Exercise causes tissue-specific enhancement of endothelin-1 mRNA expression in internal organs

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ENDOTHELIN-1 (ET-1) is a potent vasoconstrictor peptide produced by vascular endothelial cells (28, 36). It has been reported that low concentrations of ET-1, which did not produce vasoconstriction, potentiated contractions to norepinephrine in human internal mammary and coronary vessels (37). In experimental animals, it has also been demonstrated that a low dose of ET-1 enhances adrenergic vasoconstriction in perfused rat mesenteric arteries (32). Thus it was thought that endogenous ET-1 contributes to the regulation of vascular tone through its direct vasoconstrictive action and/or an indirect effect of ET-1, enhancement of the vasoconstrictive action of norepinephrine. Furthermore, it has been reported that systemic administration of an endothelin-receptor antagonist significantly decreased systemic blood pressure and peripheral vascular resistance in healthy humans, strongly suggesting that endogenously generated ET-1 contributes to basal vascular tonus in humans (8). We previously reported that the arteriovenous difference in ET-1 concentration in nonworking muscles was significantly increased after exercise, whereas that in the working muscles was not significantly different before and after exercise (17). These findings suggested that the production of ET-1 is increased in the circulation of nonworking muscles by exercise. However, it is not known how exercise affects the production of ET-1 in internal organs.

Endurance exercise results in a significant redistribution of tissue blood flow, by which the blood flow is greatly increased in the working muscles, whereas it is decreased in the splanchnic circulation (such as in the kidneys and intestines) and in the nonworking muscle circulation (1, 5, 21–23, 35). Although it has been considered that the exercise-induced redistribution of blood flow is partly caused by the increased activity of the sympathetic nerve system (3) and multiple local metabolic factors (14), the precise mechanisms are not known. It has also been demonstrated that endothelium-derived relaxing factor, which is identical to nitric oxide (NO) (9, 25), is partly involved in determining the pattern of the redistribution of tissue blood flow during exercise (31). Although this finding suggests that vascular endothelial cells may be involved in the exercise-induced redistribution of blood flow, the roles of endothelium-derived vasoconstrictor substances, such as ET-1, in exercise-induced physiological responses remain to be investigated.

We previously reported that the arteriovenous difference in ET-1 concentration in nonworking muscles was significantly increased after exercise, whereas that in the working muscles was not significantly different before and after exercise (17). However, it is unclear how exercise affects the production of ET-1 in internal organs. To answer this question, the present study was designed to investigate whether exercise affects prepro-ET-1 mRNA expression in the internal organs, such as the kidneys and lungs, in exercise-induced physiological responses remain to be investigated.
METHODS

Animals and protocol. Twelve male Wistar rats (7 wk old) were obtained from Clea Japan (Tokyo, Japan) and cared for according to the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892] based on the 1964 Helsinki Declaration. The rats were maintained on a 12:12-h light-dark cycle and received food and water ad libitum. All rats were familiarized with running on a motor-driven treadmill 5 days/wk, over a 4-wk period, until they were capable of running at a speed of 25 m/min for 45 min at no incline (0% grade). The running time and the speed of the treadmill were gradually increased in 4 wk from 10 min at 10 m/min to 45 min at 25 m/min. Systolic arterial pressure and heart rate of the animals were measured with a tail-cuff sphygmomanometer (PS-100, Riken Kaibatsu, Kanagawa, Japan) on the day before the experiment. The body weight of the animals was also measured on the day before the experiment. The day of experiment, the rats were randomly divided into two groups. In one group, six animals performed treadmill running (0% grade) for 45 min at a speed of 25 m/min (exercise group). The other six animals served as the sedentary control (sedentary control group).

Immediately after removal from the treadmill, the exercise group was anesthetized with diethyl ether. After anesthetization, a blood sample was collected from the heart and subsequently the lungs and kidneys were quickly removed, weighed, and frozen in liquid nitrogen. The plasma and tissue samples were stored at −80°C for measurement of plasma ET-1 concentration by a sandwich-enzyme immunoassay (EIA), for measurement of plasma epinephrine concentration and plasma norepinephrine concentration by radioenzymatic assay, and for determination of preproET-1 mRNA expression by RT-PCR analysis. The animals in the sedentary control group were killed ~24 h after their last bout of exercise, i.e., at the same time point as was the exercise group.

