35). These equivocal results could be explained by differences in species and modes of exercise training among studies (i.e., exercise type, intensity, and duration etc.). More importantly, since these studies of autonomic nerve stimulation did not evaluate HRV, a causal relationship between increased HRV and adaptation in peripheral autonomic HR control remains largely undetermined. Furthermore, despite the fact that HRV has been evaluated using frequency domain as well as time domain analyses, to date, there are no reports available examining the effects of exercise training on the dynamic HR response to sympathetic or vagal stimulation in the frequency domain. Analysis of peripheral autonomic regulation in the frequency domain would advance our understanding of the mechanisms responsible for the alterations in HRV that occur in response to exercise training.

We have recently developed a technique to assess the dynamic characteristics of HR control by the autonomic nervous system in rats using transfer function analysis (21). The transfer function analysis can quantitatively evaluate the HR response to autonomic nerve stimulation over a wide frequency range which is necessary for interpreting the generation of HRV. Therefore, the aims of the present study were i) to identify the dynamic characteristics of sympathetic and vagal HR control in exercise trained rats and ii) to determine whether alterations in peripheral autonomic regulation contribute to changes in the frequency components of HRV in exercise trained rats.

Materials and Methods

Animal care and training program.

Animal care was in accordance with the "Guiding Principles for Care and Use of Animals in the Field of Physiological Sciences," approved by the Physiological Society of Japan. All protocols were reviewed and approved by the Animal Subjects Committee of the National Cerebral and Cardiovascular Center. Fourteen male Sprague-Dawley rats (200 ~ 250g body weight) were fed standard laboratory chow and water ad libitum and housed (3 per cage) in a temperature-controlled room with a 12:12-h dark-light cycle. Rats were randomly assigned to one of two groups: sedentary (n = 7) and exercise trained (n = 7).

Exercise training was performed on a motor-driven treadmill, 5 days/wk for 16 wk, gradually progressing toward a speed of 15 m/min at 15° for 60 min. Sedentary rats walked (10m/min at 15°) 10 min/d once per week during the 16-week period to maintain treadmill familiarity. At the end of the 16-week period, maximal exercise capacity was measured twice in each rat in tests separated by 2 days (6). The protocol for the maximal exercise capacity test consisted of walking at 10 m/min for 5 minutes followed by 2 m/min increases in speed every 2 minutes until the rat reached exhaustion. Rats were considered exhausted when they failed to stay off of a shock bar.

Assessment of autonomic tone in the conscious resting state

After the performance test, three steel electrodes were implanted under anesthesia. These electrodes were utilized for monitoring the electrocardiogram. R-R interval was measured using a cardiotachometer (AT601G, Nihon Kohden, Tokyo, Japan). On the first day of the study, which was 24 h after electrodes had been implanted, resting HR was recorded to analyze the R-R interval variability

in the quiet unrestrained rat which was kept in a small box. In accordance with a previous study (25), autonomic tone was assessed by intraperitoneal injections of methylatropine (3 mg/kg) and propranolol (4 mg/kg). Immediately after resting HR was recorded, methylatropine was injected. Since the HR response to methylatropine reached its peak in 10-15 min, this time interval was allocated before the HR measurement. Propranolol was injected after methylatropine injection, and again the HR was measured after 10-15 min. Intrinsic HR was evaluated after simultaneous blockade by propranolol and methylatropine. Sympathetic tonus was defined as the difference between the HR after methylatropine injection and intrinsic HR. On the second day, propranolol was administered first to obtain the inverse sequence of blockade. Vagal tonus was defined as the difference between the HR after propranolol injection and intrinsic HR.

Sympathetic and vagal stimulation

Surgical Preparations. After obtaining data for the assessment of autonomic tone and HRV, rats were anesthetized by a mixture of urethane (250 $mg \cdot ml^{-1}$) and α-chloralose (40 $mg \cdot ml^{-1}$), initiated with an intraperitoneal bolus injection of 1 $ml \cdot kg^{-1}$. If additional anesthesia was needed, 0.1 $ml \cdot kg^{-1}$ was given intraperitoneally. The rats were intubated and mechanically ventilated with oxygen-enriched room air. The rats were slightly hyperventilated to suppress chemoreflexes. A catheter was placed in the right femoral artery and connected to a pressure transducer (DX-200, Nihon Kohden, Tokyo, Japan) to measure arterial pressure (AP). HR was measured using a cardiotachometer (AT601G, Nihon Kohden, Tokyo, Japan) triggered by the R wave on the electrocardiogram.

