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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 post-ganglionic 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 ($P<0.05$ 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).

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.

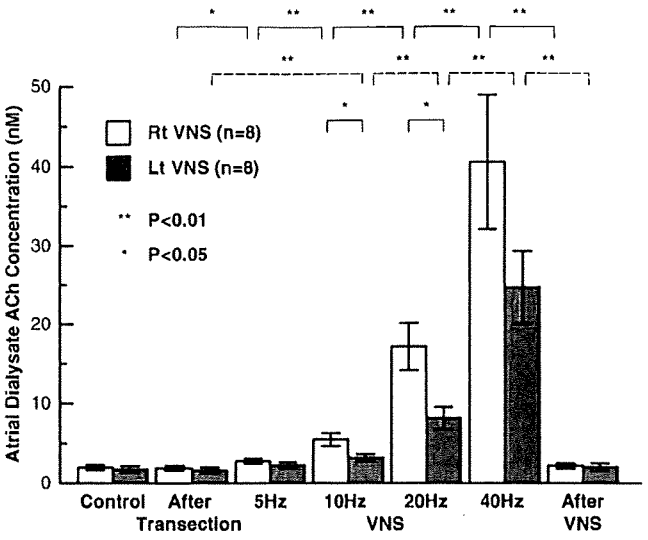

Fig. 1. Dialysate ACh concentrations of controls and during electrical vagal nerve stimulation at different frequencies. Right vagal nerve stimulation increased atrial dialysate ACh concentration from 1.9 ± 0.3 nM in the post-transection control to 2.7 ± 0.4 nM at 5 Hz, 5.5 ± 0.8 nM at 10 Hz, 17.2 ± 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.

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

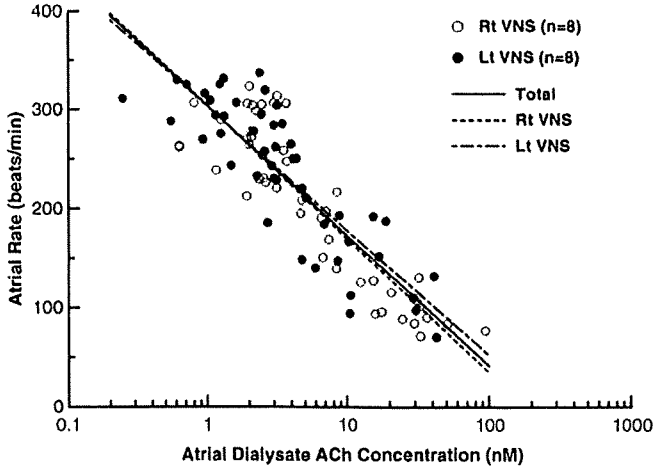

Fig. 2. Relation between dialysate ACh concentration (logarithmic scale) and atrial rate. Dialysate ACh concentration in the right atrium correlates well with atrial rate ($R^2 = 0.77$). Solid line, regression line fitting all 96 data points; dotted line, regression line fitting 48 data points of right 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.

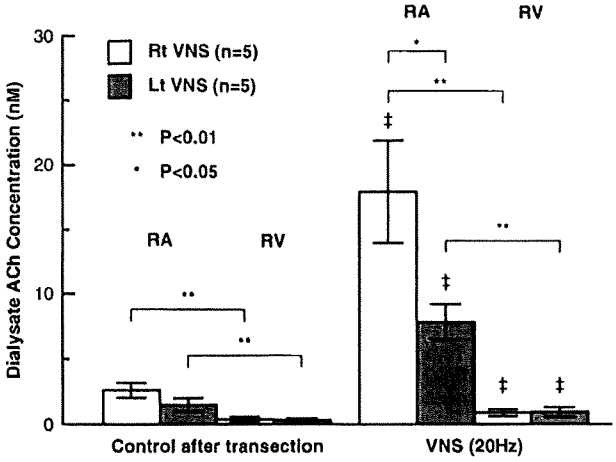

Fig. 3. Dialysate ACh concentrations in right atrium and right ventricle of controls and during electrical vagal nerve stimulation. Right vagal nerve stimulation significantly increased dialysate ACh concentration from 2.6 ± 0.6 to 17.9 ± 4.0 nM in the right atrium ($P<0.01$) and from 0.4 ± 0.2 to 0.9 ± 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.

