained from 6 segments were ensemble averaged. Prior to FFT, linear trend was removed from each segment

(5) Data selection

Even though extrasystoles are removed, segments with many deleted data cannot be expected to yield reliable power spectral analysis results. Therefore data with 40 or more extrasystoles within 6 minutes were excluded from analysis.

(6) Definition of high frequency component (HF)

In this study, the effect of bigeminy that occurs in heart failure was observed in the higher frequency range. Therefore we excluded frequency range > 1.5 Hz and HF was defined as the power from 0.5 to 1.5 Hz. Power of HF component was determined during daytime (6:00 to 18:00) and nighttime (18:00 to 6:00).

Statistical analysis

All data are presented as mean \pm SD. Continuous variables were compared using unpaired t-test between two groups. The differences were considered significant when $p < 0.05$.

Results

Hemodynamics

Figure 2 shows the results of hemodynamic parameters measured under anesthesia 6 weeks after the onset of donepezil administration. A left ventricular pressure waveform and its first derivative are exemplified in Figure 2A. In this example (in a nontreated rat) the maximal first derivative of left ventricular pressure (dP/dt_{max}) was markedly decreased. In the donepezil group, dP/dt_{max} was significantly increased compared to the nontreated group (3822 ± 389 versus 3256 ± 955 mmHg/s, $p < 0.05$, Figure 2B). Left ventricular enddiastolic pressure (LVEDP; 23.2 ± 5.7 versus 30.1 ± 5.6 mmHg, $p < 0.05$, Figure 2C) and right atrial pressure (RAP; 4.1 ± 2.9 versus 7.0 ± 4.0 mmHg, $p < 0.05$, Figure 2D) was significantly lowered by donepezil administration. The contractility index dP/dt_{max} is known as a heart rate- and preload-dependent index. Because heart rate was higher in the nontreated group (354 ± 37 vs. 324 ± 23 bpm, difference $\sim 9\%$) and LVEDP was higher in the nontreated group, the difference in heart rate and preload would have underestimated the true difference in contractility. Moreover, decreased LVEDP with decreased RAP in donepezil

group suggested that body fluid retention was suppressed.

Neurohumoral factors

Figure 3 shows the blood concentrations of norepinephrine, epinephrine and BNP measured 6 weeks after donepezil administration was started. Donepezil administration resulted in significant decreases in blood norepinephrine (316 ± 248 versus 1885 ± 1423 pg/ml, $p < 0.01$), epinephrine (347 ± 153 versus 1694 ± 1355 pg/ml, $p < 0.05$) and BNP (362 ± 80 versus 457 ± 68 ng/ml, $p < 0.05$) concentrations. These results indicated that donepezil effectively suppressed the overactive sympathetic nervous system, which is a hallmark pathophysiology of heart failure.

Infarct size and heart weight

Figure 1B shows representative ventricular sections in the nontreated and the donepezil groups. The myocardial infarction resulted from obliteration of the left coronary artery was $48 \pm 6\%$ of the left ventricular perimeter in the nontreated group and $53 \pm 3\%$ in the donepezil group, with no significant difference in infarct size between two groups. Therefore, donepezil administration starting two weeks after myocardial infarction did not reduce the infarct size, suggesting that infarct size did not account for the differences in hemodynamics and neurohumoral factors described above.

Figure 1C compares the ventricular weight per body weight between the nontreated and the donepezil groups. The combined weight of the left and right ventricles was significantly lower in the donepezil group compared to the nontreated group (3.02 ± 0.21 vs. 3.40 ± 0.13 g/kg body weight, $p < 0.05$). This result indicated that donepezil reduced cardiac remodeling after myocardial infarction was completed.

Power spectral analysis of heart rate variability

The left panel of Figure 4A shows a representative change in RR intervals with respect to time in a rat from the donepezil group. RR intervals connected with dotted lines were judged as extrasystoles or post-extrasystoles and were removed before spectral analysis. The right panel shows the result of spectral analysis from the same data. The area circumscribed by the thick lines was calculated as the HF component. The HF components during daytime (6:00 to 18:00, Figure 4B) and nighttime (18:00 to 6:00, Figure 4C) were calculated for the donepezil group (n = 6) and the nontreated group (n = 5). The log transformed HF components [$\log(\text{HF})$] of the two groups were analyzed statistically.

During the night, $\log(\text{HF})$ was significantly increased in the donepezil group compared to the untreated group. On the other hand, there was no significant difference in $\log(\text{HF})$ during the day between the two groups. These results indicated that heart rate variability at night was enhanced by donepezil administration in rats.

Discussion

Imbalance of the autonomic nervous system, particularly overactive sympathetic activity together with reduced vagal activity has been considered to be one of the major factors that aggravate heart failure. Our previous study has demonstrated that upstream treatment using electrical stimulation of the vagal nerve improves the survival rate in rats with heart failure after extensive healed myocardial infarction. Although pharmacological reproduction of the vagotonic treatment of heart failure would benefit clinically, no vagotonic drugs have successfully showed anti-remodeling, the most direct evidence against the progression of heart failure.

