Supplemental Methods

The animal experiments were approved by the National Cardiovascular Center Research Committee and were performed according to institutional guidelines.

Experimental Protocols

1) Effects of Metformin on Cardiomyocyte Viability and Apoptosis After Exposure to H_2O_2

To investigate whether metformin has a cardioprotective effect against damage due to H_2O_2 in vitro, we assessed cell viability and apoptosis in cultured cardiomyocytes using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and both the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining plus flow cytometry, respectively. The cells were cultured in serum-free media for 24 hours and then incubated in the presence of 50 μmol/L H_2O_2 for 24 hours. Cardiomyocytes were pretreated with either metformin (1 to 100 μmol/L) or 5-amino-4-imidazole-1-β-D-carboxamide ribofuranoside (AICAR; an AMPK activator) (500 μmol/L) for 60 minutes before the addition of H_2O_2. Other cells were preincubated with an AMPK inhibitor, compound-C (20 μmol/L) for 6 hours before the addition of either metformin or AICAR. Then cell viability and apoptosis were analyzed.

2) Effects of Metformin on Cardiac Performance in Dogs With Pacing-Induced Heart Failure

After pacemaker implantation, the dogs were randomly assigned to 3 groups as follows: 1) a group that received a normal diet and drinking water (Pacing group, n=8), 2) a group that received metformin orally at a dose of 100 mg/kg/day (Pacing+Met group, n=8), and 3) a group received AICAR

subcutaneously every other day at 5 mg/kg (Pacing+AICAR group, n=4). We also performed a sham operation in another 6 dogs (Sham group, n=6). The dose of metformin (100 mg/kg/day) was selected because our preliminary study showed that this was the maximum dose that did not induce hypoglycemia (data not shown). The dose of AICAR (5 mg/kg subcutaneously on alternate days) was selected because we preliminarily confirmed that phosphorylation of AMPK was elevated at least 48 hours after subcutaneous injection of AICAR, by reference to previous report in rats, due to the lack of any data for dogs (Supplemental Figures).[1] Echocardiography was performed and hemodynamic parameters were measured before and after 4 weeks of right ventricular (RV) pacing. After assessment of these parameters, each heart was excised and divided into three parts for immunoblotting, quantitative reverse-transcriptase polymerase chain reaction (PCR), and histological examination.

Materials

1, 1-Dimethylbiguanide hydrochloride (metformin hydrochloride) was a kind gift from Nippon Shinyaku Co. Ltd. (Kyoto, Japan), while AICAR (an AMPK activator) and compound-C (an AMPK inhibitor) were purchased from Calbiochem (California, USA). Antibodies directed against endothelial nitric oxide synthase (eNOS) were obtained from Affinity BioReagents (Colorado, USA). Other antibodies were purchased from Cell Signaling Technology (Massachusetts, USA).

Cell Culture

Primary cultures of cardiomyocytes were prepared from ventricles of 1-day-old Wistar rats, as described previously.[2] In brief, cardiomyocytes were plated at a density of 5×10^5 cells/mL on

collagen-coated culture dishes and incubated in standard medium (DMEM with 10% FBS) for 72 hours, after which incubation was continued under serum-free conditions for 48 hours.

Cell Viability Assay (MTT Assay)

Cell viability was analyzed by a nonradioactive cell proliferation assay using MTT, as described previously with minor modifications[3].

Assessment of Cardiomyocyte Apoptosis

To investigate the influence of metformin on cardiomyocyte viability, TUNEL assay was performed as reported previously.[3] Apoptosis was also quantified by flow cytometry (FACScan; Becton, Dickinson and Company, New Jersey, USA) after cells were stained with annexin V and propidine iodide (PI) according to the manufacturer's instructions (Annexin V-FITC Apoptosis Detection Kit; Sigma, Saint Louis, USA).

Canine Pacing Model

Beagle dogs (Oriental Yeast Co. Ltd, Tokyo, Japan) weighing 8 to 10 kg were sedated with intravenous sodium pentobarbital at a dose of 25 mg/kg. After intubation with a cuffed endotracheal tube, anesthesia was maintained with 0.5 % to 1% isoflurane and an equal mixture of air and oxygen. Ventilation was provided with a tidal volume of 22 mL/kg at a rate of 15 times per minute. A bipolar pacing lead (Model BT-45P, Star Medical Inc., Tokyo, Japan) was advanced under fluoroscopic guidance through the right jugular vein to the RV apex and was connected to a programmable pacemaker (VOO mode; Model SIP-501, Star Medical Inc., Tokyo, Japan) that was implanted in a

subcutaneous pocket in the neck. The success of this procedure was confirmed by electrocardiography. Cefazolin sodium (1 g) was given intravenously after surgery, and the dogs were allowed to recover for a few hours. Then heart failure was induced by rapid RV pacing at a rate of 230 beats per minute for 4 weeks, as reported previously.[4]

Echocardiography

Transthoracic echocardiography was performed by using an echocardiographic system equipped with a 4-MHz phased-array transducer (SONOS 5500, PHILIPS, Eindhoven, the Netherlands) in conscious dogs before pacemaker implantation and 30 minutes after the cessation of right ventricular (RV) pacing at 4 weeks. A two-dimensional short-axis view of the left ventricle was obtained at the level of the papillary muscles. All measurements were made by two observers, who were blinded with respect to the source of the tracings.

Hemodynamic Studies

Both left ventricular end-diastolic pressure (LVEDP) and mean aortic pressure were measured by pressure transducers using a 5 Fr pig tail catheter (Terumo Co. Ltd., Tokyo, Japan) that was inserted into the left ventricle from the left femoral artery. The mean pulmonary artery pressure (PAP) and the pulmonary capillary wedge pressure (PCWP) were measured using a 7 Fr Swan-Ganz catheter (American Edwards Laboratories, California, USA). Cardiac output (CO) was determined at least three times by the thermodilution technique. Systemic vascular resistance (SVR) was calculated as follows: (mean aortic pressure-right atrial pressure) × 80/CO.

Histological examination

The collagen volume fraction was examined in sections of the left ventricular (LV) free wall, after excluding vessels, artifacts, minor scars, and incomplete tissue. Specimens were stained with Masson's trichrome stain to evaluate the extent of interstitial fibrosis, as described previously.[5] The area of stained tissue was calculated as a percentage of the total area within a field by using Scion image software (Beta 4.0.2).

Quantitative Reverse-Transcriptase PCR

The quantitative reverse-transcriptase PCR was performed as described previously.[6] Total RNA was extracted from LV myocardium with RNA-Bee-RNA Isolation Reagent (Tel-Test, Texas, USA). Then 1,000 ng of total RNA was reverse transcribed and amplified with an Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Oligonucleotide primers and TaqMan probes for canine atrial natriuretic peptide (ANP) (Cf 02705687_g1), canine transforming growth factor-β1 (TGF-β1) (Cf 02741608_m1), and canine ribosomal protein S18 (Cf 02681523_g1) were purchased from Applied Biosystems (California, USA). Both Taqman probe and primer designs were optimized to enhance stability on the basis of the known sequences of canine brain natriuretic peptide (BNP)[7] and canine endothelial NO synthase (eNOS).[8] We used the following probes, sense primers, and antisense primers: 5'-FAM-CAGTTGGCCCTGGAA-MGB-3', 5'-GAAGGACGCAGTTTCAGAGCTG -3' and 5'-AAAGCACCCTGACTTGTGCATC-3' for canine BNP; and

5'-FAM-CCTGGAGGATGTGGC-MGB-3', 5'-AACCTGTGTGACCCTCATCGAT-3' and 5'-TCACTTTGGCCAGCTGGTAACT-3' for canine eNOS, respectively.

Immunoblotting

Immunoblotting was performed as described previously.[9] A Bio-Rad ChemiDoc XRS system (Bio-Rad Laboratories, Inc., California, USA) was used for chemiluminescence imaging and immunoreactive bands were quantified with Bio-Rad Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc., California, USA).

Measurement of Nitric Oxide End-Products

The plasma level of nitric oxide (NO) metabolic end-products (nitrite + nitrate) was measured by the Griess method, as reported previously.[10] Subsequently, ΔNO was defined as the difference between the plasma NO level before and after 4 weeks of RV pacing.