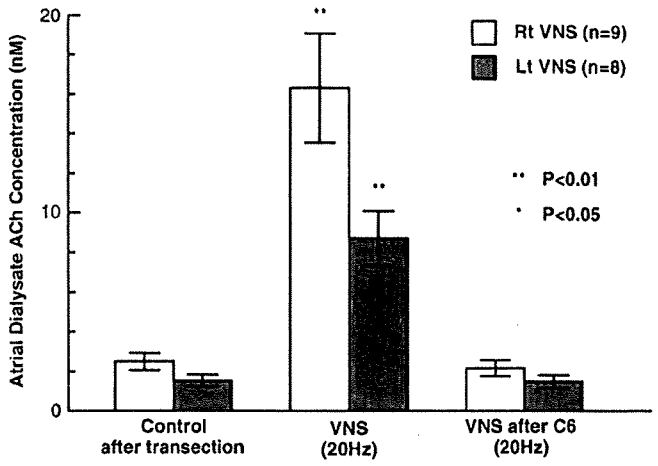

Fig. 4. Influence of ganglionic blocker on vagal nerve stimulation-induced ACh release. Right vagal nerve stimulation significantly increased atrial dialysate ACh concentration from 2.5 ± 0.4 to 16.3 ± 2.8 ($P<0.01$), and intravenous administration of hexamethonium suppressed the ACh concentration to 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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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

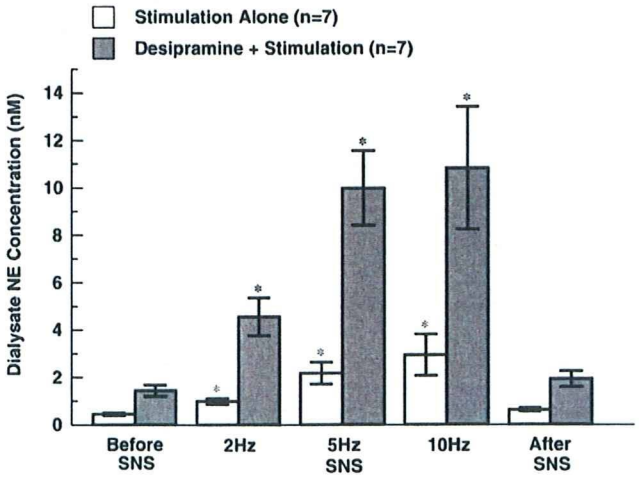

Fig. 1. Dialysate NE concentrations of controls and during electrical stimulation of right cardiac sympathetic nerve at different frequencies. The two-way analysis of variance (ANOVA) revealed the significant effect of sympathetic nerve stimulation on dialysate NE concentration ($P < 0.001$) and the 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.

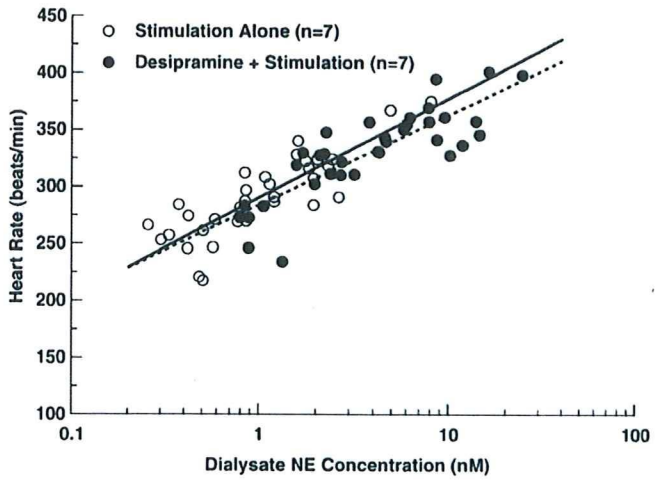

Fig. 2. Relation between dialysate NE concentration (logarithmic scale) and heart rate. Dialysate NE concentration in the right atrial myocardium correlates well with heart rate. Solid line: regression line fitting 35 data points obtained from Protocol 1 (stimulation alone) ($R^2 = 0.71$); dotted line: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

an important role in the regulation of synaptic NE concentration in the SA node. Microdialysis is a powerful tool to assess the changes of synaptic NE concentration in the SA node.

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Coronary Artery Volume Noninvasively Measured With Multislice Computed Tomography

— Definition, Accuracy and Implication —

Masaru Sugimachi, MD; Toru Kawada, MD

In this issue of *Circulation Journal*, Ehara et al[1] describe a new concept of measuring 'coronary artery volume' (CAV) to examine the balance between coronary vasculature and myocardial mass. They have developed a method of measuring CAV as accurately as possible using 64-slice computed tomography (64-MSCT). An adaptive threshold value was used to detect the coronary artery border to improve the accuracy of CAV. Ehara et al have exemplified the usefulness of CAV by examining the relationship between CAV and left ventricular mass (LVM) in consecutive patients undergoing MSCT without significant coronary artery stenosis or left ventricular wall motion abnormality. The authors concluded that CAV increases with LVM, but that the increase was not sufficient for the increase in LVM.

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What is CAV?

The authors have defined CAV as the sum of the small volumes opacified by the contrast medium. The opacified small volumes were detected by the difference of radiodensity or Hounsfield unit (an index showing the degree of transparency to X-ray) using 64-MSCT (see below for details). Because the authors have analyzed data of routine 64-MSCT for the evaluation of coronary artery disease, the image is taken when the arterial side is mainly opacified, during the diastolic cardiac phase, and under coronary vasodilatation. Therefore, CAV mainly represents the sum of volumes of epicardial coronary arteries larger than the arteries undetectable due to the limited resolution of MSCT (see below).

