Effects of exercise training on expression of endothelin-1 mRNA in the aorta of aged rats

Seiji MAEDA*, Takashi MIYAUCHI*, Motoyuki IEMITSU*, Takumi TANABE†, Tomoko YOKOTA*, Katsutoshi GOTO‡, Iwao YAMAGUCHI* and Mitsuo MATSUDA†

*Cardiovascular Division, Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan, †Department of Sports Medicine, Institute of Health and Sport Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8574, Japan and ‡Department of Pharmacology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

ABSTRACT

Aging impairs endothelial function and the vascular tone regulation, although the precise mechanism remains unclear. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by vascular endothelial cells. Because ET-1 has a potent vasoconstrictor effect on vessels, it may be involved in the regulation of vascular tone. We hypothesized that aging causes a decrease in ET-1 expression in aorta, and that exercise training improves the aging-induced decrease in ET-1 expression in aorta. This study was performed to examine whether gene expression of ET-1 in the aorta of rats is altered by aging and subsequent exercise training. We studied expression of ET-1 mRNA in the aortas of sedentary young rats (Sedentary young group, 4 months old), sedentary aged rats (Sedentary aged group, 23 months old), and swim trained aged rats (Training aged group, 23 months old; swimming training for 8 weeks, 5 days/week, 90 min/day). The expression of ET-1 mRNA in the aorta was analysed by real-time quantitative PCR. Body weight and resting heart rate were significantly lower in the Training aged group compared with the Sedentary aged group. These results suggest that the Training aged rats exhibited physiological effects from exercise training. The expression of ET-1 mRNA in the aorta was markedly lower in Sedentary aged group compared with the Sedentary young group, whereas it was significantly higher in Training aged group compared with the Sedentary aged group. These results show that the expression of ET-1 mRNA in the aorta is decreased by aging, and that the expression is increased by exercise training. Therefore, the present study provides a possibility that exercise training improves endothelial function through up-regulation of the aging-induced decrease in ET-1 expression in the aorta.

INTRODUCTION

It is well demonstrated that loss of endothelial function not only is characteristic of diseases such as essential or secondary hypertension [1,2], hypercholesterolaemia [3,4], and atherosclerosis [4], but also has been associated with advancing age [5]. Experimental data indicate that, independent of the presence of other pathologies, aging alters endothelial function in both aorta and small resistance arteries in rats [6–10]. The alteration of endothelial function occurring with aging may have important clinical implications for the pathogenesis of cardiovascular disease. Vascular endothelial cells play an important role in the regulation of vascular activity by producing vasoactive substances [11,12]. It has been suggested that a decrease in the bioavailability of NO, an endothelium-derived relaxing factor, may occur with aging [10,13–15]. However, the actions of endothelium-derived vasoconstrictor substances with aging remain to be investigated.

Key words: aging, aorta, endothelin-1, exercise training.
Abbreviations: ET, endothelin; Ct, number of PCR cycles to reach significant threshold; ΔRn, fluorescence intensity of PCR product.
Correspondence: Dr T. Miyauchi (e-mail t-miyauc@md.tsukuba.ac.jp).
Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by vascular endothelial cells [12,16–18], and has been reported to be involved in the regulation of vascular tone [19]. We previously reported that acute exercise significantly increases the circulating plasma concentration of ET-1 [20]. However, it is not known whether the expression of ET-1 in the aorta is altered by exercise training.

Because aging has been reported to produce an impairment in endothelial function, and thus vascular tone regulation, firstly, we hypothesized that aging causes a decrease in ET-1 expression in aorta. Furthermore, because exercise training has been reported to produce an alteration in the function of vascular endothelial cells [21], secondly, we hypothesized that exercise training improves the aging-induced decrease in ET-1 expression in aorta. The purpose of the present study was to investigate whether gene expression of ET-1 in the aorta of rats is altered by aging and subsequent exercise training. In the present study, we studied expression of ET-1 mRNA in the aortas of sedentary young rats (Sedentary young group, 4 months old), sedentary aged rats (Sedentary aged group, 23 months old), and swim trained aged rats (Training aged group, 23 months old; swimming training for 8 weeks, 5 days/week, 90 min/day). The expression of ET-1 mRNA in the aorta was analysed by real-time quantitative PCR.