Metabolic Parameters

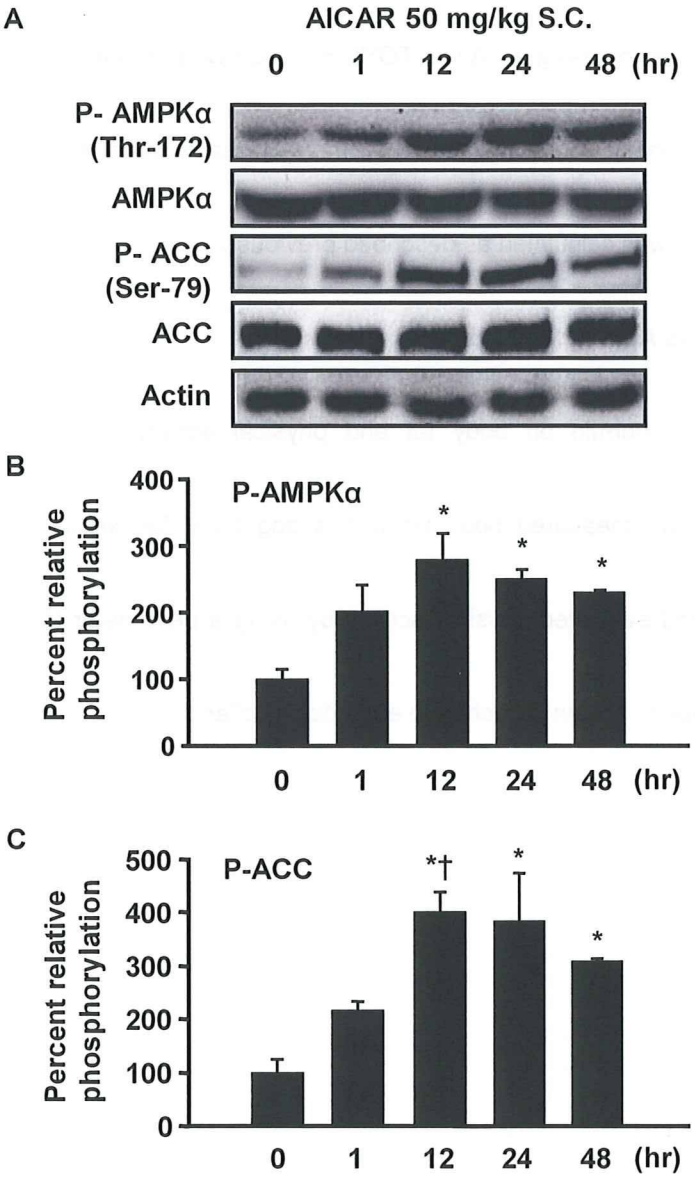
All dogs were fed a standard diet with a fixed carbohydrate and fat content (DS-A, Oriental Yeast Co. Ltd, Tokyo, Japan). After fasting for 14 hours, metabolic parameters such as the plasma levels of glucose, lactate, free fatty acids (FFA), and insulin were measured with a quick-auto-neo-GLU-HK (Shino-Test Corporation, Tokyo, Japan.), Determiner LA (KYOWA MEDEX Co., Ltd., Tokyo, Japan.), NEFA-SS Eiken, Eiken Chemical Co., Ltd., Tokyo, Japan.), and YK060 Insulin ELISA Kit (Yanaihara Institute Inc. Shizuoka, Japan), respectively. Insulin resistance was assessed from the fasting insulin and glucose levels by the homeostasis model assessment-insulin resistance (HOMA-IR) method, i.e.,

HOMA-IR is [fasting glucose (mmol/L) × fasting insulin (μU/mL)] / 22.5.[11] The levels of norepinephrine and angiotensin II were measured by using a CA test TOSOH (Tosoh Corporation, Tokyo, Japan.) and a NEX-105 (125I)-Tyr4-Angiotensin II test (PerkinElmer Inc., Massachusetts, USA.), respectively. Myocardial substrate extraction was calculated as described previously.[12]

Measurement of Body Fat and Activity in Dogs

To examine the effects of metformin on body fat and physical activity in this dog model of pacing-induced heart failure, we measured body fat with a dog body fat counter (IBF-D02, Kao Corporation, Tokyo, Japan) and evaluated physical activity by using a pedometer (SE-MG10, SATO KEIRYOUKI MFG. Co., Ltd., Tokyo, Japan) attached to each dog's collar.

Supplemental Figures



Changes in the phosphorylation of AMPKα and ACC in canine hearts after subcutaneous administration of AICAR. A) Representative immunoblots of phospho-AMPKα and ACC. **B)** and **C)** The percent relative phosphorylation of AMPKα and ACC, respectively. Values are the mean±SEM. *P<0.05 vs. no treatment; †P<0.05 vs. one hour after subcutaneous administration of AICAR. Representative results from 3 independent experiments are shown.

Supplemental References :

1. Li HL, Yin R, Chen D, Liu D, Wang D, Yang Q, Dong YG. Long-term activation of adenosine monophosphate-activated protein kinase attenuates pressure-overload-induced cardiac hypertrophy. *J Cell Biochem.* 2007;100:1086-1099.

2. Asakura M, Kitakaze M, Takashima S, Liao Y, Ishikura F, Yoshinaka T, Ohmoto H, Node K, Yoshino K, Ishiguro H, Asanuma H, Sanada S, Matsumura Y, Takeda H, Beppu S, Tada M, Hori M, Higashiyama S. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat Med.* 2002;8:35-40.

3. Okada K, Minamino T, Tsukamoto Y, Liao Y, Tsukamoto O, Takashima S, Hirata A, Fujita M, Nagamachi Y, Nakatani T, Yutani C, Ozawa K, Ogawa S, Tomoike H, Hori M, Kitakaze M. Prolonged endoplasmic reticulum stress in hypertrophic and failing heart after aortic constriction: possible contribution of endoplasmic reticulum stress to cardiac myocyte apoptosis. *Circulation.* 2004;110:705-712.

4. Shinbane JS, Wood MA, Jensen DN, Ellenbogen KA, Fitzpatrick AP, Scheinman MM. Tachycardia-induced cardiomyopathy: a review of animal models and clinical studies. *J Am Coll Cardiol.* 1997;29:709-715.

5. Wakeno M, Minamino T, Seguchi O, Okazaki H, Tsukamoto O, Okada K, Hirata A, Fujita M, Asanuma H, Kim J, Komamura K, Takashima S, Mochizuki N, Kitakaze M. Long-term stimulation of adenosine A2b receptors begun after myocardial infarction prevents cardiac

remodeling in rats. *Circulation.* 2006;114:1923-1932.

6. Fujita M, Okuda H, Tsukamoto O, Asano Y, Hirata YL, Kim J, Miyatsuka T, Takashima S, Minamino T, Tomoike H, Kitakaze M. Blockade of angiotensin II receptors reduces the expression of receptors for advanced glycation end products in human endothelial cells. *Arterioscler Thromb Vasc Biol.* 2006;26:e138-142.

7. Lisy O, Redfield MM, Schirger JA, Burnett JC, Jr. Atrial BNP endocrine function during chronic unloading of the normal canine heart. *Am J Physiol Regul Integr Comp Physiol.* 2005;288:R158-162.

8. Fulton D, Papapetropoulos A, Zhang X, Catravas JD, Hintze TH, Sessa WC. Quantification of eNOS mRNA in the canine cardiac vasculature by competitive PCR. *Am J Physiol Heart Circ Physiol.* 2000;278:H658-665.

9. Tsukamoto O, Minamino T, Okada K, Shintani Y, Takashima S, Kato H, Liao Y, Okazaki H, Asai M, Hirata A, Fujita M, Asano Y, Yamazaki S, Asanuma H, Hori M, Kitakaze M. Depression of proteasome activities during the progression of cardiac dysfunction in pressure-overloaded heart of mice. *Biochem Biophys Res Commun.* 2006;340:1125-1133.

10. Asanuma H, Node K, Minamino T, Sanada S, Takashima S, Ueda Y, Sakata Y, Asakura M, Kim J, Ogita H, Tada M, Hori M, Kitakaze M. Celiprolol increases coronary blood flow and reduces severity of myocardial ischemia via nitric oxide release. *J Cardiovasc Pharmacol.* 2003;41:499-505.

11. Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, Muggeo M. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care.* 2000;23:57-63.

12. Nikolaidis LA, Elahi D, Hentosz T, Doverspike A, Huerbin R, Zourelias L, Stolarski C, Shen YT, Shannon RP. Recombinant glucagon-like peptide-1 increases myocardial glucose uptake and improves left ventricular performance in conscious dogs with pacing-induced dilated cardiomyopathy. *Circulation.* 2004;110:955-961.

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In vivo direct monitoring of vagal acetylcholine release to the sinoatrial node

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ABSTRACT

To directly monitor vagal acetylcholine (ACh) release into the sinoatrial node, which regulates heart rate, we implanted a microdialysis probe in the right atrium near the sinoatrial node and in the right ventricle of anesthetized rabbits, and perfused with Ringer's solution containing eserine. (1) Electrical stimulation of right or left cervical vagal nerve decreased atrial rate and increased dialysate ACh concentration in the right atrium in a frequency-dependent manner. Compared to left vagal stimulation, right vagal nerve stimulation decreased atrial rate to a greater extent at all frequencies, and increased dialysate ACh concentration to a greater extent at 10 and 20 Hz. However, dialysate ACh concentration in the right atrium correlated well with atrial rate independent of whether electrical stimulation was applied to the right or left vagal nerve (atrial rate $= 304 - 131 \times \log[\mathrm{ACh}]$, $R^2 = 0.77$). (2) Right or left vagal nerve stimulation at 20 Hz decreased atrial rate and increased dialysate ACh concentrations in both the right atrium (right, 17.9 ± 4.0 nM; left, 7.9 ± 1.4 nM) and right ventricle (right, 0.9 ± 0.3 nM; left, 1.0 ± 0.4 nM). However, atrial dialysate ACh concentrations were significantly higher than ventricular concentrations, while ventricular dialysate ACh concentrations were not significantly different between right and left vagal nerve stimulation. (3) The response of ACh release to right and left vagal nerve stimulation was abolished by intravenous administration of a ganglionic blocker, hexamethonium bromide. In conclusion, ACh concentration in dialysate from the right atrium, sampled by microdialysis, is a good marker of ACh release from postganglionic vagal nerves to the sinoatrial node.