How Accurate and Reproducible is CAV Measurement?

In this article, the authors have established a method of measuring CAV with every attempt to improve the accuracy and reproducibility for their MSCT device. These procedures are worthy of being discussed for other researchers who are interested in and would like to reproduce CAV measurement.

Inaccuracies and variability of CAV measurement would arise from (1) an arbitrary cut-off value for border detection, (2) partial volume effect, (3) motion artifact and (4) possible variable resolution of various MSCT devices. The authors have wisely minimized the errors introduced by the first 3 factors.

It is usually difficult to determine the border of the coronary arteries with a reasonable criterion. This may be because opacification of arteries is incomplete, or the opacification is thinner near the border than the center, resulting in a gradual decrease in radiodensity at the border, rather than a clear-cut abrupt change in radiodensity. In addition, at the border of small arteries, a voxel (the smallest size identified by 64-MSCT) may contain both arterial lumen (which is opacified) and arterial wall (which is not opacified). A voxel has a radiodensity of an intermediate value between an opacified and unopacified voxel, which is known as the 'partial volume effect'.

To minimize the errors introduced by an arbitrary cut-off value and the partial volume effect, the authors have developed a way of reasonably determining the cut-off value for border detection, based on preliminary phantom experiments with moving cylinders containing various concentrations of contrast medium. The results of these preliminary experiments are summarized in Figures 1–3 in Ehara et al[1] Figure 2 clearly shows that a cut-off value that exactly reproduces the phantom cylinder volume can be determined. The cut-off value is, however, not fixed, but changes with the true radiodensity of the contrast medium in the cylinder. Based on this, the authors determined the cut-off value for CAV measurement, adaptively in each subject, in reference to the radiodensity of the proximal region of the left and right coronary arteries. The cut-off value was not relatively influenced by different heart rates, which also decreased the degree of error by motion artifacts. Similar procedures may be applicable to quantitative coronary angiography.

The determined threshold is, however, only valid for the specific MSCT device used in the study by Ehara et al[1] If other researchers are to reproduce their CAV measurement, another attempt to determine the threshold for their device is necessary.

The limited resolution of MSCT would determine the definition of CAV. The authors used MSCT with an isotropic resolution of 400μm. This indicates that CAV in the paper by Ehara et al would be the sum of volume of the arteries >400μm. If MSCT is used with a different resolution, the definition of CAV would be different and CAV would be systematically different.

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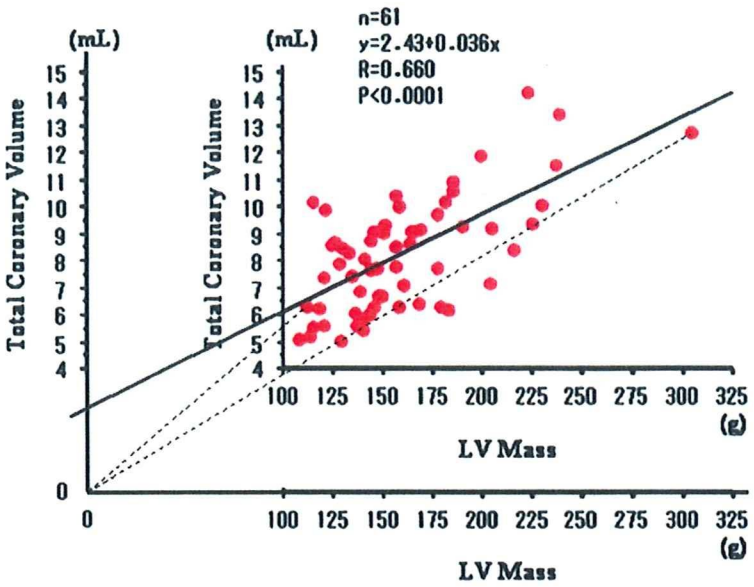

Figure. Linear regression between coronary artery volume (total coronary volume) and left ventricular (LV) mass (reproduced and modified from Ehara et al[1]). The axes are extended and the regression line is extrapolated to show a positive offset of coronary artery volume. Schematically, the authors have compared the slopes of dashed lines.

Is CAV a Proxy for Capillary Density or Coronary Flow Reserve?

The relation between coronary vasculature and myocardial mass, or more specifically inappropriate perfusion of the myocardium, has been traditionally examined histologically[2] by capillary density. Later, similar information was obtained in vivo by the measurement of coronary flow reserve. In fact, some have described the relationship between coronary capillary density and coronary flow reserve in patients with hypertrophic cardiomyopathy[3] in patients with idiopathic dilated cardiomyopathy[4] or in mini pigs with hypercholesterolemia[5]

In contrast, the way in which CAV correlates with coronary capillary density or coronary flow reserve is yet to be determined. As CAV measures the volume of arteries far larger than capillaries, these problems need to be resolved (eg, by animal experiments) before we can measure CAV in patients with a wide variety of cardiovascular diseases.