A catheter was introduced into the right femoral vein for drug administration. Sinoaortic baro-denervation was performed bilaterally to minimize changes in sympathetic efferent nerve activity via arterial baroreflexes. The vagi were sectioned bilaterally at the neck. A pair of bipolar stainless steel electrodes was attached to the right cervical sympathetic nerve for efferent sympathetic stimulation or the right cervical vagus for efferent vagal stimulation. The stimulation electrodes and nerve were secured with silicon glue (Kwik-Sil, World Precision Instruments, Sarasota, FL). Body temperature was monitored with a thermometer placed in the rectum, and was maintained at 38°C with a heating pad throughout the experiment.

Experimental Procedures. The pulse duration was set at 2 ms and the stimulation amplitude was fixed at 10 V for both sympathetic and vagal nerve stimulation. To allow for stabilization of hemodynamics, sympathetic and vagal nerve stimulations were started ~1 h after the end of surgical preparations. Between sympathetic and vagal stimulation protocols, > 15 min elapsed to allow AP and HR to return to their respective baseline values.

To estimate the dynamic transfer characteristics from sympathetic stimulation to HR response, the sectioned end of the right cervical sympathetic nerve was stimulated employing a frequency-modulated pulse train for 10 min. The stimulation frequency was switched every 1000 ms to either 0 or 5 Hz according to a binary white-noise signal. The power spectrum of the stimulation signal was reasonably constant up to 0.5 Hz. The transfer function was estimated up to 0.5 Hz because the reliability of estimation decreased due to the diminution of input power above this frequency. The selected frequency range sufficiently spanned the range of physiological interest (21). For

estimation of the static transfer characteristics from sympathetic stimulation to HR response, stepwise sympathetic stimulation was performed. Sympathetic stimulation frequency was increased from 1 to 5 Hz in 1-Hz increments. Each frequency step was maintained for 60 s.

To estimate the dynamic transfer characteristics from vagal stimulation to HR response, the right vagus was stimulated employing a frequency-modulated pulse train for 10 min. The stimulation frequency was switched every 500 ms to either 0 or 10 Hz according to a binary white-noise signal. The power spectrum of the stimulation signal was reasonably constant up to 1 Hz. The transfer function was estimated up to 1 Hz because the reliability of estimation decreased due to the diminution of input power above this frequency. The selected frequency range sufficiently spanned the range of physiological interest (21). For estimation of the static transfer characteristics from vagal stimulation to HR response, stepwise vagal stimulation was performed. Vagal stimulation frequency was changed among 2, 4, 8, 16, and 32 Hz. Each frequency step was maintained for 60 s.

Data analysis

Spectral analysis of HRV. Data obtained during the conscious resting state were digitized at 200 Hz utilizing a 12-bit analog-to-digital converter and stored on the hard disk of a dedicated laboratory computer system. Beat-by-beat time series of R-R interval were interpolated every 130 ms (Δt). Twelve data segments of 512 (N) points overlapping half of the preceding data were processed. For each data segment, after the linear trend was removed and Hanning window applied, power spectral density was computed using the fast

Fourier transform algorithm. The frequency resolution was $\Delta f = 1/(N\ \Delta t)$, i.e., 0.015 Hz, and the highest frequency was $\Delta f = 1/2\Delta t$, i.e., 3.85 Hz, where f is frequency. The very low frequency (VLF) band ranged between 0.017 and 0.27 Hz, the low frequency (LF) band between 0.27 and 0.75 Hz, and the high frequency (HF) band between 0.75 and 3.3 Hz, according to an earlier report (8). The percentage of LF or HF power relative to the sum of LF and HF powers, and the ratio of LF to HF power were also calculated.

