The in vivo regulation of heart rate in the murine sinoatrial node by stimulatory and inhibitory heterotrimeric G proteins

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1William Harvey Heart Centre, Barts and The London School of Medicine and Dentistry, London, United Kingdom; 2Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina; 3Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health, Bethesda, Maryland; and 4Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Erlangen-Nuernberg, Erlangen, Germany

Submitted 21 January 2013; accepted in final form 15 May 2013

Sebastian S, Ang R, Abramowitz J, Weinstein LS, Chen M, Ludwig A, Birnbaumer L, Tinker A. The in vivo regulation of heart rate in the murine sinoatrial node by stimulatory and inhibitory heterotrimeric G proteins. Am J Physiol Regul Integr Comp Physiol 305: R435–R442, 2013. First published May 22, 2013; doi:10.1152/ajpregu.00037.2013.—Recent physiological modulation of heart rate is controlled by the sympathetic and parasympathetic systems acting on the sinoatrial (SA) node. However, there is little direct in vivo work examining the role of stimulatory and inhibitory G protein signaling in the SA node. Thus, we designed a study to examine the role of the stimulatory (Gs) and inhibitory G protein (Gi2) in vivo heart rate regulation in the SA node in the mouse. We studied mice with conditional deletion of Gs and Gi2 in the conduction system using cre-loxP technology. We crossed mice in which cre recombinase expression was driven by a tamoxifen-inducible conduction system-specific construct with “Gs floxed” and “Gi2 floxed” mice. We studied the heart rate responses of adult mice compared with littermate controls by using radiotelemetry before and after administration of tamofoxen. The mice with conditional deletion of Gs and Gi2 had a loss of diurnal variation and were bradycardic or tachycardic, respectively, in the daytime. In mice with conditional deletion of Gs, there was a selective loss of low-frequency power, while with deletion of Gi2, there was a loss of high-frequency power in power spectral analysis of heart rate variability. There was no evidence of pathological arrhythmia. Pharmacological modulation of heart rate by isoproterenol was impaired in the Gs mice, but a muscarinic agonist was still able to slow the heart rate in Gi2 mice. We conclude that Gs- and Gi2-mediated signaling in the sinoatrial node is important in the reciprocal regulation of heart rate through the autonomic nervous system.

G protein; heart rate; sinoatrial node

CARDIAC AUTOMATICITY IS MODULATED by both sympathetic and parasympathetic systems, and they are opposing in nature. Noradrenaline from sympathetic nerve efferents and adrenaline released into the circulation from the adrenal medulla accelerate heart rate, while ACH from vagal nerve efferents slows it down. The neurotransmitters and hormones act on the sinoatrial (SA) node to modulate an intrinsic pacemaking clock, the nature of which is controversial. The two main ideas are cycles of membrane pacemaker depolarization generated by hyperpolarization-activated cyclic nucleotide-gated cation channels (also known as I(f) or I(p)) and/or the cyclical release of Ca2+ from intracellular stores via ryanodine receptors increasing inward sodium-calcium exchanger currents during the diastolic depolarization (3, 6, 11, 13). Noradrenaline and adrenaline bind to β1- and β2-adrenoreceptors and activate the stimulatory G protein (Gs). Activated Gs stimulates adenylyl cyclase, leading to an increase in cAMP level, which directly activates I(f). This also leads to increased activity of PKA, resulting in modulation of downstream effectors, such as intracellular Ca2+-handling pathways, for example, phospholamban and the ryanodine receptor. In contrast, slowing of pacemaker depolarization occurs because of ACh binding to muscarinic M2 receptors and the activated Gq/11 antagonizes the above pathways. In addition, the free Gβγ subunit released from Gq/11 directly activates the G protein-gated inwardly rectifying K+ channel leading to membrane hyperpolarization. It is generally thought that all of these mechanisms contribute to some extent to modulating heart rate (1). These responses are all predicated on signaling networks initiated via Gs and Gq/11, and the evidence of their importance is largely based on ex vivo studies of the sinoatrial node and pacemaker cells (17). Furthermore, in vivo heart rate shows variability not reproduced in such studies, and specific frequency components are attributed to sympathetic and vagally mediated cardiovascular reflexes (22). In addition, the issue is intricate at the molecular level. Gnas is a complex gene with the potential for generation of long and extralong isoforms (27). Furthermore, there are four main isoforms of Gαi (Gαi1, Gαi2, Gαi3, and Gαo), and these are all widely expressed in most tissues with significant possibilities for redundancy (5). We have previously shown that mice with global genetic deletion of Gαi2 have abnormalities of heart rate regulation compatible with a role for that protein in the SA node, and thus, we focus here on that isoform (29). We have not previously investigated the contribution that Gs makes to heart rate regulation, and this would not be possible in a strictly analogous fashion to our studies on the inhibitory G proteins as global genetic deletion of Gαi2 is embryonically lethal (27). Thus, we refined our strategy and sought to investigate the significance of Gs and Gαi2 for physiological heart rate regulation in the sinoatrial node of the adult mouse using a conditional gene-targeting approach.