It is also reasonable to assume CAV may provide information other than coronary capillary density or coronary flow reserve. In Ehara et al, CAV is only measured under nitroglycerine. The response of CAV to increased coronary flow or to endothelium-dependent vasodilatation may be of clinical value. If better accuracy and reproducibility is established, CAV may potentially replace quantitative coronary angiography for this purpose because of its noninvasive nature.

Is CAV Really Unmatched With LVM?

The authors' conclusion of unmatched CAV with LVM should be discussed. **Figure** shows the linear regression between CAV and LVM reproduced and modified from Figure 6 of Ehara et al. The modified figure has extended axes and the extrapolated regression line has been added.

Even though there is only a single data set for each patient, the authors assumed that the line started at the origin and calculated the slope. Schematically, they have compared the slopes of dashed lines.

Figure, however, indicates that the CAV–LVM relationship obtained from pooled data has a positive CAV offset, but does note indicate that the slope is shallow. Because there is no reason to deny the presence of a positive CAV offset, and because the slope was not compared with a standard slope, the conclusion of unmatched CAV with LVM is not solid.

This question may be resolved by comparing the CAV–LVM relationship obtained by sequential CAV measurement during physiological growth and that obtained during the progression of pathological hypertrophy of the heart in animal experiments.

Advantage of CAV Measurement

The noninvasive nature of CAV measurement enhances its clinical usefulness because it enables sequential evaluation and may help to bring evaluations still in the investigational stage into routine bedside practice. Similar technological developments (eg, coronary flow reserve by cine magnetic resonance[6]) may be combined and eventually enable the detailed pathophysiology of cardiovascular disease to be described.

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Feedback Control of Multiple Hemodynamic Variables with Multiple Cardiovascular Drugs

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Abstract— **The ultimate goal of disease treatment is to control the biological system beyond the native regulation to combat pathological process. To maximize the advantage of drugs, we attempted to pharmacologically control the biological system at will, e.g., control multiple hemodynamic variables with multiple cardiovascular drugs. A comprehensive physiological cardiovascular model enabled us to evaluate cardiovascular properties (pump function, vascular resistance, and blood volume) and the feedback control of these properties. In 12 dogs, with dobutamine (5±3 μg·kg^{-1}·min^{-1}), nitroprusside (4±2 μg·kg^{-1}·min^{-1}), dextran (2±2 ml·kg^{-1}), and furosemide (10 mg in one, 20 mg in one), rapid, sufficient and stable control of pump function, vascular resistance and blood volume resulted in similarly quick and stable control of blood pressure, cardiac output and left atrial pressure in 5±7, 7±5, and 12±10 minutes, respectively. These variables remained stable for 60 minutes (RMS 4±3 mmHg, 5±2 ml·min^{-1}·kg^{-1}, 0.8±0.6 mmHg, respectively).**

I. Introduction

THE ultimate goal of disease treatment is to control the biological system beyond the native regulation to combat pathological process. This control may be partly achieved by native regulatory systems, but these frequently fail when disease progresses.

Many pharmacological treatments have provided us with control measures that may act in ways not possible by native regulators. To fully take advantage of these medicines, we must establish ways of using these agents to control the biological system at our will. As an example, we tried to control multiple hemodynamic variables with multiple cardiovascular drugs.

Several closed-loop systems have succeeded in directly controlling a single hemodynamic variable [1,2]. Multiple-variable control, however, has been unsuccessful [3-5].

Multiple-input multiple-output feedback control remains a challenge if the input-output relationships for all combinations are of equal significance. We therefore tried to decouple the input-output relationships by using a comprehensive physiological cardiovascular model. The model enabled us to define a set of parallel independent relationships between cardiovascular properties and drugs: pump function / inotrope, vascular resistance / vasodilator, and blood volume / volume expander. The model also provided us with a method to quantitatively calculate cardiovascular properties.

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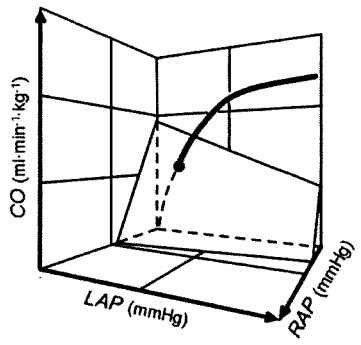

Fig. 1. Extended Guyton's model.
Thick curve, pump function of left and right heart; shaded surface, capacitive function of total vascular beds; CO, cardiac output; LAP, left atrial pressure; RAP, right atrial pressure.

II. Model and Methods

A. Cardiovascular property identification

Abnormalities of hemodynamic variables arise from abnormalities of cardiovascular properties, including pump function, vascular resistance, and blood volume. We identified these properties using an extended version of Guyton's circulatory equilibrium framework (Fig. 1) [6,7].