Measurement of plasma ET-1 concentration by sandwich-EIA. Each blood sample was placed into a chilled tube containing aprotinin (300 kallikrein-inhibiting units/ml) and EDTA (2 mg/ml) and centrifuged at 3,000 g for 15 min at 4°C. The plasma was stored at −80°C until use. Plasma ET-1 concentration was measured by a sandwich-EIA as previously described in our papers (16, 17, 19, 20, 24, 29). In brief, plasma (1 ml) was acidified with 3 ml of 4% acetic acid, and immunoreactive ET-1 was extracted with a Sep-Pak C18 cartridge (Waters, Milford, MA). The elutes were reconstituted with 0.25 ml of assay buffer and subjected to a sandwich-EIA for ET-1. A sandwich-EIA for ET-1 was carried out as previously described with the immobilized mouse monoclonal antibody AwETN40, which recognizes the NH₂-terminal portion of ET-1, and peroxidase-labeled rabbit anti-ET-1 COOH-terminal peptide (15–21) Fab’ (16–19, 29). The coefficient of variation of the ET-1 assay for the intra-assay variation was 11%, and the coefficient of variation for the interassay variation was 13% (18).

Measurement of plasma epinephrine and norepinephrine concentrations. Plasma norepinephrine concentration and plasma epinephrine concentration were measured by using a radioenzymatic assay on the basis of the method of Peuler and Johnson (26). Plasma samples from each animal were diluted of the each positive-control cDNA were amplified by PCR and quantified by scanner.

Verification of the semi-quantitative PCR analysis. We performed quantitative PCR analysis to evaluate the expression level of preproET-1 mRNA and GAPDH mRNA. To demonstrate that our quantitative PCR strategy was valid, serial dilutions of the each positive-control cDNA were amplified by PCR and quantified by scanner.

Total tissue RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer’s instructions. The tissue was homogenized in Isogen (100 mg tissue/1 ml Isogen) with a polytron tissue homogenizer (PT105K/35, Kinematica, Lucerne, Switzerland). The chloroform extraction, isopropanol precipitation, and 75% (vol/vol) ethanol washing of the precipitated RNA were subsequently performed. The obtained RNA was resolved in pyrocarnic acid diethyl ester-treated water and treated with DNaseI (Takara, Shiga, Japan) and extracted again by Isogen to eliminate the genomic DNA. The RNA concentration was determined spectrophotometrically at 260 nm.

Total tissue RNA (10 μg) was primed with 0.05 μg oligo(dT) 12–18 and reverse transcribed by avian myeloblastosis virus reverse transcriptase by using a first-strand cDNA synthesis kit (LifeSciences). The reaction was performed at 43°C for 60 min.

The cDNA was diluted in a 1:10 ratio, and 1 μl was used for PCR. Each PCR reaction contained 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 0.5 μM of each gene-specific primer, and 0.025 U/μl Taq polymerase (Takara). The gene-specific primers were synthesized according to the published cDNA sequences for each of the following: preproET-1 (30) and GAPDH (34). The sequences of the oligonucleotides were as follows: preproET-1 (sense): 5’TTCTCTTCTTCTGCTTTGTG3’; preproET-1 (antisense): 5’TAGTTTTTTTTCCTCCACC3’; GAPDH (sense): 5’GCCATCAACGACCCCTTCATTG3’; and GAPDH (antisense): 5’TGCAGTCACCTGGTCCTC3’.

PCR was carried out by using a PCR thermal cycler (TP-5000, Takara). The cycle profile included denaturation for 15 s at 94°C, annealing for 15 s at each suitable temperature, and extension for each suitable time at 72°C. The annealing temperature was set as follows: preproET-1, 54°C and GAPDH, 58°C. The extension time was set as follows: preproET-1, 1 min and GAPDH, 30 s. The reaction cycles of PCR were performed in the range that demonstrated a linear correlation between the amount of cDNA and the yield of PCR products (45 cycles: preproET-1 mRNA in kidney, 28 cycles: preproET-1 mRNA in lungs, 24 cycles: GAPDH mRNA in kidney, and 26 cycles: GAPDH mRNA in lungs). The PCR products were found to be of the expected size, as shown by 1.5% agarose gel electrophoresis. In addition, the specificity of the amplified sequences was confirmed by restriction enzyme analysis and DNA sequencing. The DNA sequence of each amplicon was perfectly matched to each published sequence.

Quantitative analysis of PCR products. The amplified PCR products were electrophoresed on a 1.5% agarose gels, stained with ethidium bromide, visualized by a ultraviolet transilluminator, and photographed. The photographs were scanned by a scanner (CanoScan 600, Canon, Tokyo, Japan), and quantification was performed by computer with MacBAS software (Fuji Film, Tokyo, Japan).