MATERIALS AND METHODS

Murine husbandry. Mice were maintained in an animal core facility under U.K. Home Office guidelines relating to animal welfare. All maintenance, breeding, and procedures were covered by project license PPL 70/6732. All mice were kept in a temperature-controlled environment (21–23°C) with 12:12-h dark-light cycles. Animals were...
allowed access to standard rodent chow and water ad libitum. Mice were studied between 8 and 12 wk of age. Mouse strains and breeding and genotyping. We interbred several mouse lines. We used mice in which the tamoxifen-inducible CreERT2 construct was “knocked” into the pacemaker channel HCN4 locus (HCN4-KiT, referred to as cre+/H11001 here), allowing for selective expression of cre recombinase in the cardiac conduction system following intraperitoneal administration of tamoxifen (12). We interbred these with "Gsg floxed" (Gsgfx/H11001) and “Gsi2 floxed” (Gsi2fx/H11001) mice. Gsgfx/H11001 mice have loxP sites placed in introns upstream and downstream of exon 1. Gsi2fx/H11001 mice have loxP sites placed in introns upstream of exon 2 and downstream of exon 4, and, thus, cre-mediated excision deletes exons 2, 3, and 4, which is equivalent to amino acids 40–155. A description of the targeting strategy to produce both lines of fx/fx mice has previously been published (8, 26). After successive rounds of breeding, we generated Gsgfx/H11001 cre/H11001 mice, Gsi2fx/H11001 cre/H11001 mice, and littermate “controls”, which consisted of wild-type, cre+/H11001 only, and Gsgfx/H11001 or Gsi2fx/H11001 only genotypes. We observed no prominent differences among the latter groups and thus pooled them into single littermate control populations. Mice of both sexes were used in this study. The cre recombinase-expressing line is on a C57/BL6 background, Gsgfx/H11001 is on a C57/BL6 background, and the Gsi2fx/H11001 is on the 129/Sv background. Tamoxifen (Sigma) was freshly dissolved in sunflower oil, and 1 mg tamoxifen/25 g body wt ip was injected on five consecutive days. Mice were studied 10 days after the last dose. Mice were tail clipped (~2 mm) at 3–4 wk of age. Genomic DNA was isolated from tail clips. PCR-based genotyping was then performed on isolated DNA to confirm genotype of individual mice. One-hundred-and-fifty microliters of tail lysis buffer [3.35 ml 2 M Tris-HCl pH 8.8, 1.66 ml 1 M (NH4)2SO4, 1.34 ml 0.5 M MgCl2, 0.5 ml Triton X-100, 92.2 ml H2O, and 1 ml β-mercaptoethanol] was added, and the samples were heated to 100°C for 10 min in a heating block to denature mouse proteins. Samples were then cooled, and 5 μl of proteinase K (20 mg/ml) was added at 55°C for 12 h. Samples were then reheated to 100°C for a further 10 min and then spun at 13,000 rpm for 30 s in a tabletop centrifuge to sediment tail debris. One microliter of sample (mouse genomic DNA) was used per genotyping PCR. Identification of cardiac Cre expression was determined using the following primer sets: HCN4Ki F5=−CCCGCGCTGGAGTTTCAATA−3 and HCN4Ki R5=−CTTCGCCCAGTTGATCATGTG−3. The presence of the Cre transgene was determined by the presence or absence of a Cre band (383bp). Gsg lox-P (floxed allele) genotyping was performed using the following primers: Gsg LoxF 5=−TTCGGTCTCGTCCTCTTAGTTG−3 and Gsg LoxR 5=−AACAAATCGCACCCACCAGTGAGG−3 using betaine (1.5 M) in the PCR master mix to obtain a WT band corresponding to ~216 bp and a Gsg floxed band corresponding to ~264 bp, respec-