Pump function of the left heart (S_L) can be quantified as the ratio of cardiac output (CO) to the logarithm of left atrial pressure (LAP) (S_L = CO / [ln (LAP - 2.03) + 0.80]). Systemic vascular resistance (R) can be calculated as blood pressure (BP) minus right atrial pressure (RAP) divided by CO. Stressed total blood volume (V) is obtained by V = (CO + 19.61 RAP + 3.49 LAP) × 0.129.

B. Autopilot System

Autopilot controller of multiple hemodynamic variables consisted of multiple feedback loops. We designed these

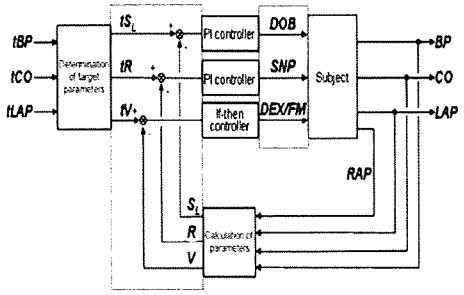

Fig. 2. Autopilot controller.
Calculated cardiovascular properties, rather than hemodynamic variables, were feedback-controlled to achieve multiple independent control of variables.

feedbacks as being independent of each other. The selection and the combination of controlled property and the controlling drugs enabled the independent operation (Fig. 2) [8].

S_L and R were controlled by proportional-integral (PI) feedback, with infusion of dobutamine (DOB) and sodium nitroprusside (SNP), respectively. Proportional and integral gain values were calculated using Chien-Hrones-Reswick's method [9] from gain, time constant, and dead-time delay of the approximated first-order step responses of S_L to DOB and R to SNP. We infused 10% dextran 40 solution (DEX, 10 $ml \cdot min^{-1}$) as long as V was <1 $ml \cdot kg^{-1}$ than the target, and injected furosemide (FM, 10 mg) every 20 minutes while V was >2 $ml \cdot kg^{-1}$ than the target.

C. *Animal Experiments*

We evaluated the performance of the autopilot controller in 12 adult anesthetized mongrel dogs (both sexes, 25±4 kg). We measured BP, CO, LAP and RAP. DOB, SNP, and DEX were automatically administered into the femoral vein through independent infusion routes, using either a computer-controlled roller pump or an infusion pump. FM was given through the jugular vein manually according to computer instructions.

These dogs underwent coronary microembolization, resulting in left ventricular failure. After hemodynamic stabilization, we began implementing control using the autopilot system.

III. Results

	Proportional gain (K_p) $\mu g \cdot ml^{-1}$	Integral gain (K_i) sec^{-1}
S_L control	0.06	0.01
R control	-1.37	0.007

Table 1. Selected gain parameters for designed controller.
Dose ($\mu g \cdot kg^{-1} \cdot min^{-1}$) of drugs for the control of S_L (DOB) or R (SNP) is determined as (Dose) = $K_p(1 + K_i / s)\,\Delta$(Controlled variable)

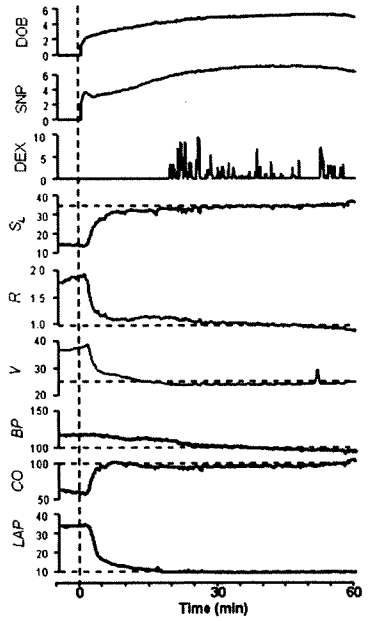

Fig. 3. An example of the automatic control of hemodynamics.
Feedback control was rapid, sufficient, and stable. DOB, dobutamine ($\mu g \cdot kg^{-1} \cdot min^{-1}$); SNP, sodium nitroprusside ($\mu g \cdot kg^{-1} \cdot min^{-1}$); DEX, dextran 40 solution ($ml \cdot min^{-1}$); S_L, pump function ($ml \cdot kg^{-1} \cdot min^{-1}$); R, resistance ($mmHg \cdot ml^{-1} \cdot kg \cdot min$); V, blood volume ($ml \cdot kg^{-1}$); BP, blood pressure (mmHg); CO, cardiac output ($ml \cdot kg^{-1} \cdot min^{-1}$); LAP, left atrial pressure (mmHg)

Based on the step response from coronary microembolized dogs, we determined the proportional and integral gain as shown in Table 1.

Similar to the example shown in Figure 3, in 12 dogs, by administering DOB (5±3 $\mu g \cdot kg^{-1} \cdot min^{-1}$), SNP (4±2 $\mu g \cdot kg^{-1} \cdot min^{-1}$), DEX (2±2 $ml \cdot kg^{-1}$), and FM (10 mg in one, 20 mg in one), rapid, sufficient and stable control of S_L, R and V. This resulted in corresponding appropriate control of BP, CO and LAP in 5±7, 7±5, and 12±10 minutes, respectively. These remained stable for 60 minutes (RMS BP=4±3 mmHg, CO=5±2 $ml \cdot min^{-1} \cdot kg^{-1}$, LAP=0.8±0.6 mmHg).