**METHODS**

**Animals and protocol**

The experimental protocols were approved by the Committee on Animal Research at the University of Tsukuba. Male 21-month old and 2-month old Wistar rats were obtained from Institute for Animal Reproduction (Ibaraki, Japan) and cared for according to the Guiding Principles for the Care and Use of Animals based on the Helsinki Declaration of 1964. These rats were maintained on a 12:12-h light-dark cycle and received food and water ad libitum. Six 21-month old rats were exercised by swimming for 5 days/week (Training aged group), in a tank of water at 35 °C with a surface area of 2830 cm² and a depth of 60 cm. The rats swam for 15 min/day for the first 2 days, then the swimming time was increased gradually over the 1-week period from 15 min/day to 90 min/day. Thereafter, the Training aged group continued swimming training for 7 weeks. Therefore, the Training aged group received 8 weeks of swimming training. Six 21-month old rats (Sedentary aged group) and six 2-month old rats (Sedentary young group) remained confined to their cages for 8 weeks, but were handled daily. After swimming training for 8 weeks, systolic blood pressure, diastolic blood pressure, and heart rate of the animals were measured with a tail-cuff sphygmomanometer (PS-100, Riken Kaihatsu, Kanagawa, Japan). The body weight of the animals was also measured after swimming training for 8 weeks. The day of the experiment, rats were anaesthetized with diethyl ether. After anaesthetization, the abdominal aorta was removed, weighed, and frozen in liquid nitrogen. The abdominal aorta samples were stored at −80 °C for determination of ET-1 mRNA expression by real-time quantitative PCR analysis. Sedentary aged rats and Sedentary young rats were killed at the same time point as the Training aged rats (Sedentary aged rat, 23 months old; Training aged rat, 23 months old; Sedentary young rat, 4 months old).

**cDNA synthesis**

Total tissue RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction with Isogen (Nippon Gene, Toyama, Japan) according to the method described in our previous papers [22–24]. Briefly, the tissue was homogenized in Isogen (100 mg of tissue/1 ml of Isogen) with a Polytron tissue homogenizer (model PT10/35, Kinematica, Lucerne, Switzerland). The precipitated RNA was extracted with chloroform, precipitated with isopropanol, and washed with 75% (v/v) ethanol. The resulting RNA was re-dissolved in diethyl pyrocarbonate-treated water, treated with DNase I (Takara, Shiga, Japan), and extracted again by Isogen to eliminate the genomic DNA. The RNA concentration was determined spectrophotometrically at 260 nm.

Total RNA (2 µg) was reverse transcribed using MultiScribe Reverse Transcriptase from the Taq Man Reverse Transcription with random hexamer primers (Perkin Elmer Applied Biosystems, Foster, CA, U.S.A.). Final reaction concentrations were as follows: 1 × TaqMan RT buffer, 5.5 mM MgCl₂, 500 mM of each dNTP, 2.5 µM random hexamer, 0.4 unit/µl RNase inhibitor, and 1.25 units/µl Multi Scribe Reverse Transcriptase. The reaction of reverse transcription was performed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min.