Fig. 1. ECG traces and diurnal variation of heart rate. A and C: representative ECG recordings and mean data for Gsgfx/H11001 cre+ (n=8) and control (n=8). B and D: for Gsi2fx/H11001 cre+ (n=12) and control mice (n=9). Values in B and D are expressed as means ± SE.
tively. Cre-mediated excision was confirmed by Gas cre F 5'-GAGAGCGGAGGAGGACGC-3' and Gas cre R 5'-AGCCTAATCTGTGCGATGC-3' primers, which gives a band of ~250 bp upon cre-mediated excision of exon 1. Gas2 lox-P (floxed allele) genotyping was performed using the following primers: Gas2 loxF 5'-GGA GCC TGG ACT TTG CTT CTG ACC-3' and Gas2 loxR 5'-GGC TAT GAT CCC AAA ACT CCC CG-3'. Then, to further examine heart rate modulation in vivo, we used pharmacological surgery. After a minimum of a week-long period of aseptic surgical technique. After a minimum of a week-long period of surgical recovery, ECG signals were acquired by radio-telemetry. To acquire R-R interval variability signal from ECG data streamed over 30 min at high sampling frequency (2 kHz), digitized and analyzed by 10.220.32.246 on July 4, 2017 http://ajpregu.physiology.org/ Downloaded from

Table 1. Summarized ECG and HRV parameters for Gas/(n = 8) and for Gas/(n = 9)

<table>
<thead>
<tr>
<th>Animal</th>
<th>PR, ms</th>
<th>QRS, ms</th>
<th>QTc, ms</th>
<th>RR, ms</th>
<th>SD nn, ms</th>
<th>RMS SD, ms</th>
<th>TP, ms</th>
<th>2 VLF, ms2</th>
<th>LF, ms2</th>
<th>HF, ms2</th>
<th>nLF, nu</th>
<th>nHF, nu</th>
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</thead>
<tbody>
<tr>
<td>before</td>
<td>17.5</td>
<td>2.17</td>
<td>0.96</td>
<td>5.8</td>
<td>42.9</td>
<td>3.74</td>
<td>150</td>
<td>3.09</td>
<td>150</td>
<td>5.72</td>
<td>1.17</td>
<td>34.97</td>
</tr>
<tr>
<td>after</td>
<td>20.0</td>
<td>2.66</td>
<td>1.12</td>
<td>6.3</td>
<td>47.2</td>
<td>3.95</td>
<td>160</td>
<td>3.55</td>
<td>160</td>
<td>6.11</td>
<td>1.32</td>
<td>37.35</td>
</tr>
</tbody>
</table>

Table 1.

Heart rate and G proteins

Parameter | Before | After
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>PR (ms)</td>
<td>17.5</td>
<td>20.0</td>
</tr>
<tr>
<td>QRS (ms)</td>
<td>2.17</td>
<td>2.66</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>0.96</td>
<td>1.12</td>
</tr>
<tr>
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<td>6.3</td>
</tr>
<tr>
<td>SD nn (ms)</td>
<td>42.9</td>
<td>47.2</td>
</tr>
<tr>
<td>RMS SD (ms)</td>
<td>3.74</td>
<td>3.95</td>
</tr>
<tr>
<td>TP (ms)</td>
<td>150</td>
<td>160</td>
</tr>
<tr>
<td>2 VLF (ms²)</td>
<td>3.09</td>
<td>3.55</td>
</tr>
<tr>
<td>LF (ms²)</td>
<td>150</td>
<td>160</td>
</tr>
<tr>
<td>HF (ms²)</td>
<td>5.72</td>
<td>6.11</td>
</tr>
<tr>
<td>nLF (nu)</td>
<td>1.17</td>
<td>1.32</td>
</tr>
<tr>
<td>nHF (nu)</td>
<td>34.97</td>
<td>37.35</td>
</tr>
</tbody>
</table>