Transfer function analysis. The dynamic characteristics of the HR response to sympathetic or vagal stimulation were estimated by a transfer function analysis (see Appendix for details). Dynamic sympathetic control of HR was quantified by fitting a second-order low-pass filter with pure delay to the estimated transfer function. The dynamic vagal control of HR was quantified by fitting a first-order low-pass filter with pure delay to the estimated transfer function. To facilitate the intuitive understanding of the system's dynamic characteristics, we calculated the system step response of HR to 1-Hz nerve stimulation as follows:

The system impulse response was derived from the inverse Fourier transform of the transfer function. The system step response was then obtained from the time integral of the impulse response. The length of the step response was 51.2 s. The 80% rise time for the sympathetic step response or the 80% fall time for the vagal step response was estimated as the time at which the step response reached 80% of the steady-state response calculated by averaging the last 10 s of data of the step response.

Statistical analysis

All data are represented as mean ± SD. Data were analyzed using unpaired t-tests (sedentary vs. exercise trained) or two-way repeated measures ANOVA. Values of $P < 0.05$ were considered to be significant.

Results

Physical characteristic

Morphometric characteristics and exercise capacity for sedentary and exercise trained rats are presented in Table1. The mean body weight of the exercise trained rats was significantly smaller than that of the sedentary rats. The mean ventricular weight of the exercise trained rats was slightly but significantly smaller than that of the sedentary rats. Consequently, the ventricular weight normalized by body weight was significantly greater in the exercise trained compared to the sedentary group. The lung weight to body weight ratio was not different between the groups. Exercise capacity was 64% greater in the exercise trained than in the sedentary group. The reproducibility of measuring the maximal exercise capacity was reasonably high ($y = 1.2x - 226.1$, $R^2 = 0.79$, x and y represent the first and second measurements).

Spectral analysis of HRV and autonomic tone in the conscious resting state

The power spectral densities of R-R interval are shown in Table 2. The percentage of LF power was significantly smaller and the percentage of HF power was significantly greater in the exercise trained rats than in the sedentary rats. The LF/HF ratio in the exercise trained rats was significantly smaller compared with that in the sedentary rats. HR at rest was significantly lower in

the exercise trained compared to the sedentary group (Fig. 1*A*). The intrinsic HR was similar between the groups (Fig. 1*A*). Although the sympathetic tonus was comparable between the groups, the vagal tonus tended to be greater (P = 0.08) in the exercise trained compared to the sedentary group (Fig. 1*B*).

Dynamic sympathetic and vagal transfer functions

Table 3 summarizes hemodynamics during dynamic sympathetic stimulation. Sympathetic stimulation significantly increased mean HR in both sedentary and exercise trained groups. Mean HR and AP did not differ between the groups, before and during sympathetic stimulation. Figure 2*A* illustrates the dynamic transfer function characterizing sympathetic HR control. The frequency band effect was significant ($P < 0.0001$) but the group effect was insignificant (P = 0.5461) in the dynamic gain values of the sympathetic transfer function by two-way repeated measures ANOVA. The parameters of the sympathetic transfer function were comparable between the groups (Table 4). Figure 2*B* shows the calculated step response of HR to sympathetic stimulation. The steady–state response and the 80% rise time did not differ significantly between the groups (Table 4).

Table 5 summarizes hemodynamics during dynamic vagal stimulation. Vagal stimulation significantly decreased mean HR in both sedentary and exercise trained groups. Mean HR and AP did not differ between the groups, before and during vagal stimulation. Figure 3*A* illustrates the dynamic transfer function characterizing vagal HR control. The frequency band effect ($P < 0.0001$) and the group effect ($P < 0.0001$) were both significant in the dynamic gain values of the vagal transfer function by two-way repeated measures ANOVA. The estimated

dynamic gain (*see Appendix*) tended to be greater in the exercise trained compared to the sedentary group (P = 0.06, Table 6). Other parameters did not differ between the groups. Figure 3*B* shows the calculated step response of HR to vagal stimulation. The calculated steady–state response in the exercise trained rats also tended to be greater than that in the sedentary rats (P = 0.06, Table 6). There was no significant difference in the 80% fall time between the groups.