IV. Discussion

We have shown that by evaluating cardiovascular properties (pump function, vascular resistance, and blood volume), and then controlling these properties with individually selected drugs, we were able to automatically control multiple hemodynamic abnormalities rapidly, stably, and simultaneously.

Direct control of multiple hemodynamic variables, however, likely fails because each drug affects more than one variable. Direct control remains unfeasible even with more complicated methods developed in control engineering; appropriate physiological modeling and precise evaluation of cardiovascular properties are essential to achieving adequate control.

V. CONCLUSION

Calculating cardiovascular properties (pump function, vascular resistance, and blood volume) based on a comprehensive cardiovascular model and feedback control of these properties are required for the accurate control of multiple hemodynamic variables (BP, CO, LAP).

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Macroscopic Two-Pump Two-Vasculature Cardiovascular Model to Support Treatment of Acute Heart Failure

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Abstract— **Comprehensive understanding of hemodynamics remains a challenge even for expert cardiologists, partially due to a lack of an appropriate macroscopic model. We attempted to amend three major problems of Guyton's conceptual model (unknown left atrial pressure, unilateral heart damage, blood redistribution) and developed a comprehensive macroscopic model of hemodynamics that provides quantitative information. We incorporated a third axis of left atrial pressure, resulting in a 3D coordinate system. Pump functions of left and right heart are expressed by an integrated cardiac output curve, and the capacitive function of total vasculature by a venous return surface. The equations for both the cardiac output curve and venous return surface would facilitate precise diagnosis (especially evaluation of blood volume) and choice of appropriate treatments, including application to autopilot systems.**

I. Introduction

COMPREHSNSIVE understanding of hemodynamics remains a challenge even for specialist clinicians including cardiologists. This is in part attributed to a lack of an appropriate macroscopic model of hemodynamics that would facilitate reasoning. Most cardiologists relied only on, if at all, the classical Guyton's circulatory equilibrium framework [1].

Guyton's model consists of only two subdivisions of the whole circulation: the cardiopulmonary component (in which both hearts and pulmonary vasculature are lumped) and the systemic vascular bed. These two subdivisions are characterized by the 'cardiac output curve' and 'venous return curve', respectively. The 'cardiac output curve' approximated the (total) pump function, and the 'venous return curve' approximated the capacitive function of systemic vasculature. The intersection of these curves coincides with the operating point of the circulation.

Guyton's model is, however, inappropriate (see Model and Methods) for the understanding of hemodynamics in patients with, for example, acute myocardial infarction, where only one ventricle is preferentially damaged. That is why many cardiologists gradually abandoned using Guyton's model for their reasoning.

If we can amend the shortcomings of Guyton's model and develop a more appropriate model, the new model would obviously help diagnosis procedures and treatment selection. Furthermore, the model may be able to quantify the hemodynamic abnormalities rather than just to identify them.

Therefore, the aim of this study was to develop a comprehensive macroscopic model of hemodynamics that would provide quantitative information and aid diagnosis and treatments.

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II. Model and Methods

A. Shortcomings of Guyton's Model

Guyton's model has a number of problems when used in patients with unilateral heart failure.

First, the model does not provide left atrial pressure (LAP) values directly. LAP indicates the degree of pulmonary congestion and blood desaturation, and is as important as cardiac output (CO) and blood pressure.

Second, it is impossible to precisely model unilateral heart failure, which is frequently seen in patients with ischemic heart disease.

Third, in unilateral heart failure, the relative blood volumes in pulmonary and systemic vascular beds vary. As Guyton's model assumes only blood volume within the systemic vascular bed, such redistribution would shift the venous return curve even though the total blood volume remains the same.

B. Development of Comprehensive Cardiovascular Model

To solve the above problems, we extended Guyton's model.

First, a third axis of LAP was introduced in our new model (Fig. 1) [2], [3], so that LAP can be obtained directly. The pumping ability of the heart and the capacitive function of the vasculature are expressed simultaneously in the 3D space (RAP-LAP-CO coordinate system).

Second, the pumping abilities of the left and right heart are expressed separately by the respective cardiac output surfaces that are independent of each other. In an equilibrium state, by matching the cardiac output of both sides, the pumping ability of the whole heart can be integrated and expressed by a curve

expressing the intersection of the two surfaces (integrated cardiac output curve, Fig. 1, thick curve).

Third, the capacitive function of total vasculature (including both systemic and pulmonary vasculatures) is expressed by the venous return surface (Fig. 1, shaded surface), which is an extension of the venous return curve. This surface expresses the changes in LAP and right atrial pressure (RAP) in response to CO change, while the total intravascular blood volume remains constant. In addition, blood redistribution between systemic and pulmonary vasculatures (without change in total blood volume) will be expressed by movement within the surface rather than by deviation from the surface.