**Real-time quantitative PCR**

The expression levels of ET-1 were measured by real-time quantitative PCR using a TaqMan SYBER Green quantitative PCR assay kit (Perkin Elmer Biosystems, Foster, CA, U.S.A.) and ABI Prism 7700 Sequence Detector (Perkin Elmer Applied Biosystems) [25,26]. The expression of (18S rRNA was determined as an internal control. The PCR amplification reaction and the detection of PCR product was monitored by measuring the increase in fluorescence intensity caused by the binding of SYBER Green dye to double-stranded (ds) DNA [25–28]. The gene-specific primers were synthesized from Primer Express v. 1.0 software (Perkin Elmer Applied Biosystems) according to the published cDNA sequences for each of the following: ET-1 [29] and 18S rRNA [30]. The sequences of the oligonucleotides were as follows:
ET-1 forward, 5'-TTGCTCCTGCTCCTCCTGTGATT3'; ET-1 reverse, 5'-TAGACCTAGAGGGCCCTCC-TAGT-3'; 18 S rRNA forward, 5'-GAGGTTGAATTTCTTGGACCGG-3'; 18 S rRNA reverse, 5'-CGAACTCTCCGACTTTCGTTCT-3'.

The 18 S rRNA expression was used as the internal control. The size of each amplicon was established as 119 bp for ET-1 and 93 bp for 18 S rRNA. The PCR mixture (25 µl total volume) consists of primers ET-1 (300 nM each) and 18 S rRNA (200 nM), SYBER Green Master mix (containing with SYBER Green dye, AmpliTaqGold DNA polymerase, dNTP with dUTP, Passive Reference and optimized buffer; Perkin Elmer Applied Biosystems), 0.25 unit of AmpErase uracil N-glycosidase (Perkin Elmer Applied Biosystems). Each PCR amplification was performed in triplicate, using the following profile: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min.

For the standard curve the real-time quantitative PCR, serial dilutions of a rat aorta cDNA performed within the range of various concentrations (1 ×, 2 ×, 4 ×, 8 × and 16 ×). No-template (water) reaction mixture was prepared as a negative control. From the negative control, the primers used in the present study confirmed an effect of the binding to the primer dimers, because this condition is the most suitable for making primer dimers [25,27].

Quantitative analysis of PCR products

During the PCR amplification, fluorescence emission was measured and recorded in real-time using ABI Prism 7700 sequence detector. These fluorescence intensity of PCR product (ARn) data were stored in the Macintosh computer and were analysed by the Sequence Detector v1.6.3 program (Perkin Elmer Applied Biosystems) of the ABI Prism 7700 detection system. The amplification plots produced automatically created a baseline, exponential phase, and plateau in a logarithmic plot of cycle number and ARn (see Figure 1A). The algorithm from the graph calculates the cycle at which each PCR amplification reaches a significant threshold (Ct; i.e., usually ten times the standard deviation of the baseline) [26,27]. The calculated Ct values are a quantitative measurement for the mRNA levels of various genes tested [26,27]. The standard curve was created automatically by the ABI Prism 7700 detection system by plotting the Ct values corresponding to five standard dilutions. The Ct values for standards and samples were usually in the range of between 15 and 35 cycles of amplification. The correlation coefficient of linear regression for the standard curve was calculated automatically (see Figure 1B). Ct is inversely proportional to the starting cDNA quantity, i.e. the higher the starting template copy number, the lower the Ct. The PCR amplification in unknown samples was plotted on the standard curve and the relative starting quantity was calculated. The quantitative value of ET-1 mRNA expression was normalized to that of 18 S rRNA mRNA expression.

Statistical analysis

Values are expressed as means ± S.E.M. Statistical analysis was carried out by ANOVA followed by Schefé’s F-test for multiple comparisons. P < 0.05 was accepted as significant.

RESULTS

Body weight was significantly lower in the Training aged group compared with the Sedentary aged group (Table 1). Resting heart rate was significantly lower in the Training aged group compared with the Sedentary aged group, and that in the Sedentary aged group was significantly higher compared with the Sedentary young group (Table 1). There were no significant differences among the Sedentary young group, Sedentary aged group and Training aged group in systolic blood pressure or diastolic blood pressure (Table 1). These results suggest that the Training aged rats exhibited physiological effects from exercise training.