*P < 0.05, repeated-measures one-way ANOVA. The comparisons are between mice before and after tamoxifen treatment in both groups, with a Holm-Sidak adjusted p-value at 0.05. HF: high frequency; LF: low frequency; nLF: normalized low frequency; nHF: normalized high frequency; nR: normalized units.
30-min time course and was measured when it had reached a steady state. The chronotropic effect of isoprenaline and carbachol was expressed as a percentage change of heart rate relative to baseline heart rate prior to drug administration. Intrinsic heart rate was determined in a similar way by administering atropine (1 mg/kg) and propanolol (1 mg/kg) together to achieve autonomic blockade in awake conscious mice with telemetry probes in situ. We also examined the effects of the drugs on HRV by recording 10 min of ECG tracing prior to drug administration and another 10 min after the heart rate had reached a steady state following drug administration.

Statistical analysis. Data are reported as means ± SE. Statistical significance of multiple treatments was determined by one- and two-way ANOVA followed by Dunnett’s post hoc analysis, and statistical significance between two groups was determined by two-tailed Student’s t-tests, as appropriate (GraphPad Prism v4). In all instances, \( P < 0.05 \) was considered significant.

RESULTS

Generating mice with deletion of Gas and Gai2 in the conduction system. Gnaisflx/dx cre+, Gai2flx/dx cre+ mice and littermate controls were generated as described above. We performed quantitative real-time RT-PCR on RNA extracted from the SA node and ventricle. We normalized measurements in the Gnasflx/dx cre+ and Gai2flx/dx cre+ mice to expression in control mice (both after the administration of tamoxifen) in ventricle and SA node. There was a significant reduction in relative Gas and Gai2 RNA expression in the SA node but not ventricle (\( \text{Gnais}^{\text{flx/dx}} \text{ cre+} \text{ SA node} = 0.18 \) [95% confidence interval (CI) 0.17–0.18], ventricle = 1.01, [95% CI 0.99–1.03]; and Gai2flx/dx cre+ SA node = 0.11 [95% CI 0.09–0.24], ventricle = 1.04 [95% CI 0.80–1.36]; \( n = 2 \) and 3 mice, respectively, in triplicate for all groups). We also isolated genomic DNA from the tail, cardiac ventricle, and SA node and performed PCR, which confirmed cre-mediated deletion of exon 1 of \( \text{Gnas} \) and exons 2–4 in \( \text{GnaI2} \) in the SA node but not in the ventricle or tail (not shown).

Effects on mean heart rate and diurnal variation. Significant diurnal variation in heart rate (HR) was observed in \( \text{Gnas}^{\text{flx/dx}} \text{ cre+} \) and \( \text{Gai2}^{\text{flx/dx}} \text{ cre+} \) mice before tamoxifen treatment but not after (Fig. 1). There was a significant decrease in night-time HR in \( \text{Gnas}^{\text{flx/dx}} \text{ cre+} \) mice, while there was a significant increase in day-time HR after tamoxifen treatment in \( \text{Gai2}^{\text{flx/dx}} \text{ cre+} \) mice (Fig. 1). In contrast, control mice had preserved diurnal variation, and there was no significant change after tamoxifen treatment (Fig. 1). We examined ECG parameters, and there was no significant difference in PR interval, QRS duration, or any evidence of spontaneous arrhythmia, such as heart block (Fig. 1 and Table 1). However, QTc was shorter in the \( \text{Gnas}^{\text{flx/dx}} \text{ cre+} \) mice, but this was not investigated further in this study.

Heart rate variability (HRV) analysis. In both \( \text{Gnas}^{\text{flx/dx}} \text{ cre+} \) and \( \text{Gai2}^{\text{flx/dx}} \text{ cre+} \) mice, HRV was altered after tamoxifen treatment. This was detectable in an individual mouse in power spectral density analysis (Fig. 2). Specifically, there was a selective loss of normalized LF power in \( \text{Gnas}^{\text{flx/dx}} \text{ cre+} \) mice, while, in contrast, in \( \text{Gai2}^{\text{flx/dx}} \text{ cre+} \) mice, there was a selective loss in normalized HF power (Fig. 2 and Table 1).