Dynamic gain values of sympathetic and vagal transfer function corresponding to HRV frequency bands

When dynamic gain values of the sympathetic transfer function were averaged for the VLF and LF (up to 0.5 Hz, *see Methods*) bands, the frequency band effect was significant but the group effect was insignificant by two-way repeated measures ANOVA (Fig. 4*A*). When dynamic gain values of the vagal transfer function were averaged for the VLF, LF, and HF (up to 1 Hz, *see Methods*) bands, the frequency band effect was insignificant but the group effect was significant such that the dynamic gain was significantly greater in the exercise trained compared to the sedentary group (Fig. 4*B*).

Static sympathetic and vagal transfer function

The increase in HR with stepwise sympathetic stimulation was similar between groups (Fig. 5*A*). The stimulation frequency effect was significant while the group effect was insignificant by two-way repeated measures ANOVA. In contrast, the decrease in HR with stepwise vagal stimulation was greater in the exercise trained compared to sedentary rats (Fig. 5*B*). Both the stimulation

frequency effect and the group effect were significant.

Discussion

We have examined the dynamic transfer function of autonomic HR control by using random binary sympathetic and vagal nerve stimulation in sedentary and exercise trained rats. The major findings in the present study are i) that the exercise training did not alter the sympathetic transfer function substantially but augmented the dynamic gain of the vagal transfer function and ii) in the frequency domain, exercise training increased the dynamic HR response to vagal stimulation but not sympathetic stimulation regardless of the frequency band. These findings are the first quantitative data on the effect of exercise training on the dynamic characteristics of peripheral HR control by the sympathetic and vagal systems.

Validity of exercise training

The relative ventricular hypertrophy and higher exercise capacity in the exercise trained compared to the sedentary group suggested that exercise program used in the present study was sufficient to induce physiological adaptations commensurate with an effective training stimulus. As is well known, exercise training induces bradycardia at rest (Fig. 1*A*). Moreover, changes in the spectral parameters for R-R interval (Table 2) and autonomic tone (Fig. 1*B*) induced by the exercise training are consistent with earlier studies in rats (30, 31).

Effect of exercise training on sympathetic and vagal transfer function

Exercise training altered neither dynamic (Fig. 2) nor static sympathetic transfer function (Fig. 5*A*). These results are different than those reported in a previous study in which swim training significantly reduced the HR response to sympathetic nerve stimulation in a double atrial/right stellate ganglion preparation in guinea pigs (22). The discrepancy between investigations may have arisen from differences in the nerves experimentally stimulated (cervical sympathetic nerve vs. stellate ganglion), animal species studied (rats vs. guinea pigs), and/or experimental preparation utilized (*in vivo* vs. *ex vivo*). The mechanisms underlying the sympathetically mediated exercise training effect on HR are also controversial. For instance, chronotropic responsiveness to isoproterenol has been reported to be decreased in one study (15) but unchanged in another (22) by exercise training. Further, in response to exercise training, the density and affinity of β-adrenoceptors in the heart have been shown to be reduced in some reports (26, 33) while unchanged in others (3, 34, 35).

Exercise training augmented the dynamic gain of the vagal transfer function (Fig. 2). The effect of exercise training was also significant for static vagal transfer function (Fig. 5*B*). These results are in agreement with previous studies showing that exercise training significantly augmented the HR response to vagal nerve stimulation in a double atrial/right vagal nerve preparation using mice (9, 10). In contrast, Negrao et al (25) demonstrated that the HR response to vagal stimulation was depressed in exercise trained rats. A possible explanation for this disparate result is that the arterial baroreflexes remained intact in the experimental preparation used in the study (25). In contrast, sinoaortic baro-denervation was performed in the present investigation to

minimize baroreflex-mediated changes in sympathetic efferent nerve activity. Exercise training has been shown to attenuate the baroreflex-mediated sympathetic nerve response to hypotension (11). Although speculative, in the study by Negrao et al. (25), baroreflex-mediated sympathetic activation in response to vagally-induced hypotension might have been less in exercise trained compared to sedentary rats. Consequently, the gain of vagal stimulation might have been attenuated in exercise trained animals relative to sedentary rats. This suggestion is reasonable given that accentuated antagonism is indicative of a diminution in background sympathetic tonus which can decrease the gain of the vagal transfer function (17).