1. Introduction

Parasympathetic nerves play an important role in the regulation of heart rate under physiological conditions. To better understand the parasympathetic control of heart rate, it is important to quantitatively assess the efferent cardiac vagal nerve activity. Several methods have been used to assess this activity. Efferent cardiac vagal nerve electrical activity has been measured directly at the preganglionic site in several studies (Jewett, 1964; Kunze, 1972). We have developed a microdialysis technique which is used with high-performance liquid chromatography (HPLC) to monitor in vivo endogenous acetylcholine (ACh) release in the heart (Akiyama et al., 1994). Using this technique, we were able to monitor endogenous ACh release into the ventricular myocardium (Akiyama et al., 1994; Kawada et al., 2001). This technique permits the estimation of relative changes in postganglionic efferent cardiac vagal nerve activity in the ventricle.

However, vagal innervation is known to be heterogeneous in the heart. Kilbinger and Löffelholz (1976) reported that the ACh content of the ventricle was 41% and 19% of the atrial content in chicken and rabbit, respectively. Brown (1976) reported that ACh concentration was higher in the atrium than the ventricle, and that ACh content was higher in the right than the left portions in both the atrium and ventricle of the cat. Thus, to better understand the parasympathetic control of heart rate, which is the sinus rate under physiological conditions, we need information about the activities of postganglionic vagal nerves innervating the sinoatrial (SA) node.

In this study, we developed a dialysis probe using shorter dialysis fiber, which was suitable for implantation into the atrium. Using this dialysis probe, we tried to monitor myocardial interstitial ACh levels in the right atrium, especially near the SA node. Furthermore, we investigated whether the myocardial interstitial ACh levels reflect relative changes in activity of postganglionic vagal nerves innervating the SA node.

2. Materials and methods

2.1. Surgical preparation

Animal care was provided in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*

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approved by the Physiological Society of Japan. All protocols were approved by the Animal Subject Committee of the National Cardiovascular Center. Forty-three Japanese white rabbits weighing from 2.2 to 2.9 kg were anesthetized using an intravenous injection of pentobarbital sodium (50 mg/kg) via the marginal ear vein, followed by a continuous intravenous infusion of α-chloralose and urethane (16 mg/kg/h and 100 mg/kg/h) through a catheter inserted into the femoral vein to maintain an appropriate level of anesthesia. The animals were intubated and ventilated mechanically with room air mixed with oxygen. Systemic arterial pressure was monitored by a catheter inserted into the femoral artery. Esophageal temperature, which was measured by a thermometer (CTM-303, TERUMO, Japan), was maintained between 38 and 39 °C using a heating pad. In all protocols, bilateral vagal nerves were exposed through a midline cervical incision and sectioned at the neck after the control dialysate sampling. A pair of bipolar stainless steel electrodes was attached to the efferent side of the right or left vagal nerve. The nerve and electrode were covered with warmed mineral oil for insulation. When vagal stimulation was required, the efferent vagal nerve was stimulated by a digital stimulator (SEN-7203, Nihon Kohden, Japan). The pulse duration and amplitude of nerve stimulation were set at 1 ms and 10 V.

With the animal in the lateral position, right lateral thoracotomy was performed and the right 3rd to 5th ribs were partially resected to expose the heart. After incision of the pericardium, stainless steel wires were attached to the apex and the anterior wall of the left ventricle for ventricular pacing. To prevent severe bradycardia and cardiac arrest induced by vagal stimulation, left ventricular pacing was performed at the same frequency as the heart rate before vagal stimulation. The ventricular rate was determined from the electrocardiogram using a cardiotachometer. Another pair of stainless steel wires was attached to the appendage of the right atrium for recording atrial electrocardiogram, from which atrial rate was determined. Heparin sodium (100 IU/kg) was administered intravenously to prevent blood coagulation. At the end of the experiment, animals were killed with an overdose injection of pentobarbital sodium. A postmortem examination confirmed that the dialysate probe did not penetrate into the atrial or ventricular cavity and the dialysis membrane was positioned totally within the atrial or ventricular wall.

2.2. Dialysis technique

The materials and properties of the dialysis probe have been described previously (Akiyama et al., 1994). Briefly, we designed a handmade transverse dialysis probe. A dialysis fiber of semipermeable membrane (4 mm length, 310 μm outer diameter, 200 μm inner diameter; PAN-1200, 50,000 molecular weight cutoff; Asahi Chemical, Tokyo, Japan) was attached at both ends to polyethylene tubes (25 cm length, 500 μm outer diameter, 200 μm inner diameter). A fine guiding needle (30 mm length, 510 μm outer diameter, 250 μm inner diameter) with a stainless steel rod (5 mm length, 250 μm outer diameter) was used for the implantation of the dialysis probe. In protocol 1 and 3, a dialysis probe was implanted in the right atrium near the junction between the superior vena cava and the right atrium. In protocol 2, a dialysis probe was also implanted in the right ventricular free wall. After implantation, the dialysis probe was perfused with Ringer's solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 3 mM) containing the cholinesterase inhibitor eserine (100 μM) at a speed of 2 μl/min, using a microinjection pump (CMA/100, Carnegie Medicin, Sweden). Experimental protocols were started 120 min after implantation of the dialysis probe. We took account of the dead space between the dialysis membrane and the sample tube at the start of each dialysate sampling. Phosphate buffer (4 μl) containing an internal standard (isopropylhomocholine chloride) was transferred into each sample tube before dialysate sampling. Dialysate sampling periods were set at 10 min (1 sample volume = 20 μl).

2.3. Analytic procedure

Dialysate ACh was assayed using HPLC with electrochemical detection. An autosampler (CMA/200, Carnegie Medicin) was used. The HPLC system consisted of a pump with a pulse dumper (EP-300, Eicom, Japan), a separation column (AC-Gel, styrene polymer, 4 μm particle size, 2 mm inner diameter × 150 mm length, Eicom), an immobilized enzyme column (AC-Enzymepack, 1 mm inner diameter × 4 mm length, Eicom), an electrochemical detector (ECD-300, Eicom), and a degasser (DG-300, Eicom). The electrochemical detector was operated with a platinum working electrode at + 0.45 V vs. an Ag/AgCl reference electrode. The mobile phase was 50 mM potassium bicarbonate solution containing 400 mg/L of sodium 1-decansulfonate and 50 mg/L of disodium-EDTA. The pump flow rate was 0.15 ml/min.

Chromatograms were recorded and analyzed by an analog-to-digital converter (Power Chrom EPC-300, AD Instruments, Australia) with a computer. Concentrations of ACh and isopropylhomocholine chloride were determined by measuring the peak areas. The absolute detection limit of ACh was 10 fmol/injection (signal-to-noise ratio = 3).

2.4. Experimental protocols

2.4.1. Protocol 1

To examine whether atrial dialysate ACh concentration reflects ACh release from cardiac vagal nerves, we investigated the relationship between the dialysate ACh concentration in the right atrium and the frequency of right and left vagal nerve stimulation. We sampled control dialysate before and after vagal transection. Then we stimulated the right ($n = 8$) or left ($n = 8$) efferent vagal nerves for 10 min at frequencies of 5, 10, 20 and 40 Hz, and sampled dialysate during each stimulation. Ten minutes after vagal nerve stimulation, we sampled the dialysate again to check the recovery of ACh levels.

2.4.2. Protocol 2

To investigate the difference in vagal innervation density between the right atrium and right ventricle, we compared the atrial and ventricular dialysate ACh concentrations under control condition and during electrical vagal nerve stimulation. Control dialysates were sampled after vagal transection. Then the right ($n = 5$) or left ($n = 5$) efferent vagal nerve was stimulated for 10 min at a frequency of 20 Hz, and dialysates were collected during vagal stimulation.

2.4.3. Protocol 3

ACh is released from both pre- and post-ganglionic vagal nerves as a primary neurotransmitter. The cardiac vagal nerve ganglia are localized near the atrium (Löffelholz and Pappano, 1985). Electrical stimulation of cervical vagal nerves activates the entire efferent parasympathetic pathway, including both preganglionic and postganglionic nerves in the atrium. Thus it is possible that pre- and/or post-ganglionic nerves serve as the source of dialysate ACh. To determine whether pre- or post-ganglionic nerves are the source of atrial dialysate ACh, we observed ACh release in response to nerve stimulation before and after blockade of ganglionic transmission. We sampled control dialysate after vagal transection. Then we stimulated the right ($n = 9$) or left ($n = 8$) vagal nerve at a frequency of 20 Hz before and after intravenous administration of hexamethonium bromide (30 mg/kg) and sampled dialysate during vagal stimulation. To prevent severe hypotension induced by hexamethonium, arterial pressure was maintained by continuous intravenous infusion of phenylephrine (17.2 ± 1.6 μg/kg/min).