Figure 1(A) shows the representative semi-log view of the real-time PCR amplification plots of ET-1 mRNA during 40 PCR cycles. The threshold is set at ten times the standard deviation of the mean baseline emission calculated for PCR cycles 3 to 22. The Ct values for standards and samples in the present study were in the range of between 22 and 33 cycles of amplification. The ET-1 and 18 S rRNA primer set produced the primer dimers that the SYBER Green dye could be bind to, but the amount of combination was too low to be taken account of (2−18). Figure 1(B) shows the representative semi-log view of standard curve of the ET-1 on a plot of

Table I  Body weight, heart rate and blood pressure in Sedentary young group, Sedentary aged group and Training aged group

<table>
<thead>
<tr>
<th></th>
<th>Sedentary young group</th>
<th>Sedentary aged group</th>
<th>Training aged group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>423 ± 6</td>
<td>757 ± 25*</td>
<td>543 ± 16*†</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>343 ± 7</td>
<td>395 ± 13*</td>
<td>292 ± 11*†</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>138 ± 2</td>
<td>136 ± 8</td>
<td>143 ± 7</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>84 ± 5</td>
<td>97 ± 9</td>
<td>87 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.; n, number of rats; *P < 0.01, significant difference versus Sedentary young group; †P < 0.01, significant difference versus Sedentary aged group.
Exercise training and endothelin-1 in aorta of aged rat

Figure 1 Amplification plots (A) and standard-curve (B) for ET-1 mRNA in the abdominal aorta
(A) Representative semi-log view of real-time amplification plots from the extension phase fluorescence emission data of ET-1 in the aorta of the sedentary young rats, sedentary aged rats, swim trained aged rats, and standard samples during 40 PCR cycles by using ABI Prism 7700 detection system. The standard deviation is determined from the data points collected from the baseline (2 to 22 cycles) of the amplification plot. The arrow indicates the Ct. The Ct values are calculated by determining the point at which the fluorescence exceeds a threshold limit (usually 10 times the standard deviation of the baseline). (B) Representative semi-log view of the standard curve of the ET-1 on a plot of the Ct value against input target quantity. All points represent the means of triplicate PCR amplifications. The slope of the standard curve was $-3.356$ and the correlation coefficient was 0.990.

The expression of ET-1 mRNA in the aorta was markedly lower in the Sedentary aged group compared with Sedentary young group, whereas that was significantly higher in the Training aged group compared with the Sedentary aged group (Figure 2). Therefore, the expression of ET-1 mRNA in the aorta is decreased by aging, and this expression is increased by exercise training.

DISCUSSION

In the present study, we determined ET-1 mRNA expression in the aortas of sedentary young rats, sedentary aged rats and swim trained aged rat. The expression of ET-1 mRNA in the aorta was markedly lower in sedentary aged rats than in sedentary young rats, the Ct against input target quantity. The slope of the standard curve was $-3.356$ and the correlation coefficient was 0.990.

The expression of ET-1 mRNA in the aorta was analysed by real-time quantitative PCR. We studied the expression of 18 S mRNA as an internal control. The ratio of ET-1 mRNA to 18 S mRNA was calculated. Thus, the value of expression of ET-1 mRNA was normalized by that of 18 S mRNA. Data are expressed as means $\pm$ S.E.M.