It has been documented that the intensity of exercise as well as the duration of exercise training are related to the autonomic adaptation to exercise training (28). These factors have been shown to be largely variable among different studies. A well controlled experimental setup is needed to clarify these issues.

Dynamic gain values of sympathetic and vagal transfer functions corresponding to HRV frequency bands

HRV is considered to reflect autonomic tone (19). The VLF component is likely to reflect changes in vasomotor tone in relation to thermoregulation and local adjustment of resistance in individual vascular beds; the LF component is considered to be a marker of sympathetic activity although it remains a matter of debate; and the HF component mainly originates from respiratory activity and is considered to be mediated by vagal input (27). In rats, Cerutti et al. (8)

determined that the LF component ranged between 0.27 and 0.74 Hz, and the HF component was above 0.75 Hz.

Averaged dynamic gain values of sympathetic transfer function for VLF and LF bands did not differ between the sedentary and exercise trained groups (Fig. 4*A*). These results suggest that changes in the peripheral sympathetic control of HR likely do not contribute significantly to training induced alterations in HRV. Therefore, the lower percentage of LF power and LF/HF ratio in the exercise trained group (Table 2) may indicate reduced activation of sympathetic outflow from autonomic centers (23). In contrast, averaged dynamic gain values of vagal transfer function for VLF, LF, and HF bands (Fig. 4*B*) as well as the percentage of HF power (Table 2) were significantly greater in the exercise trained compared to the sedentary group. These results suggest that the augmentation in HRV induced by exercise training is, at least in part, mediated by augmentations in the peripheral vagal control of HR.

What are the possible mechanisms underlying augmentations in the peripheral vagal control of HR? Danson and Paterson (10) have presented evidence that neuronal nitric oxide synthase (nNOS) may be a key enzymatic protein underlying such training induced increases in cardiac vagal function. This group has also demonstrated that HR changes in response to vagal stimulation are enhanced by exercise training in wild-type mice but not in heterozygous nNOS knockout mice (9). Another candidate for augmentations in the peripheral vagal control of HR is muscarinic receptors, which play a fundamental role in HR control via vagally mediated regulation. However, the effects of exercise training have been inconsistent among studies, showing both increases (12) and no change (2, 3) in muscarinic receptors in the myocardium

of rats. The possibility cannot be dismissed that training-induced changes in the activity of afferent inputs mediating vagal outflow may also contribute to the alterations in HRV (4). Further investigation is needed to clarify these issues.

Perspectives

To date, the mechanisms underlying increased HRV after exercise training remain to be elucidated. HRV may reflect both the autonomic outflow from the central nervous system and the peripheral autonomic regulation of atrial pacemaker cells. In human studies, it is difficult to separately examine each factor. The findings of the present study suggest that the augmentation in HRV induced by exercise training is, at least in part, mediated by augmentations in the peripheral vagal control of HR. In other words, even if vagal outflow from the central nervous system remains unchanged after exercise training, HRV could be increased by an enhanced responsiveness in the peripheral vagal, but not sympathetic, regulation of HR.

It has been well documented that decreased HRV is observed in heart failure (18) as well as in a variety of life-style related diseases such as diabetes (16), hypertension (24), and obesity (1). Further, reductions in HRV are related to increases in mortality rates as well as the occurrence of adverse cardiac events (32). Exercise training induced augmentations in HRV maintain the potential to partially correct or normalize the autonomic dysfunction manifest in these disease states (4). Understanding the mechanisms contributing to the alterations in HRV induced by exercise training may significantly impact the development of novel therapeutic strategies for the treatment of autonomic dysfunction.