2.5. Statistical analysis

All data are presented as mean ± SE. For each protocol, heart rate and mean arterial pressure were compared by one-way repeated measures analysis of variance followed by a Dunnett's test against

control (Glantz, 2005). In protocol 1, we compared vagal stimulation-induced ACh release among the seven groups by one-way repeated measures analysis of variance followed by Tukey's test. Heart rates (atrial rate) and dialysate ACh concentrations during right and left vagal stimulation were compared by unpaired *t*-test. After logarithmic transformation of atrial dialysate ACh concentration, a linear regression analysis was performed to examine the relation between dialysate ACh concentration and atrial rate. In protocol 2, we compared atrial and ventricular dialysate ACh concentrations during vagal stimulation by two-way repeated measures analysis of variance. We also compared the effects of right and left vagal stimulation on atrial and ventricular dialysate ACh concentrations using an unpaired *t*-test. In protocol 3, we compared stimulation-induced ACh release with and without hexamethonium using one-way repeated measures analysis of variance followed by a Dunnett's test against control. Differences were considered significant at $P<0.05$.

3. Results

3.1. Protocol 1

Responses of heart rate and mean arterial pressure to electrical vagal nerve stimulation are shown in Table 1. Transection of bilateral vagal nerves did not change heart rate or mean arterial pressure significantly. While both right and left vagal stimulation decreased heart rate in proportion to the frequency of the stimulus, right vagal nerve stimulation decreased the heart rate to a greater extent than left vagal nerve stimulation at all stimulus frequencies tested ($P<0.05$ at 5 Hz, $P<0.01$ at 10 Hz, $P<0.05$ at 20 Hz and $P<0.05$ at 40 Hz). Heart rate recovered to the pre-stimulation levels after stimulation. Both right and left vagal nerve stimulation with ventricular pacing decreased mean arterial pressure. Mean arterial pressure recovered partially but remained lower than the pre-stimulation levels 10 min after stimulation.

Transection of bilateral vagal nerves did not change dialysate ACh concentration (Fig. 1). Both right and left vagal stimulation increased the dialysate ACh concentration in proportion to the stimulus frequency. Right vagal stimulation increased the dialysate ACh concentration from 1.9 ± 0.3 nM in the post-transection control to 2.7 ± 0.4 nM at 5 Hz ($P<0.05$ vs. control), 5.5 ± 0.8 nM at 10 Hz ($P<0.01$ vs. 5 Hz), 17.2 ± 3.0 nM at 20 Hz ($P<0.01$ vs. 10 Hz) and 40.4 ± 8.4 nM at 40 Hz ($P<0.01$ vs. 20 Hz). Dialysate ACh concentration recovered to 2.2 ± 0.3 nM 10 min after stimulation. Left vagal stimulation increased dialysate ACh concentration from 1.6 ± 0.3 nM in the post-transection control to 2.2 ± 0.4 nM at 5 Hz (N.S. vs. control), 3.2 ± 0.5 nM at 10 Hz ($P<0.01$ vs. control), 8.2 ± 1.4 nM at 20 Hz ($P<0.01$ vs. 10 Hz) and 24.7 ± 4.6 nM at 40 Hz ($P<0.01$ vs. 20 Hz). Dialysate ACh concentration recovered to 2.0 ± 0.5 nM 10 min after stimulation. While both right and left vagal stimulation increased dialysate ACh concentration in a frequency-dependent manner, right vagal nerve stimulation increased dialysate ACh concentration to a greater extent than left vagal nerve stimulation at 10 and 20 Hz (N.S. at 5 Hz, $P<0.05$ at 10 Hz, $P<0.05$ at 20 Hz and N.S. at 40 Hz).

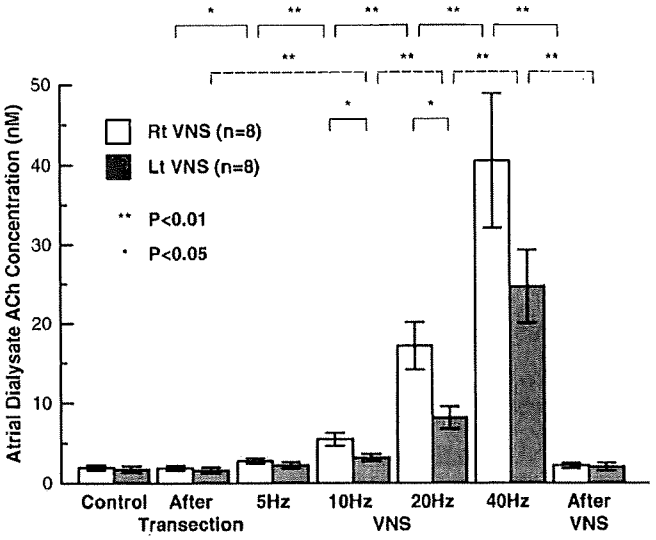

Fig. 1. Dialysate ACh concentrations of controls and during electrical vagal nerve stimulation at different frequencies. Right vagal nerve stimulation increased atrial dialysate ACh concentration from 1.9 ± 0.3 nM in the post-transection control to 2.7 ± 0.4 nM at 5 Hz, 5.5 ± 0.8 nM at 10 Hz, 17.2 ± 3.0 nM at 20 Hz and 40.4 ± 8.4 nM at 40 Hz. Left vagal nerve stimulation increased atrial dialysate ACh concentration from 1.6 ± 0.3 nM in the control to 2.2 ± 0.4 nM at 5 Hz, 3.2 ± 0.5 nM at 10 Hz, 8.2 ± 1.4 nM at 20 Hz and 24.7 ± 4.6 nM at 40 Hz. Values are means ± SE; Rt: right; Lt: left; VNS: electrical vagal nerve stimulation; *n*: number of rabbits: $^{**}P<0.01$, $^{*}P<0.05$.

The relationship between dialysate ACh concentration and atrial rate ($n=16$) is shown in Fig. 2. Dialysate ACh concentration in the right atrium correlated well with atrial rate (AR; $AR=304-131\times\log[ACh]$, $R^2=0.77$). There was no significant difference in the intercept or slope of regression line between right and left vagal nerve stimulation (right: $AR=304-135\times\log[ACh]$, $R^2=0.79$; left: $AR=303-126\times\log[ACh]$, $R^2=0.73$) (Glantz, 2005). The correlation between dialysate ACh concentration and atrial rate was independent of the side of vagal nerve stimulation.

Table 1
Responses of heart rate and mean arterial pressure to electrical vagal nerve stimulation (protocol 1).

	Heart rate (bpm)	Mean arterial pressure (mm Hg)
Rt vagal stimulation ($n=8$)	Atrial rate (pacing rate)	
Control before transection	298 ± 8	83 ± 4
Control after transection	293 ± 7	85 ± 6
VNS (5 Hz)	246 ± 5** (296 ± 5)	71 ± 7
VNS (10 Hz)	201 ± 6** (296 ± 5)	77 ± 6
VNS (20 Hz)	121 ± 7** (296 ± 5)	72 ± 8
VNS (40 Hz)	88 ± 4** (296 ± 5)	65 ± 7**
After VNS	287 ± 10	70 ± 9
Lt vagal stimulation ($n=8$)	Atrial rate (pacing rate)	
Control before transection	305 ± 8	89 ± 4
Control after transection	308 ± 5	92 ± 6
VNS (5 Hz)	267 ± 6* (309 ± 4)	79 ± 6**
VNS (10 Hz)	236 ± 10** (309 ± 4)	82 ± 6
VNS (20 Hz)	165 ± 13** (309 ± 4)	77 ± 5**
VNS (40 Hz)	129 ± 16** (309 ± 4)	67 ± 6**
After VNS	305 ± 13	75 ± 8**

Values are means ± SE; *n*: numbers of rabbits; Rt: right; Lt: left; VNS: electrical vagal nerve stimulation; $^{**}P<0.01$ vs. control; $^{*}P<0.05$ vs. control.