Figure 2 Expression of ET-1 mRNA in the aorta of Sedentary young group (open bar; $n = 6$), Sedentary aged group (solid bar; $n = 6$) and Training aged group (hatched bar; $n = 6$, swim trained for 8 weeks)

The expression of ET-1 mRNA in the aorta was analysed by real-time quantitative PCR. We studied the expression of 18 S mRNA as an internal control. The ratio of ET-1 mRNA to 18 S mRNA was calculated. Thus, the value of expression of ET-1 mRNA was normalized by that of 18 S mRNA. Data are expressed as means $\pm$ S.E.M.
whereas it was significantly higher in swim trained aged rats than in sedentary aged rats. These findings suggested that the expression of ET-1 mRNA in the aorta is decreased by aging, and that the expression is increased by exercise training. It has been proposed that endogenously generated ET-1 contributes to basal vascular tone, because it was found that the systemic administration of the endothelin-receptor antagonist TAK-044 significantly decreased systemic blood pressure and peripheral vascular resistance [31]. Aging impairs endothelial function and the vascular tone regulation [5–10]. On the basis of the results from past studies plus the present results, it was considered that the decreased ET-1 production in the aorta may cause the decrease in vascular tone and the consequent loss of endothelial function. Taken together, the present study provides a possibility that although aging produces an impairment in endothelial function partly by the decreased ET-1 production in the aorta, exercise training improves endothelial function through up-regulation of the aging-induced decrease in ET-1 expression in the aorta.

Aging is associated with a number of anatomic and haemodynamic changes in the vascular system, including collagen degeneration, loss of elastin, increased intima-media thickness of the arteries and reduced vascular compliance [32,33]. The alterations of endothelial function also have been associated with advancing age [5–10]. Furthermore, it has been reported that the vasodilating response to acetylcholine, an endothelium-dependent relaxant agent, decreased with advancing age [5]. Vascular endothelial cells play an important role in the regulation of vascular activity by producing vasodilating and vasoconstricting substances [11,12]. It has been suggested that a decrease in the bioavailability of NO, an endothelium-derived relaxing factor, may occur with aging [10,13–15]. ET-1, an endothelium-derived relaxant peptide, is a potent vasoconstrictor peptide [12,16–18], and has been reported to be involved in the regulation of vascular tonus [19,31]. The present study showed that the expression of ET-1 mRNA in the aorta was markedly lower in Sedentary aged group compared with Sedentary young group, suggesting that the ET-1 production in the aorta is decreased by aging. Therefore, it is considered that aging-induced loss of endothelial function not only may be caused by an alteration of the NO system, but also may be associated with a decrease in ET-1 production.

Exercise training induced an improvement in the vascular system. Exercise training also has been reported to produce an alteration in the function of vascular endothelial cells [21]. Sessa et al. [34] reported that exercise training increased expression of NO synthase mRNA in the dog aorta. Furthermore, Delp and Laughlin [35] demonstrated that the expression of NO synthase protein in the rat aorta increased due to exercise training. The present study revealed that the expression of ET-1 mRNA in the aorta is increased by exercise training. Therefore, it is considered that exercise training induced an improvement in endothelial function through alterations of the NO and ET systems. We previously reported that acute exercise significantly increases the circulating plasma concentration of ET-1 [20]. Therefore, it is possible that the repeated bouts of exercise causes an up-regulation of ET-1 production in the aorta after exercise training.

In summary, we have demonstrated that the expression of ET-1 mRNA in the aorta was markedly lower in sedentary aged rats compared with sedentary young rats, whereas it was significantly higher in swim trained aged rats compared with sedentary aged group. These results show that the expression of ET-1 mRNA in the aorta is decreased by aging, and that the expression is increased by exercise training. Therefore, the present study provides a possibility that ET-1 participates in aging-induced loss of endothelial function, and that the exercise training improves endothelial function through up-regulation of the aging-induced decrease in ET-1 expression in the aorta.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (00006781, 11357019, 11480003, 11557047, 12470147), a grant from University of Tsukuba Research Projects, and a grant from the project of TARA (Tsukuba Advanced Research Alliance) in University of Tsukuba.

REFERENCES

Exercise training and endothelin-1 in aorta of aged rat

123


24 Iemitsu, M., Miyachi, T., Maeda, S. et al. (2000) Intense exercise causes decrease in expression of both endothelial NO synthase and tissue NOx level in hearts. Am. J. Physiol. 279, R951–R959