Preparation of positive-control cDNAs of preproET-1 and GAPDH. The cDNAs for the verification of the semi-quantitative PCR analysis were prepared from each gene PCR product of rat cDNA. Each PCR product was purified, quantified, and used as positive-control cDNAs.
Table 1. Body weight and tissue weights in exercise group and sedentary control group

<table>
<thead>
<tr>
<th></th>
<th>Sedentary Control Group (n = 6)</th>
<th>Exercise Group (n = 6)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>330.0 ± 7.3</td>
<td>340.0 ± 3.7</td>
<td>NS</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>1.13 ± 0.03</td>
<td>1.13 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>1.20 ± 0.05</td>
<td>1.24 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Kidney weight/body weight, mg/g</td>
<td>3.43 ± 0.13</td>
<td>3.35 ± 0.09</td>
<td>NS</td>
</tr>
<tr>
<td>Lung weight/body weight, mg/g</td>
<td>3.63 ± 0.16</td>
<td>3.65 ± 0.08</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. NS, not significant.

RESULTS

There were no significant differences between the sedentary control group and the exercise group in systolic arterial pressure (123 ± 3 vs. 120 ± 3 mmHg) or heart rate (386 ± 17 vs. 385 ± 7 beats/min) on the day before death. There was no significant difference in body weight between the two groups (Table 1). Neither the kidney wet weight nor the lung wet weight differed significantly between the two groups (Table 1). These results indicated that the physical changes induced by 4 wk of training (familiarization with running on a treadmill) were of the same degree in the sedentary control group and the exercise group.

Immediately after the 45-min exercise or rest, the plasma concentrations of epinephrine and norepinephrine were significantly greater in the exercise group than in the sedentary control group (Fig. 1). Thus the plasma epinephrine concentration and the plasma norepinephrine concentration were increased by the acute exercise.

The expression of preproET-1 mRNA in the kidneys and lungs of both the sedentary control group and the exercise group was evaluated by RT-PCR analysis. The expression of GAPDH mRNA was also determined as an internal control. The expression of preproET-1 mRNA was markedly increased in the kidneys of the exercise group (Fig. 3). Therefore, the expression of preproET-1 mRNA in the kidneys was increased by the acute exercise. However, in the lungs, the expression of preproET-1 mRNA did not differ significantly between the two groups (Fig. 4). Therefore, the expression of preproET-1 mRNA in the lungs was not altered by the acute exercise.

The plasma concentration of ET-1 after acute exercise/rest was slightly but significantly higher in the exercise group than in the sedentary control group (Fig. 5). Therefore, the plasma ET-1 concentration was increased by the acute exercise.

DISCUSSION

In the present study, we determined preproET-1 mRNA expression in the lungs and kidneys of rats after acute exercise. The expression of preproET-1 mRNA in the kidneys was markedly higher in the exercise rats than in the sedentary control rats, whereas that in the lungs did not differ between the two groups. These findings suggested that the production of ET-1 was tissue specifically increased in the kidneys by acute exercise. 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 (8). Exercise results in a marked decrease in renal blood flow (6, 15, 33), whereas it increases cardiac output and pulmonary blood flow. On the basis of the results from past studies plus the present results, it was considered that the increased ET-1 production in the kidneys may cause the increase in vascular tone and the consequent decrease in blood flow in the kidneys, which are helpful in increasing blood flow in exercising muscles or in lungs, thereby contributing to the redistribution of blood flow during exercise. Such a decrease in renal blood flow would maximize the blood flow available to the active muscles.
The present study showed that the plasma concentrations of epinephrine and norepinephrine were far greater in the exercise rats than in the sedentary control rats, suggesting that sympathetic nerve activity was greatly augmented during acute exercise. In addition to the vasoconstrictor effect of ET-1, low concentrations of ET-1, which do not produce vasoconstriction, potentiate contractions to norepinephrine in arteries (37). It has also been demonstrated that a low dose of ET-1 enhances adrenergic vasoconstriction in perfused rat mesenteric arteries (32). Therefore, in blood vessels in the kidneys, it is possible that ET-1 potentiates norepinephrine-induced vasoconstriction. The increase in ET-1 in the kidneys may cause vasoconstriction through its direct action and/or by enhancing the vasoconstriction to norepinephrine and may contribute to the exercise-induced redistribution of blood flow.

In our previous study, the arteriovenous difference in the ET-1 concentration in nonworking muscles was significantly increased after exercise, whereas that in the working muscles was not significantly different before and after exercise (17). These findings suggested that the production of ET-1 was increased in the circulation of nonworking muscles by exercise. In that study, however, the following mechanism was also possible. During and immediately after exercise, the tissue blood flow in nonworking muscles may be decreased by vasoconstriction (3, 14). The reductions in blood flow could be mediated by sympathetic mechanisms and/or other vasoconstrictors (i.e., angiotensin II). When the local circulating blood flow is decreased, the level of local venous plasma ET-1 concentration could be elevated due to accumulation of ET-1 without an increase in endothelial ET-1 production. Therefore, it is possible that ET-1 was accumulated during exercise in the tissue of nonworking muscles because of a decreased blood flow and that the venous plasma concentration of ET-1 in the nonworking muscles was elevated after exercise by a washout effect. Accordingly, the increases in plasma ET-1 induced by exercise may...
not be due to the increases in ET-1 production and may be the result of decreases in blood flow to the various organs found in the gut and elsewhere. The most appropriate answer to this question would be provided from the direct measurement of preproET-1 mRNA expression in various tissue after exercise. The present study showed for the first time that preproET-1 mRNA expression in the internal organs is tissue specifically increased by exercise. The expression of preproET-1 mRNA in the kidneys was markedly higher in the exercise rats than in the sedentary control rats, suggesting that the production of ET-1 was actually increased in the kidney by acute exercise.