3.2. Protocol 2

Responses of heart rate and mean arterial pressure were similar to the responses to vagal stimulation at 20 Hz in protocol 1 (Table 2). Responses of ACh release in the right atrium and right ventricle to vagal stimulation are shown in Fig. 3. Right vagal stimulation increased the atrial dialysate ACh concentration from 2.6 ± 0.6 nM in the post-transection control to 17.9 ± 4.0 nM ($P<0.01$) and the ventricular dialysate ACh concentration from 0.4 ± 0.2 nM to 0.9 ± 0.3 nM ($P<0.01$). Left vagal stimulation also increased the atrial dialysate ACh concentration from 1.5 ± 0.4 nM to 7.9 ± 1.4 nM ($P<0.01$) and the ventricular dialysate ACh concentration from 0.3 ± 0.1 nM in the control to 1.0 ± 0.4 nM ($P<0.01$). Atrial dialysate ACh concentrations were higher than ventricular dialysate ACh concentrations in both right and left vagal stimulation ($P<0.01$). The interaction between the stimulation and the position of probe (atrium or ventricle) was significant ($P<0.01$). There was no difference in ventricular dialysate ACh concentration between right and left vagal stimulation, but atrial dialysate ACh concentration was significantly

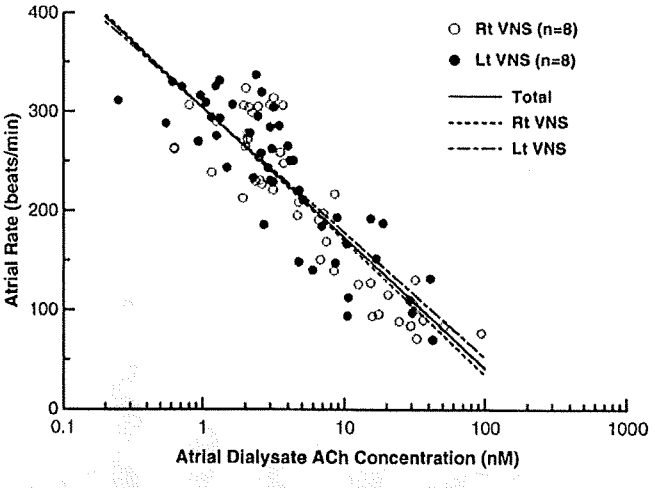

Fig. 2. Relation between dialysate ACh concentration (logarithmic scale) and atrial rate. Dialysate ACh concentration in the right atrium correlates well with atrial rate ($R^2=0.77$). Solid line, regression line fitting all 96 data points; dotted line, regression line fitting 48 data points of right vagal nerve stimulation; dot-dashed line, regression line fitting 48 data points of left vagal nerve stimulation. Rt: right; Lt: left; VNS: electrical vagal nerve stimulation.

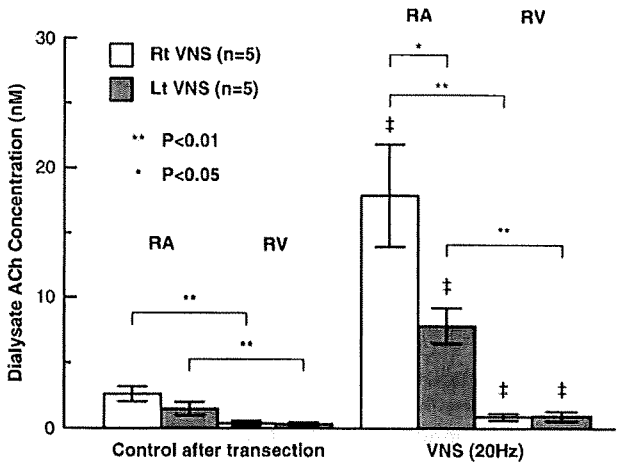

Fig. 3. Dialysate ACh concentrations in right atrium and right ventricle of controls and during electrical vagal nerve stimulation. Right vagal nerve stimulation significantly increased dialysate ACh concentration from 2.6±0.6 to 17.9±4.0 nM in the right atrium ($P<0.01$) and from 0.4±0.2 to 0.9±0.3 nM in the right ventricle ($P<0.01$). Left vagal nerve stimulation also increased dialysate ACh concentrations from 1.5±0.4 to 7.9±1.4 nM in the right atrium ($P<0.01$) and from 0.3±0.1 to 1.0±0.4 nM in the right ventricle ($P<0.01$). Dialysate ACh concentrations in the right atrium were significantly higher than those in the ventricle ($P<0.01$). Right vagal nerve stimulation increased atrial dialysate ACh concentration more than left vagal nerve stimulation ($P<0.05$). Values are means±SE; Rt: right; Lt: left; RA: right atrium; RV: right ventricle; VNS: electrical vagal nerve stimulation; *n*: number of rabbits; ‡$P<0.01$ vs. control; **$P<0.01$, *$P<0.05$.

higher during right vagal stimulation compared to left vagal stimulation ($P<0.05$).

3.3. Protocol 3

Responses of heart rate and mean arterial pressure are shown in Table 3. Both right and left vagal nerve stimulation decreased heart rate markedly before administration of hexamethonium. Administration of hexamethonium decreased heart rate significantly but mildly compared to control. Mean arterial pressure was maintained at pre-stimulation levels by continuous intravenous infusion of phenylephrine. After administration of hexamethonium, both right and left vagal nerve stimulation did not change the heart rate. Right vagal stimulation increased dialysate ACh concentration from 2.5±0.4 to 16.3±2.8 nM ($P<0.01$), but right vagal stimulation after administration of hexamethonium failed to increase ACh concentration (2.2±0.4 nM) compared to control. Likewise, left vagal stimulation increased dialysate ACh concentration from 1.5±0.3 to 8.7±1.4 nM ($P<0.01$), but left vagal stimulation after administration of hexamethonium did not increase ACh concentration (1.5±0.3 nM) compared to control (Fig. 4).

4. Discussion

We demonstrated that the microdialysis technique permitted in vivo monitoring of ACh release into the sinoatrial node from postganglionic cardiac vagal nerves. Dialysate ACh concentration in the right atrium correlated well with atrial rate and this correlation was independent of the side of vagal stimulation. These results indicate that in vivo monitoring of the myocardial interstitial ACh levels in the right atrium by microdialysis provides a useful strategy to obtain insights into the physiological roles of the vagal system in regulating heart rate.

4.1. Characteristics of atrial dialysate ACh concentration

With both right and left vagal nerve stimulation, the dialysate ACh concentration in the right atrium increased with increasing stimulus frequency and decreased to prestimulation levels after stimulation (Fig. 1). These results indicate that atrial dialysate ACh reflects ACh release from cardiac vagal nerves innervating the right atrium. Right vagal nerve stimulation decreased the atrial rate more than left stimulation at all stimulus frequencies, and right vagal nerve stimulation increased dialysate ACh concentration more than left stimulation at 10- and 20-Hz. The right atrium, including the SA node, is innervated not only by the right but also by the left vagal nerve. Ardell and Randall (1986) reported that supramaximal right and left

Table 2
Responses of heart rate and mean arterial pressure to electrical vagal nerve stimulation (protocol 2).

	Heart rate (bpm)	Mean arterial pressure (mm Hg)
Rt vagal stimulation ($n=5$)	Atrial rate (pacing rate)	
Control after transaction	305±3	74±8
VNS (20 Hz)	122±4** (304±4)	65±9*
Control after VNS	300±3	68±8
Lt vagal stimulation ($n=5$)	Atrial rate (pacing rate)	
Control after transaction	306±5	95±3
VNS (20 Hz)	168±19** (308±5)	83±1**
Control after VNS	316±8	82±2**

Values are means±SE; *n*, numbers of rabbits; Rt: right; Lt: left; VNS: electrical vagal nerve stimulation; **$P<0.01$ vs. control; *$P<0.05$ vs. control.

Table 3
Responses of heart rate and mean arterial pressure to electrical vagal nerve stimulation (protocol 3).

	Heart rate (bpm)	Mean arterial pressure (mm Hg)
Rt vagal stimulation ($n=9$)	Atrial rate (pacing rate)	
Control after transaction	292±9	70±8
VNS (20 Hz)	116±7** (299±5)	69±7
Hexamethonium iv	257±4**	84±7*
VNS after hexamethonium iv	257±4**	83±8*
Lt vagal stimulation ($n=8$)	Atrial rate (pacing rate)	
Control after transaction	317±3	79±3
VNS (20 Hz)	173±13** (313±4)	81±3
Hexamethonium iv	273±4**	87±5
VNS after hexamethonium iv	273±4**	87±4

Values are means±SE; *n*, numbers of rabbits; Rt: right; Lt: left; VNS: electrical vagal nerve stimulation; iv: intravenous administration; **$P<0.01$ vs. control; *$P<0.05$ vs. control.