Limitations

There are several limitations to this study. First, the rats were slightly hyperventilated throughout the stimulation protocol. We cannot rule out the possibility that the hyperventilation might have affected the results reported. Second, dynamic sympathetic stimulation lowered mean AP in sedentary rats although sinoaortic baro-denervation was performed. This may be explained by a possible difference in left ventricular functional capacity. For example, under conditions of equivalent HR, changes in systolic blood pressure were smaller in sedentary rats compared to exercise trained rats (13). Third, the stimulation amplitude was fixed at 10 V for both sympathetic and vagal nerve stimulation. It should be noted, however, that our preliminary results indicated that 10 V was sufficiently large enough to evoke maximal HR responses. Fourth, transfer function data were obtained from anesthetized animals. This must be taken into account when interpreting the present results as anesthesia may affect the peripheral autonomic regulation of atrial pacemaker cells. Finally, we stimulated the sympathetic and vagal nerves according to a binary white-noise signal. Although this method of stimulation is quite different from the physiological pattern of neuronal discharge, the coherence was near unity over the frequency range of interest. This finding indicates that the system properties do not vary considerably in response to different patterns of stimulation.

Conclusion

In the present study, it was demonstrated for the first time that exercise training did not alter dynamic sympathetic control of HR while it did augment

dynamic vagal control of HR. In addition, the group effect was significant with regard to the dynamic gain values for the vagal transfer functions corresponding to VLF, LF, and HF bands. This finding suggests that enhancements in the peripheral vagal control of HR may, at least in part, contribute to the exercise induced augmentation in HRV in healthy rats.

Appendix: Transfer function analysis

The dynamic transfer function from binary white-noise stimulation to the HR response was estimated based on the following procedure. Input–output data pairs of the stimulation frequency and HR were resampled at 10 Hz in order to be consistent with our previous study (21). Subsequently, data pairs were partitioned into eight 50%-overlapping segments consisting of 1,024 data points each. For each segment, the linear trend was subtracted and a Hanning window was applied. A fast Fourier transform was then performed to obtain the frequency spectra of nerve stimulation [$N(f)$] and HR [$HR(f)$]. Over the eight segments, the power of the nerve stimulation [$S_{N\cdot N}(f)$], the power of the HR [$S_{HR\cdot HR}(f)$], and the cross-power between these two signals [$S_{N\cdot HR}(f)$] were ensemble averaged. Finally, the transfer function [$H(f)$] from nerve stimulation to the HR response was determined using the following equation (20):

$$H(f) = \frac{S_{N\cdot HR}(f)}{S_{N\cdot N}(f)} \qquad [1]$$

To quantify the linear dependence of the HR response on vagal or sympathetic stimulation, the magnitude-squared coherence function [$Coh(f)$] was estimated employing the following equation (20):

$$\mathrm{Coh}(f) = \frac{|S_{N\cdot HR}(f)|^2}{S_{N\cdot N}(f)\cdot S_{HR\cdot HR}(f)} \qquad [2]$$

Coherence values range from zero to unity. Unity coherence indicates perfect linear dependence between the input and output signals; in contrast, zero coherence indicates total independence between the two signals.

Since the transfer function from sympathetic stimulation to HR response in rats approximated a second-order low-pass filter with pure delay (21), we determined the parameters of the sympathetic transfer function using the following equation:

$$H(f) = \frac{K}{1+2\zeta\frac{f}{f_N}j+\left(\frac{f}{f_N}j\right)^2} e^{-2\pi fjL} \qquad [3]$$

where K is dynamic gain (in beats·min^{-1}·Hz^{-1}), f_N is the natural frequency (in Hz), ζ is the damping ratio, L is lag time (in s), and f and j represent frequency and imaginary units, respectively. These parameters were estimated by means of an iterative nonlinear least-squares regression.

Since the transfer function from vagal stimulation to HR response in rats approximated a first-order low-pass filter with pure delay (21), we determined the parameters of the vagal transfer function using the following equation:

$$H(f) = \frac{-K}{1+\frac{f}{fc}j} e^{-2\pi fjL} \qquad [4]$$

where K represents the dynamic gain (in beats·min^{-1}·Hz^{-1}), fc denotes the corner frequency (in Hz), L denotes the lag time (in s), and f and j represent frequency and imaginary units, respectively. The negative sign in the numerator

indicates the negative HR response to vagal stimulation. These parameters were estimated by means of an iterative nonlinear least-squares regression.

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