Our study results clearly demonstrated that donepezil treatment improved hemodynamics, ameliorated cardiac remodeling, and prevented neurohumoral activation. Because donepezil exerted no significant effects on infarct size, and donepezil was

administered after infarction had been established, these effects cannot be attributed to the reduction in ischemic insult. Although we have not shown the benefits on survival in this study, the similar hemodynamic, anti-remodeling and neurohumoral effects as electrical vagal stimulation may also be translated to survival. Further studies on survival are needed for its clinical application.

We failed to prepare sham-operated rats that would serve as a true control. To make up for this, we have shown historical control values for hemodynamic measurements (dP/dt_{max} , 11237 ± 1389 mmHg/s; LVEDP, 6.5 ± 2.3 mmHg; RAP 1.9 ± 1.3 mmHg), neurohumoral factor measurements (NE, 392 ± 205 pg/ml; Epi, 164 ± 46 pg/ml; BNP 62 ± 7 pg/ml), and biventricular weight (2.22 ± 0.11 g/Kg) obtained from the same strain and similar age of rats. These control values indicate that hemodynamic deterioration, neurohumoral activation, and cardiac remodeling were only partially reversed except for NE. Notwithstanding, the results with the electrical stimulation of vagal nerves indicate that these small benefits may accompany a larger improvement in survival.

We selected donepezil, a novel cholinesterase inhibitor to maximize its inhibitor action on neuronal acetylcholinesterase but not on hepatic butyrylcholinesterase inhibitor [14]. We intentionally used donepezil, a drug acting both peripherally and centrally, to simulate electrical stimulation of the vagus nerve. Electrical stimulation affected both the afferent and efferent pathways of the vagus nerve, and the detailed therapeutic mechanisms including which of the two pathways plays a greater role in the therapeutic effect has remained unclear. However, the drug with dual central and peripheral action was certainly inappropriate for deepening mechanistic insights.

Mechanistic study would be important as donepezil itself may not be clinically applicable. The dose in rats, which we aimed at decreasing heart rate by 10 %, was 50 times larger than dose used for Alzheimer's disease. Although the present study does not elucidate how large is the contribution of each of the effect of donepezil on the peripheral

vagus nerve, ganglion, and central nervous system, we would like to add some mechanistic discussion for designing future studies.

Regarding the mechanism downstream of the neuro-effector junction, the neurotransmitter acetylcholine per se may have some protective effect for cardiomyocytes. In fact, Sato et al. have obtained several lines of evidence supporting this hypothesis from acute studies. First, acetylcholine promotes the phosphorylation of connexin 43, a gap junction molecule located between cardiomyocytes. This normalizes the intercellular ion flow and prevents the occurrence of fatal arrhythmia [19]. Second, acetylcholine directly enhances the phosphorylation of Akt via PI3K in the cardiomyocytes, and activates the PI3/Akt pathway to enhance the expression of hypoxia-inducible factor-1 α (HIF-1 α), which may protect the cardiomyocytes from the hypoxic state induced by ischemia [20]. As shown by these findings, the acetylcholine increased in the neuro-effector junction by vagal efferent activation possesses various functions that support the survival of cardiomyocytes. Further studies are required to study the contribution of acetylcholine in cardiomyocytes at molecular levels. Vagal enhancement at effector site may potentiate its anti-inflammation effects [21] and may ameliorate progression of heart failure through alpha 7-nicotinic receptors.

On the other hand, experiments using rat and canine models of heart failure have suggested the presence of abnormalities in the ganglia of the vagus nerve. For example, in rats with heart failure following myocardial infarction, the bradycardiac response to pre-ganglionic vagus stimulation was attenuated, while the bradycardiac response to acetylcholine was unchanged compared to control rats [22]. Furthermore, in dogs with heart failure induced by high frequency pacing, with pre-ganglionic vagus stimulation heart rate responses were attenuated, while postganglionic stimulation at the fat pad showed no difference in heart rate response compared to control dogs [23]. Taking together the above observations, donepezil may act on the ganglia of the vagus nerve in the present study.

Also, as donepezil passes the blood-brain barrier, the drug can act on the central nervous system. To gain an insight into the central effect, we conducted an analysis of heart rate variability. Heart rate variability, especially its high-frequency component (at respiratory frequency) reflected background vagal tone, and has been shown to be a strong prognostic determinant [15, 16]. Our results revealed that donepezil increased high frequency component (HF) of heart rate variability during the night, indicating enhanced vagal activity. On the other hand, HF of the heart rate variability tended to increase but not significantly during the day. These finding may suggest a central effect of donepezil, but again a secondary effect of improved hemodynamics cannot be ruled out. Regardless of the detailed mechanism, increased HF may be associated to better outcome in these rats, as shown in e.g., the ATRAMI study [24, 25]. These issues require further investigations.

In summary, the present study suggests that donepezil treatment, similar to electrical stimulation of the vagus nerve, confers beneficial effects in the prevention of cardiac remodeling in rats with heart failure following myocardial infarction. It is worthy to examine if survival would be improved by the administration of donepezil in rats with healed myocardial infarction.