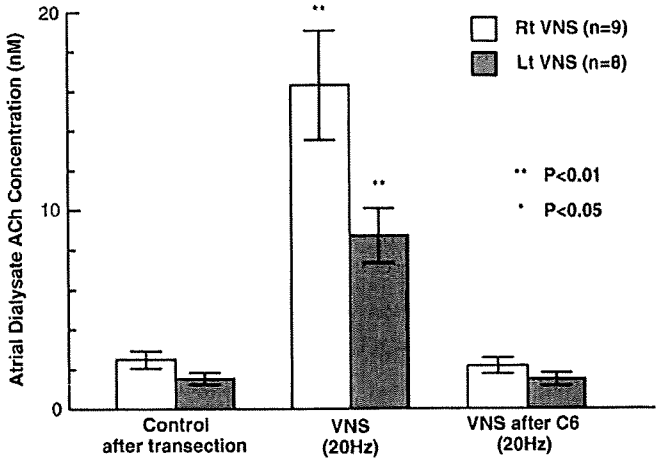

Fig. 4. Influence of ganglionic blocker on vagal nerve stimulation-induced ACh release. Right vagal nerve stimulation significantly increased atrial dialysate ACh concentration from 2.5 ± 0.4 to 16.3 ± 2.8 ($P<0.01$), and intravenous administration of hexamethonium suppressed the ACh concentration to 2.2 ± 0.4 nM. Left vagal stimulation increased atrial dialysate ACh concentration from 1.5 ± 0.3 to 8.7 ± 1.4 nM ($P<0.01$), and hexamethonium suppressed the ACh concentration to 1.5 ± 0.3 nM. Values are means ± SE; Rt: right; Lt: left; VNS: electrical vagal nerve stimulation; C6: hexamethonium bromide; *n*: number of rabbits; **$P<0.01$ vs. control; *$P<0.05$ vs. control.

cervical vagal stimulation decreased the atrial rates to 16.3% and 48.7%, respectively, of prestimulation rates in dogs. In our study, right and left vagal stimulation at a frequency of 40 Hz also decreased the atrial rate to 30% and 42% of prestimulation rates. The difference in atrial rate response between right and left vagal nerve stimulation could be explained by the different innervation densities of the right and left vagal nerves in the right atrium including the SA node. The SA node is innervated by both right and left vagal nerves with a predominance of right vagal nerves (Ardell and Randall, 1986; Randall et al., 1985), and the response of atrial rate to vagal nerve stimulation could be ascribed to vagal ACh release into the SA node. The SA node is probably regulated by ACh released from the left as well as the right vagal nerves. In this study, dialysate ACh concentration in the right atrium (logarithmically transformed) correlated well with atrial rate, and this correlation was independent of right or left vagal stimulation (Fig. 2). These results suggest that dialysate ACh in the right atrium reflects ACh released into the SA node independent of whether the ACh originates from the right or left vagal nerves.

4.2. ACh release in atrium and ventricle

In this study, the mean dialysate ACh concentration in the right ventricle after transection of bilateral vagal nerves was 20 to 30% of that in the right atrium. During vagal nerve stimulation at 20 Hz, the atrial dialysate ACh concentration increased 5 to 7 times the control value but the ventricular dialysate ACh concentration increased to only 2 to 3 times the control value (Fig. 3). This difference between atrial and ventricular dialysate ACh concentrations could be related to the density of vagal innervation. These results are consistent with previous *in vitro* studies (Kilbinger and Löffelholz, 1976; Brown, 1976; Stanley et al., 1978). Kent et al. (1974) reported that the atrial myocardium of the vertebrate heart was richly innervated as identified by specific histochemical staining of acetylcholinesterase, in contrast to the scant innervation in the ventricular myocardium.

Right vagal nerve stimulation increased atrial dialysate ACh more than left stimulation. On the other hand, there was no difference in ventricular dialysate ACh concentration between right and left vagal nerve stimulation. Although the right atrium is predominantly innervated by the right vagal nerves, the right ventricle could be equally innervated by the right and left vagal nerves. When the right vagal nerve was stimulated at 20 Hz, heart rate decreased from 305 ± 3 to 122 ± 4 bpm. When the left vagal nerve was stimulated at 20 Hz, heart rate decreased from 306 ± 5 to 169 ± 19 bpm. This difference in heart rate response could be ascribed to vagal ACh release into the SA node. Atrial dialysate ACh concentrations were 17.9 ± 4.0 and 7.9 ± 1.4 nM ($P<0.05$) during stimulation of right and left vagal nerves, respectively. In contrast, there was no significant difference in ventricular dialysate ACh concentration between right and left vagal nerve stimulation. Therefore, we consider that dialysate ACh concentration in the right atrium may be a better index of ACh release into the SA node than dialysate ACh in the right ventricle.

4.3. Source of atrial dialysate ACh

In a previous study with anesthetized cats, we demonstrated that ACh in the dialysate sampled from left ventricular myocardium primarily reflects ACh released from postganglionic cardiac vagal nerves (Akiyama et al., 1994). Cardiac ganglia are located predominantly in the posterior aspect of the atria within the subepicardial connective tissue (Löffelholz and Pappano, 1985). It is possible that ACh released from stimulated preganglionic nerves contributes to ACh in the dialysate sampled from the right atrium. In this study, intravenous administration of hexamethonium bromide, a nicotinic antagonist, abolished the increase in ACh release during efferent vagal nerve stimulation. This result demonstrates that ACh in the dialysate sampled from the right atrium primarily originates from the postganglionic cardiac nerve endings.

4.4. Significance of monitoring ACh release to the SA node

Several studies have directly measured electrical efferent vagal nerve activities at the preganglionic site in vivo (Jewett, 1964; Kunze, 1972). Although this method has been used to estimate the net activity of cardiac vagal nerves, it is technically difficult to selectively measure the electrical activity of postganglionic vagal nerves innervating the SA node. Moreover, it is possible that preganglionic signals are modulated at intracardiac ganglionic sites (Gray et al., 2004). In fact, Bibevski and Dunlap (1999) have reported that attenuated vagal control in heart failure can be ascribed to attenuated ganglionic transmission. Therefore, information about postganglionic vagal nerve activity is important for understanding vagal control of heart rate.

4.5. Methodological consideration

First, we sectioned the vagi in the neck region but the sympathetic nerves were almost intact because the sympathetic nerves run separately from the vagi at the neck in rabbits. ACh released from vagal nerve terminals may interact with muscarinic receptors on postganglionic sympathetic nerve terminals to inhibit norepinephrine release prejunctionally (Levy, 1984).

Second, ACh is degraded by ACh esterase immediately after its release. Therefore to detect ACh release in vivo, addition of a specific ACh esterase inhibitor eserine into the perfusate is necessary. We used eserine at a concentration 10–100 times higher than that required in *in vitro* experimental settings because distribution of eserine across the semipermeable membrane is required, based on previous results (Akiyama et al., 1994). Eserine should spread around the semipermeable membrane, thereby affecting the ACh release in the vicinity of the dialysis membrane. Eserine may have increased the ACh level in the synaptic cleft and enhanced heart rate response by nerve stimulation, and may have also activated regulatory pathways such as autoinhibition of ACh release via muscarinic receptors.

5. Conclusion

We were able to monitor myocardial interstitial ACh levels in the right atrium around the SA node using a microdialysis technique.

Myocardial interstitial ACh level in the right atrium correlates well with atrial rate. Microdialysis combined with HPLC will become a powerful tool for understanding the parasympathetic control of heart rate.

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References

Akiyama, T., Yamazaki, T., Ninomiya, I., 1994. In vivo detection of endogenous acetylcholine release in cat ventricles. Am. J. Physiol. 266, H854–860.
Ardell, J.L., Randall, W.C., 1986. Selective vagal innervation of sinoatrial and atrioventricular nodes in canine heart. Am. J. Physiol. 251, H764–773.
Bibevski, S., Dunlap, M.E., 1999. Ganglionic mechanisms contribute to diminished vagal control in heart failure. Circulation 99, 2958–2963.
Brown, O.M., 1976. Cat heart acetylcholine: structural proof and distribution. Am. J. Physiol. 231, 781–785.
Glantz, S.A., 2005. Primer of Biostatistics, 6th ed. McGraw-Hill, New York.
Gray, A.L., Johnson, T.A., Ardell, J.L., Massari, V.J., 2004. Parasympathetic control of the heart. II. A novel interganglionic intrinsic cardiac circuit mediates neural control of heart rate. J. Appl. Physiol. 96, 2273–2278.
Jewett, D.L., 1964. Activity of single efferent fibres in the cervical vagus nerve of the dog, with special reference to possible cardio-inhibitory fibres. J. Physiol. 175, 321–357.
Kawada, T., Yamazaki, T., Akiyama, T., Shishido, T., Inagaki, M., Uemura, K., Miyamoto, T., Sugimachi, M., Takaki, H., Sunagawa, K., 2001. In vivo assessment of acetylcholine-releasing function at cardiac vagal nerve terminals. Am. J. Physiol. Heart Circ. Physiol. 281, H139–145.
Kent, K.M., Epstein, S.E., Cooper, T., Jacobowitz, D.M., 1974. Cholinergic innervation of the canine and human ventricular conducting system. Anatomic and electrophysiologic correlations. Circulation. 50, 948–955.
Kilbinger, H., Löffelholz, K., 1976. The isolated perfused chicken heart as a tool for studying acetylcholine output in the absence of cholinesterase inhibition. J. Neural Transm. 38, 9–14.
Kunze, D.L., 1972. Reflex discharge patterns of cardiac vagal efferent fibres. J. Physiol. 222, 1–15.
Levy, M.N., 1984. Cardiac sympathetic–parasympathetic interactions. Fed. Proc. 43, 2598–2602.
Löffelholz, K., Pappano, A.J., 1985. The parasympathetic neuroeffector junction of the heart. Pharmacol. Rev. 37, 1–24.
Randall, W.C., Ardell, J.L., Becker, D.M., 1985. Differential responses accompanying sequential stimulation and ablation of vagal branches to dog heart. Am. J. Physiol. 249, H133–140.
Stanley, R.L., Conaster, J., Dettbarn, W.D., 1978. Acetylcholine, choline acetyltransferase and cholinesterases in the rat heart. Biochem. Pharmacol. 27, 2409–2411.