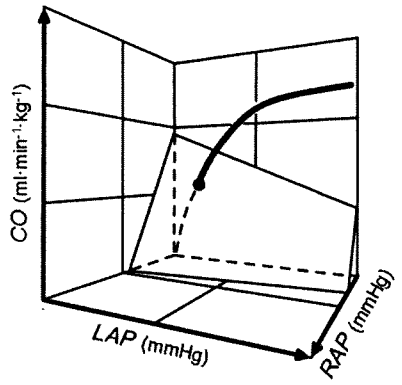

Fig. 1. An original macroscopic model of hemodynamics (an extended Guyton's model). The curve expresses the integrated pumping ability of left and right heart. The shaded surface characterizes the capacitive function of the total (systemic + pulmonary) vasculatures. The surface remains constant as long as the total intravascular blood volume remains the same. CO, cardiac output; LAP, left atrial pressure; RAP, right atrial pressure.

C. Animal Experiments to Characterize Venous Return Surface

Figure 2 depicts the scheme of an experiment to characterize the venous return surface. We replaced the left and right heart with roller pumps, which allows us to change CO of the right heart or left heart independently.

By adjusting the flow (i.e., CO) of the two pumps to the same level, the changes in RAP and LAP in response to a change in CO can be observed. Blood redistribution between systemic and pulmonary vasculatures can be reproduced by transiently unbalancing the flow of the two pumps.

From each dog (n = 6), we obtained 6 different sets of data (CO, RAP, LAP). These data were subjected to bivariate linear regression using RAP and LAP as independent variables and CO as the dependent variable.

III. Results

Figure 3 illustrates the venous return surfaces obtained from 6 dogs. Bivariate linear regression in each animal yielded a flat surface in 3D space. The surface is shown as a line in Fig. 3, because we have projected the surface in a

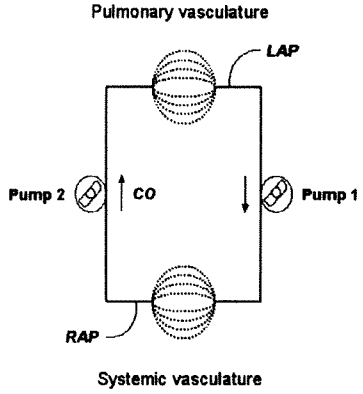

Fig. 2. An experimental scheme to characterize venous return surface. By replacing the left and right heart with roller pumps, one can change cardiac output of the right heart or left heart independently.

direction parallel to the surface. The experimental data obtained from each of the 6 animals showed good fit with the surface. In addition, the surfaces obtained from 6 animals were almost parallel, as shown by the nearly parallel 3D coordinate axes. These experimental results indicated that the venous return surface is linear and can be expressed by a common equation for all animals.

Further, by infusing or withdrawing known amounts of blood, we were able to derive an equation for the venous return surface as follows:

$$CO = V / 0.129 - 19.61\ RAP - 3.49\ LAP$$

where V is total intravascular stressed blood volume. This formula [$V = (CO + 19.61\ RAP + 3.49\ LAP) \times 0.129$] can be used to quantify V from CO, RAP and LAP.

We also succeeded to quantify the integrated cardiac output curve by logarithmic functions as follows:

$$CO = S_L\ [\ln(LAP-2.03)+0.80]$$

$$CO = S_R\ [\ln(RAP-2.13)+1.90]$$

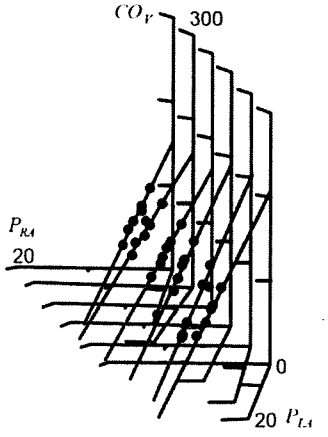

Fig. 3. Superimposed venous return surfaces obtained from 6 dogs. For each dog, the venous return surface (RAP-LAP-CO relationship) in 3D coordinate system was projected in a direction parallel to the surface, and was superimposed with each other.

where S_L and S_R are parameters expressing the pumping ability of the left and right heart, respectively. These equations are also useful for quantifying the pumping ability of right and left heart (S_L = CO / [ln(LAP - 2.03) + 0.80], S_R = CO / [ln(RAP - 2.13) + 1.90]).

Using this model, we are able to predict with acceptable precision the hemodynamics after infusion or withdrawal of known amounts of blood (CO: $y = 0.93x + 6.5$, $r^2 = 0.96$, SEE = 7.5 ml·min^{-1}·kg^{-1}; LAP: $y = 0.90x + 0.5$, $r^2 = 0.93$, SEE = 1.4 mmHg; RAP: $y = 0.87x + 0.4$, $r^2 = 0.91$, SEE = 0.4 mmHg) (Fig. 4) [3].