Pharmacological modulation of heart rate. We measured the chronotropic response after the administration of isoprenaline and carbachol in \( \text{Gnas}^{\text{flx/dx}} \text{ cre+} \) and \( \text{Gai2}^{\text{flx/dx}} \text{ cre+} \) mice, respectively, after treatment with tamoxifen and compared this to control littermate mice (Fig. 3). The positive chronotropic response to isoprenaline was abrogated but not abolished in \( \text{Gnas}^{\text{flx/dx}} \text{ cre+} \) mice, and there was only a small nonsignificant reduction in the negative chronotropic response to carbachol in...
Goi2^{flx/flx} cre+ mice. There was no evidence of conduction block or arrhythmias occurring after drug administration.

**Intrinsic heart rate determination and HRV after atropine and propranolol administration.** For complete autonomic blockade, atropine and propranolol were administered together in Goi2^{flx/flx} cre+ and Goi2^{flx/flx} cre+ mice before and after treatment with tamoxifen, and we compared in intrinsic heart rate and HRV to control littermate mice. In Fig. 4A and Table 2, we show that that there was no change in intrinsic heart rate in both Goi2^{flx/flx} cre+ and Goi2^{flx/flx} cre+. We also examined the effects of the combined atropine and propranolol treatment on HRV before and after tamoxifen treatment in the two groups of mice. We show the data after the administration of tamoxifen in Goi2^{flx/flx} cre+ and Goi2^{flx/flx} cre+ mice. The administration of the two drugs led to a decrease in HRV across all of the frequency spectra (Table 2). There was no difference between the two genotypes in this behavior before (not shown) or after tamoxifen administration (Fig. 4 and Table 2). In Fig. 4B, representative power spectra show abrogation of HF and LF power in both Goi2^{flx/flx} cre+ and Goi2^{flx/flx} cre+ mice after atropine and propranolol administration (Fig. 4B).

**DISCUSSION**

The main findings in this study are that selective deletion of Goi2 and Goi2 in the conduction system of the adult mouse impairs physiological heart rate regulation in the intact awake animal. Mice are nocturnal creatures active at night and more sedentary during the day; thus, they show a characteristic diurnal variation in heart rate. The higher mean rate at night reflects their increased movement and higher sympathetic drive. The selective deletion of Goi2 in the conduction system results in loss of normal diurnal variation with the mice no longer tachycardic at night and thus relatively bradycardic compared with control. In contrast, although mice with selective deletion Goi2 in the conduction system also lose normal diurnal variation, this occurs because they are relatively more tachycardic during the day, consistent with a decline in parasympathetic modulation of the SA node. We also determined the intrinsic heart using pharmacological blockade of the autonomic nervous system with atropine and propranolol. The intrinsic heart rate was not significantly different between the two groups of mice and the deletion of Goi2 and Goi2 in the SA node after tamoxifen administration did not change it. This confirms in vivo that the fundamental inherent pacemaker is not determined by Goi2 and Goi2 and pathways downstream of the G protein.

Heart rate shows physiological variation in rate in a number of frequency domains. In humans, a HF component (0.15 to 0.4 Hz), a LF component (0.04 to 0.15 Hz), and a very low-frequency component (<0.04 Hz) are distinguished, and in the mouse, the frequencies defining each band are 10-fold higher (22a, 24). The high-frequency component is generally thought to reflect vagal input, while the low-frequency component reflects sympathetic and vagal input. However, the latter, in particular, is controversial (22a). Our data support a position broadly in which physiological Goi2-mediated signaling in the SA node reflects the HF component and Goi2 signaling reflects the LF component, and these two are distinct with little crosstalk. In terms of autonomic input, this would correspond to delineation between parasympathetic and sympathetic, accounting largely for most of HF and LF power, respectively. It is worth noting that we use a standard normalization procedure, and once this is done, the correlation between LF and sympathetic activity is clearer (22a). Defining the molecular correlates underlying HRV is not simply of academic interest. HRV is a widely used noninvasive technique to assess autonomic tone, particularly in human studies, and has prognostic significance in patients after myocardial infarction, with hypertension and in many other diseases (22a, 23).