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Short communication

In vivo direct monitoring of interstitial norepinephrine levels at the sinoatrial node

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ABSTRACT

We assessed in vivo interstitial norepinephrine (NE) levels at the sinoatrial node in rabbits, using microdialysis technique. A dialysis probe was implanted adjacent to the sinoatrial node of an anesthetized rabbit and dialysate was sampled during sympathetic nerve stimulation. Atrial dialysate NE concentration correlated well with heart rate. Desipramine significantly increased dialysate NE concentrations both before and during sympathetic nerve stimulation compared with the absence of desipramine. However, desipramine did not affect the relation between heart rate and dialysate NE concentration. These results suggest that atrial dialysate NE level reflects the relative change of NE concentration in the synaptic cleft. Microdialysis is a powerful tool to assess in vivo interstitial NE levels at the sinoatrial node.

1. Introduction

Heart rate is determined by the frequency of depolarization of sinoatrial (SA) nodal cell during sinus rhythm. The SA node is innervated by sympathetic nerve fibers. These sympathetic nerves, together with parasympathetic nerves, play an important role in the regulation of SA node pacemaker activities. Direct measurement of electrical axonal activity of efferent cardiac sympathetic nerve (Kawada et al., 2004) and indirect measurement of norepinephrine (NE) spillover from plasma NE concentration in the coronary sinus (Meredith et al., 1993) have been used as indices of sympathetic nerve terminal activity on the effector, i.e. sinoatrial node. However, due to the heterogeneity of sympathetic innervation in the heart, quantitative assessment of sympathetic nerve terminal activities on the SA node is essential for better understanding of the sympathetic control of heart rate.

Recently we have developed a microdialysis technique that allows direct monitoring of acetylcholine release into the SA node (Shimizu et al., 2009). In the present study, we monitored interstitial NE levels in the right atrial myocardium adjacent to the SA node using the microdialysis technique and investigated the relation between interstitial NE levels and heart rate in response to sympathetic nerve stimulation. This study may prove the usefulness of microdialysis in assessing the relative change of sympathetic nerve terminal activity on the SA mode.

2. Materials and methods

2.1. Surgical preparation

Animal care was provided 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 Subject Committee of the National Cardiovascular Center. Fourteen Japanese white rabbits weighing 2.4 to 2.8 kg were used in this study. Anesthesia was initiated by an intravenous injection of pentobarbital sodium (50 mg/kg) via the marginal ear vein, and then maintained at an appropriate level by continuous intravenous infusion of α-chloralose and urethane (16 mg/kg/h and 100 mg/kg/h) through a catheter inserted into the femoral vein. The animals were intubated and ventilated mechanically with room air mixed with oxygen. Systemic arterial pressure was monitored by a catheter inserted into the femoral artery. Esophageal temperature, which was measured by a thermometer (CTM-303, Terumo, Japan), was maintained between 38 and 39 °C using a heating pad. Bilateral vagal nerves were exposed through a midline cervical incision and sectioned at the neck.

With the animal in supine position, a full median sternotomy was performed to expose the heart. The right cardiac sympathetic nerve

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was exposed through the sternotomy and sectioned intrathoracically. A pair of bipolar stainless steel electrodes was attached to the efferent side of the right cardiac sympathetic nerve. The nerve and electrode were immobilized using a quick-dry silicone gel (Kwik-Cast and Kwik-Sil, World Precision Instruments, Inc., FL, USA). When sympathetic stimulation was required, the efferent sympathetic nerve was stimulated by a digital stimulator (SEN-7203, Nihon Kohden, Japan), at a pulse duration of 1 ms and an amplitude of 5 V. Three stainless electrodes were attached around the incision of sternotomy for the body surface electrocardiogram. The heart rate was determined from the electrocardiogram using a cardiotachometer. Heparin sodium (100 IU/kg) was administered intravenously to prevent blood coagulation. A dialysis probe was implanted and dialysis was conducted as described in *Dialysis Technique* below. At the end of the experiment, the animal was euthanized with an overdose injection of pentobarbital sodium. In the postmortem examination, the right atrial wall was resected with dialysis fiber. We observed the inside of atrial wall macroscopically and confirmed that the dialysis membrane was not exposed to right atrial lumen.

2.2. Dialysis technique

The materials and properties of the dialysis probe have been described previously. (Akiyama et al., 1991; Shimizu et al., 2009) A dialysis fiber of semipermeable membrane (4 mm length, 310 μm outer diameter, 200 μm inner diameter; PAN-1200, 50,000 molecular weight cutoff; Asahi Chemical, Tokyo, Japan) was attached at both ends to polyethylene tubes (25 cm length, 500 μm outer diameter, 200 μm inner diameter). A fine guiding needle (30 mm length, 510 μm outer diameter, 250 μm inner diameter) with a stainless steel rod (5 mm length, 250 μm outer diameter) was used for the implantation of the dialysis probe. A dialysis probe was implanted into the right atrial myocardium near the junction between the superior vena cava and the right atrium. After implantation, the dialysis probe was perfused with Ringer's solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 3 mM) at a speed of 2 μl/min, using a microinjection pump (CMA/102, Carnegie Medicin, Sweden). Experimental protocols were started 120 min after implantation of the dialysis probe. We took account of the dead space between the dialysis membrane and the sample tube at the start of each dialysate sampling. Four-μl phosphate buffer (pH 3.5) was transferred into each sample tube before dialysate sampling. Dialysate sampling periods were set at 10 min (1 sample volume = 20 μl). Dialysate NE concentration was analyzed by high performance liquid chromatography (Akiyama et al., 1991).

2.3. Experimental protocols

2.3.1. Protocol 1

To examine whether atrial interstitial NE level reflects NE release from cardiac sympathetic nerve endings, we investigated the effect of sympathetic nerve stimulation on dialysate NE concentration and analyzed the relationship between the dialysate NE concentrations and heart rate ($n = 7$). We sampled control dialysate after transecting the right sympathetic nerve. Then we stimulated the right sympathetic nerve for 10 min each at frequencies of 2, 5 and 10 Hz, and collected the dialysate during each stimulation. There was a 30-min interval between the different stimulation frequencies. Twenty min after sympathetic nerve stimulation, we sampled the dialysate again to check for recovery of NE level.

2.3.2. Protocol 2

Most of the released NE is removed by neuronal uptake mechanism in the heart (Goldstein et al., 1988). To examine whether an increase in atrial interstitial NE level reflects the increase in synaptic NE levels associated with inhibition of neuronal uptake, we investigated the effects of sympathetic nerve stimulation on dialysate NE concentration in the presence of neuronal uptake inhibition and analyzed the relationship between dialysate NE concentration and heart rate ($n = 7$). After intravenous administration of a neuronal uptake inhibitor, desipramine (1.0 mg/kg), we stimulated the right sympathetic nerve and sampled the dialysate in a similar fashion as in Protocol 1.

2.4. Statistical analysis

All data are presented as means ± SE. Heart rate and dialysate NE concentrations (logarithmic transformation) in response to sympathetic stimulation were compared between the absence and presence of desipramine by two-way analysis of variance (ANOVA). If there was not a significant interaction between desipramine and stimulation effects, heart rate and dialysate NE concentrations (logarithmic transformation) in response to sympathetic stimulation were compared using Dunnett's test. After logarithmic transformation of dialysate NE concentration, a linear regression analysis was performed to examine the relation between dialysate NE concentration and heart rate. The differences in slope and intercept between two regression lines were examined. (Glantz, 2005) Differences were considered significant at $P < 0.05$.

3. Results

In Protocol 1 (stimulation alone), right cardiac sympathetic nerve stimulation significantly increased heart rate from 260 ± 8 bpm in the pre-stimulation control to 298 ± 11 bpm during stimulation at 2 Hz ($P < 0.01$ vs. control), 319 ± 10 bpm at 5 Hz ($P < 0.01$ vs. control) and 318 ± 11 bpm at 10 Hz ($P < 0.01$ vs. control) (ANOVA, $P < 0.001$). Heart rate recovered to 261 ± 9 bpm 20 min after stimulation. Right cardiac sympathetic nerve stimulation significantly increased dialysate NE concentration from 0.4 ± 0.1 nM in the pre-stimulation control to 1.0 ± 0.1 nM during stimulation at 2 Hz ($P < 0.01$ vs. control), 2.2 ± 0.5 nM at 5 Hz ($P < 0.01$ vs. control) and 2.9 ± 0.9 nM at 10 Hz ($P < 0.01$ vs. control) (ANOVA, $P < 0.001$). Dialysate NE concentration recovered to the pre-stimulation level 20 min after stimulation (0.6 ± 0.1 nM) (Fig. 1).