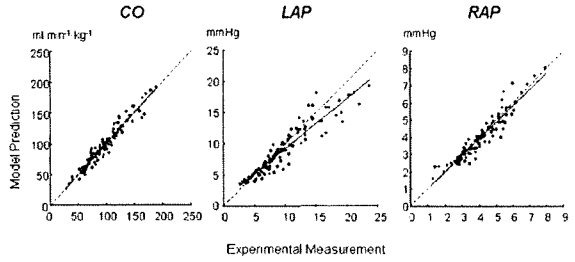

Fig. 4. Prediction of CO, LAP, and RAP based on our comprehensive macroscopic model of hemodynamics.

IV. Discussion

A. Difficulty in Decision Making of Heart Failure Treatment

Three hemodynamic variables: blood pressure, CO and LAP, appear to be the most essential factors influencing the survival of patients with heart failure. Our model clearly indicates that pump functions of left and right heart and total intravascular blood volume are determinants of CO and LAP. Systemic vascular resistance is an additional determinant of blood pressure.

For clinicians, the evaluation of blood volume is relatively difficult compared to pump functions and vascular resistance. In practice, clinicians have been using RAP as a proxy for blood volume. It is clear from our results [V = (CO + 19.61 RAP + 3.49 LAP) × 0.129] that blood volume (V) is not solely determined by RAP. Rather, all three parameters of CO, RAP and LAP are necessary to evaluate blood volume. The equation indicates that an increase of RAP by 1 mmHg is equivalent to an LAP increase of 5.6 mmHg, and a CO increase of 19.61 mL/min/kg (ca. 0.98 L/min for a 50-kg patient).

B. Application of the Model: Autopilot System

The biggest benefit of our comprehensive visual model of hemodynamics is that it enables us to diagnose the abnormality of cardiovascular system in a quantitative manner. This would lead to appropriate selection of drugs and their doses.

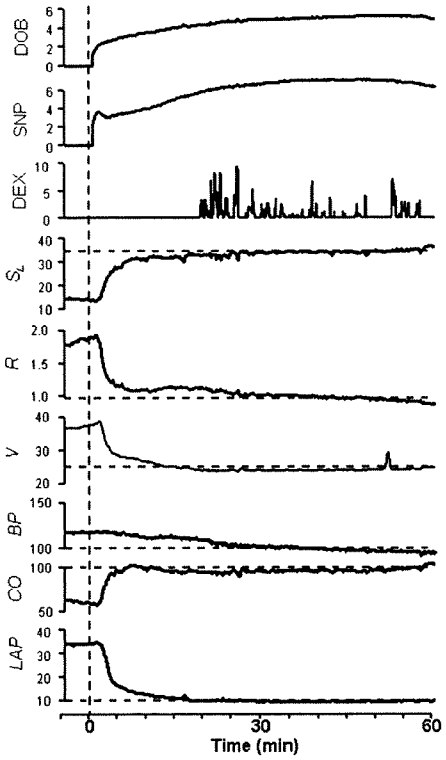

Fig. 5. An example of correction of hemodynamics with an autopilot system. By normalizing cardiovascular properties [pump function (S_L), resistance (R), blood volume (V)] with the administration of dobutamine (DOB), sodium nitroprusside (SNP), and dextran 40 solution (DEX), all the abnormal hemodynamic variables (increased blood pressure [BP], decreased cardiac output [CO], and elevated left atrial pressure [LAP]) were resolved rapidly, sufficiently, and stably.

As shown in Fig. 5, by translating hemodynamic variables into cardiovascular properties (pump function, vascular resistance, and blood volume), and by controlling each of these parameters with individual drug with preferential effect on the parameter, we are able to correct automatically all the parameters of blood pressure, CO and LAP rapidly, stably, and simultaneously.

Using an autopilot system to administer dobutamine (DOB at 5 ± 3 mg·kg^{-1}·min^{-1}), nitroprusside (SNP at 4 ± 2 mg·kg^{-1}·min^{-1}), dextran infusion (DEX at 2 ± 2 ml·kg^{-1}), and furosemide (10 mg in one, 20 mg in one) in 12 dogs with acute heart failure rapidly normalized blood pressure, CO, and LAP in 5 ± 7, 7 ± 5, and 12 ± 10 minutes, respectively. The normalized values remained stable thereafter (RMS values, blood pressure = 4 ± 3 mmHg, CO = 5 ± 2 ml·min^{-1}·kg^{-1}, LAP = 0.8 ± 0.6 mmHg).

V. Conclusion

We have successfully developed a comprehensive macroscopic model of hemodynamics that provides quantitative information. Using a 3D coordinate system, the pump functions of left and right heart are expressed by an

integrated cardiac output curve, and the capacitive function of total vasculature by a venous return surface. The equations of both the cardiac output curve and venous return surface would facilitate accurate diagnosis (especially evaluation of blood volume) and choice of appropriate treatments, including application to autopilot systems.

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