There are some interesting comparisons in HRV data between mice with conditional genetic deletion of Goi2 and Goi2 in the SA node and those after drug administration and in mice with global genetic deletion of Goi2. The global genetic deletion of Goi2 in mice is embryonically lethal, and thus, the heart rate phenotype of this animal cannot be studied (27). The global genetic deletion of Goi2 and the administration of atropine, tertiapin, propranolol, and atropine suppressed HRV in the time domain and in the frequency domain and generally had effects across all three frequency spectra (29). In contrast, the effect of conditional deletion in the SA node was more subtle and was revealed when variation in VLF was removed by the normalization of HF and LF. It is worth noting that VLF accounts for the majority of power in the HRV signal. There are some technical caveats that need to be considered. Nonstationarities in the HRV signal can be a confounding factor and, for example, monitoring HRV throughout the day as human activities change.
subjects go about their daily lives may overestimate the sympathetic contribution (4, 15). We seek to minimize using the recommended approaches, i.e., monitoring the mice at rest at a defined time during the day for a short recording period. Furthermore, it is important that the mice are allowed to recover from surgery (generally 7–10 days) and resume normal activity after probe implantation before measurements are made. These factors are the same between our current and previous studies, and thus, the differences are likely real. Factors likely responsible for the differences are those that lie outside the SA node. For example, effects in the central and peripheral nervous system also have an important influence on HRV. Gi2 is expressed in the nervous system, and drugs, such as propranolol, with a high lipophilicity cross the blood-brain barrier. In this regard, the HCN4-KiT mouse does not lead to deletion of genes in the brain after the administration of tamoxifen (12).

A potential qualification with our data is the response to the pharmacological agents. After selective deletion of Gαs in the conduction system, the response to isoprenaline was significantly attenuated but not abolished, while with Gαi2 deletion, the heart rate-slowing response to carbachol is relatively well preserved. This contrasts with the situation in the global knockout of Gαi2, where it was significantly attenuated. The tests with isoprenaline and carbachol are a pronounced pharmacological challenge in that, for example, with carbachol, the heart rate falls to levels a long way below that normally seen physiologically, even in normal situations, such as sleep. It is known that the sinoatrial node is actually quite an extensive
### Acknowledgments

This work was supported by the Wellcome Trust, Medical Research Council, The National Institute for Health Research Barts Cardiovascular Biomedical Research Unit and by the Intramural Research Program of the National Institutes of Health (project Z01-ES-101643 to LB) and National Institute of Diabetes and Digestive and Kidney Diseases to L. S. Weinstein.

### Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

### Author Contributions


### References


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### Table 2. HRV parameters after atropine and propranolol (autonomic blockade) in Gαs10/10 and Gαi2/12/12 in mice

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>HR, bpm</th>
<th>SDNN, ms</th>
<th>RMSSD, ms</th>
<th>HF, μV·ms⁻¹</th>
<th>LF, μV·ms⁻¹</th>
<th>LF/HF</th>
<th>Power at LF, μV²·ms⁻¹</th>
<th>Power at HF, μV²·ms⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gαs10/10</td>
<td>107.8 ± 7.1</td>
<td>55.9 ± 29</td>
<td>29.7 ± 19</td>
<td>0.78 ± 0.28</td>
<td>1.9 ± 0.32</td>
<td>23.1 ± 6.9</td>
<td>65.0 ± 10</td>
<td>12.3 ± 4.8</td>
</tr>
<tr>
<td>Gαi2/12/12</td>
<td>100.3 ± 9.6</td>
<td>55.9 ± 29</td>
<td>29.7 ± 19</td>
<td>0.78 ± 0.28</td>
<td>1.9 ± 0.32</td>
<td>23.1 ± 6.9</td>
<td>65.0 ± 10</td>
<td>12.3 ± 4.8</td>
</tr>
</tbody>
</table>

ΔHR, ΔSDNN, ΔRMSSD, and ΔHF were calculated from the HR before and after administration (repeated-measures one-way ANOVA).