In Protocol 2 (desipramine + stimulation), intravenous administration of desipramine significantly increased baseline heart rate (295 ± 11 vs. 263 ± 11 bpm, $P < 0.01$, paired t test) and baseline dialysate NE concentration (1.5 ± 0.2 vs. 0.8 ± 0.2 nM, $P < 0.01$, paired t test) compared

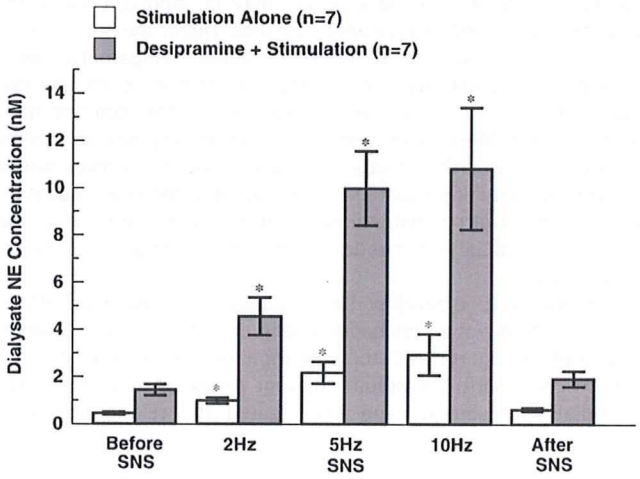

Fig. 1. Dialysate NE concentrations of controls and during electrical stimulation of right cardiac sympathetic nerve at different frequencies. The two-way analysis of variance (ANOVA) revealed the significant effect of sympathetic nerve stimulation on dialysate NE concentration ($P < 0.001$) and the significant difference in dialysate NE concentration ($P < 0.001$) between the absence and presence of desipramine. The interaction between desipramine and stimulation effects was not significant. Values are means ± SE; NE: norepinephrine; SNS: electrical sympathetic nerve stimulation; *n*: number of rabbits; *: $P < 0.01$ vs. the pre-stimulation control by Dunnett's test.

to Protocol 1. Right cardiac sympathetic nerve stimulation significantly increased heart rate from 295 ± 11 bpm in the pre-stimulation control to 349 ± 9 bpm during stimulation at 2 Hz ($P<0.01$ vs. control), 361 ± 8 bpm at 5 Hz ($P<0.01$ vs. control) and 351 ± 9 bpm at 10 Hz ($P<0.01$ vs. control) (ANOVA, $P<0.001$). Heart rate recovered to 295 ± 13 bpm 20 min after stimulation. Right sympathetic nerve stimulation also increased dialysate NE concentration from 1.5 ± 0.2 nM in the pre-stimulation control to 4.6 ± 0.8 nM during stimulation at 2 Hz ($P<0.01$ vs. control), 10.0 ± 1.6 nM at 5 Hz ($P<0.01$ vs. control) and 10.8 ± 2.6 nM at 10 Hz ($P<0.01$ vs. control) (ANOVA, $P<0.001$). Dialysate NE concentration recovered to the pre-stimulation level 20 min after stimulation (1.9 ± 0.3 nM) (Fig. 1). Heart rate and dialysate NE concentrations in Protocol 2 (desipramine + stimulation) were significantly higher than those in Protocol 1 (stimulation alone) (ANOVA, $P<0.001$). The interaction between desipramine and stimulation effects was not significant.

The relation between heart rate and dialysate NE concentration is shown in Fig. 2. Dialysate NE concentration correlated well with heart rate in both Protocols 1 and 2 (Protocol 1: $HR = 290 + 87 \times \log[NE(nM)]$, $R^2 = 0.71$; Protocol 2: $HR = 283 + 74 \times \log[NE(nM)]$, $R^2 = 0.70$) There was no significant difference in the intercept or slope between the two regression lines obtained from Protocols 1 and 2. (Glantz, 2005)

4. Discussion

We were able to monitor in vivo interstitial NE levels at the SA node using microdialysis technique. A neuronal uptake inhibitor, desipramine, significantly increased dialysate NE concentration in the right atrial myocardium. However, desipramine scarcely affected the relation between interstitial NE levels and heart rate.

4.1. Characteristics of dialysate NE concentration in right atrial myocardium

Dialysate NE concentration in the right atrial myocardium increased in response to electrical stimulation of the right cardiac sympathetic nerve and decreased to the pre-stimulation level after stimulation. These results indicate that atrial dialysate NE concentration reflects NE release from cardiac sympathetic nerve endings innervating the right atrium. Furthermore, a semi-log plot demonstrated a linear relationship between the right atrial dialysate NE concentration and heart rate. Judging from this relation, a 10-fold increase in dialysate NE concentration corresponds to an increase in heart rate of 87 bpm. The relative changes in NE release monitored by microdialysis correlate well with the frequency in depolarization of the SA nodal cell. Thus, we consider that dialysate NE concentration does reflect the relative changes in synaptic NE level. The relation between exogenous NE concentration and heart rate has been investigated in the isolated rabbit's atria (Toda, 1969). However, there is no report of a direct method to assess the endogenous NE release into the SA node. Microdialysis enables the monitoring of endogenous NE release into the SA node.

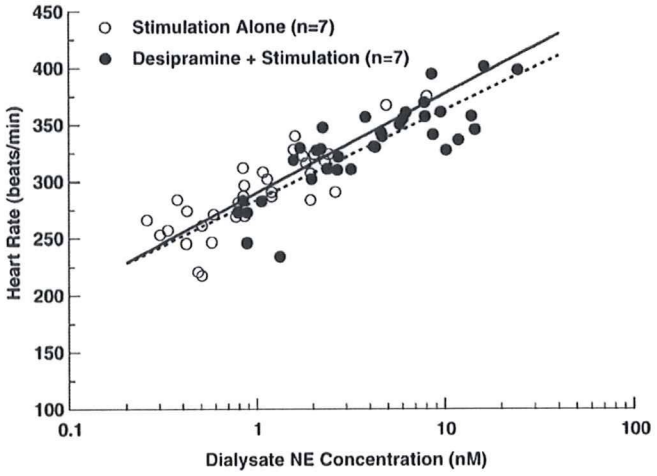

Fig. 2. Relation between dialysate NE concentration (logarithmic scale) and heart rate. Dialysate NE concentration in the right atrial myocardium correlates well with heart rate. Solid line: regression line fitting 35 data points obtained from Protocol 1 (stimulation alone) ($R^2 = 0.71$); dotted line: regression line fitting 35 data points obtained from Protocol 2 (desipramine + stimulation) ($R^2 = 0.70$). NE: norepinephrine.

4.2. Effect of neuronal uptake on dialysate NE concentration

In the presence of desipramine, a neuronal uptake inhibitor, dialysate NE concentration also increased in response to sympathetic nerve stimulation and decreased to the pre-stimulation levels after stimulation. However, dialysate NE concentrations were 3.1–4.6 times higher than the corresponding values in the absence of desipramine. These results are consistent with earlier experimental studies demonstrating that a large part of released NE is removed by neuronal uptake (Goldstein et al., 1988). In the present study, we were able to monitor the change in neuronal NE uptake function induced by desipramine using microdialysis technique.

Linked with the increase in dialysate NE concentrations in the presence of desipramine, heart rates were 33–51 bpm higher than the corresponding values in the absence of desipramine. Thus, desipramine does not alter the relation between dialysate NE concentration and heart rate. The intercept and the slope of regression line also did not differ significantly in the presence and absence of desipramine. These results indicate that neuronal uptake removes effective NE from the synaptic cleft without affecting the sensitivity of the SA nodal cell, and that neuronal NE uptake function plays an important role in the regulation of heart rate. The increase in synaptic NE concentration induced by inhibition of neuronal uptake affects the frequency of depolarization of the SA nodal cell.

Endoh (1975) reported that desipramine shifted the dose–response curve for exogenous NE to the lower NE levels. Since desipramine suppresses the neuronal uptake of both endogenous and exogenous NE, the increase in effective NE on the sinoatrial node may yield this apparent shift in the dose–response curve. Our results suggest that desipramine-inhibited neuronal uptake scarcely affects the relation between synaptic NE concentration and heart rate. Therefore, microdialysis may be a powerful tool to assess the change of synaptic NE concentration in the SA node.

4.3. Limitation

There were several limitations in the present study. First, since we did not section the left cardiac sympathetic nerve, the influence of left sympathetic nerve on the dialysate NE concentration cannot be excluded. Therefore, intravenous administration of desipramine could inhibit neuronal NE uptake at the left sympathetic nerve endings and increase dialysate NE concentration. Second, desipramine may affect the dynamic response of heart rate to sympathetic activation. We have already reported that desipramine decreases the natural frequency of the transfer function from sympathetic nerve activity to heart rate (Kawada et al., 2004). However, cardiac microdialysis using shorter dialysis fiber requires 10-min sampling time to detect changes in myocardial interstitial NE levels. Therefore, we were not able to investigate the dynamic response of heart rate to sympathetic activation in this study.

4.4. Conclusion

We were able to monitor endogenous NE release into the SA node and detect the changes in neuronal uptake function using microdialysis technique. Neuronal NE uptake together with NE release functions play