The mechanism underlying the difference in the production of ET-1 between the kidneys and lungs by exercise remains to be elucidated. It has been shown that both mechanical factors (such as hemodynamic shear stress) and neurohumoral factors (such as angiotensin II, arginine vasopressin, and so on) increase the production of ET-1 in cultured vascular endothelial cells (4, 28, 39). It has also been reported that low levels of shear stress stimulate and higher levels of shear stress depress the release of ET-1 in cultured vascular endothelial cells (10). The different alterations in blood flow between the kidneys and lungs by exercise (decrease and increase, respectively) might cause a difference in the levels of shear stress on vascular endothelial cells of the kidneys and lungs. Therefore, it is possible that a difference in the level of shear stress on vascular endothelial cells between the kidneys and lungs is one of the causal factors for the difference in ET-1 production seen in these organs. Because exercise increases cardiac output and hence greatly increases pulmonary blood flow, we chose to use the lung to represent active tissue during exercise. Alternatively, the following mechanism is also possible. It has been reported that NO and other vasodilating factors, such as prostacyclin, inhibit the production of ET-1 in vascular endothelium (27, 28). It also has been reported that hemodynamic shear stress increases production of NO from the vascular endothelium (2). Therefore, it is possible that NO or prostacyclin inhibits the production of ET-1 in the vascular endothelium of the lungs.

It has been reported that, in deoxycorticosterone acetate-salt hypertensive rats, the tissue ET-1 concentration in the vessels is higher than that in normotensive rats without a change in plasma ET-1 levels (11). Furthermore, we have reported that, in rats with cardiac hypertrophy due to aortic banding, tissue ET-1 concentration in the heart is higher than that in control sham-operated rats without a change in plasma ET-1 levels (38). These findings suggest that ET-1 plays a role in cardiovascular tissues as a local hormone, i.e., in an autocrine/paracrine fashion, rather than as a systemic hormone. Although increased levels of plasma ET-1 found in the present study are still below the concentration that causes pharmacological action re-
ported by Yang et al. (37) and Tabuchi et al. (32), it is possible that an increase in local ET-1 levels around the vessels (especially around vascular smooth muscles) in the kidney by exercise is far greater than that in plasma.

We chose the present intensity and duration of exercise in rats on the basis of the following reasons. Laughlin and Armstrong (12) have shown that during treadmill locomotion blood flows increase at 15 m/min, a fast walk just below the transition to trotting for rats, in most active muscles. They also reported that most active muscles in rats showed gradual increases in blood flow that were linearly related to time from 5 through 54 min of low-intensity treadmill exercise (13). Furthermore, it has been demonstrated that treadmill running at 21.4 m/min (70 feet/min) or 30 m/min in rats results in a significant redistribution of tissue blood flow, in which blood flow is greatly increased in the active muscles and decreased in the kidney (6, 15). Therefore, we chose the present condition of treadmill running at 25 m/min for a duration of 45 min as exercise intensity and duration, which cause the redistribution of blood flow in rats.

The present study has the following study limitations. First, although it has been reported that changes in mature levels of ET-1 mainly originated from changes in preproET-1 mRNA expression (28), it is unclear whether the increase in preproET-1 mRNA in the kidney found in the present study contributes to an increase in mature peptide level of ET-1 in the kidney. Second, because changes in blood flow by exercise has been reported to be great in the kidney (6, 15, 33), we investigated expression of preproET-1 mRNA in this organ. However, although the decreases in blood flow during exercise occur in the stomach, spleen, liver, pancreas, and small and large intestines, we did not measure the expression of preproET-1 mRNA in these organs. Therefore, it is unclear whether the expression of preproET-1 mRNA in these organs is altered by exercise. Third, the plasma norepinephrine concentrations shown in Fig. 1 for sedentary control rats are excessively high compared with those found in the literature for awake, resting, nonrestrained rats (7). This could be due to the stress produced by the induction of anesthesia. In addition, plasma norepinephrine concentrations may or may not be indicative of sympathetic activation because plasma norepinephrine concentrations are the consequence of release and reuptake. Fourth, we expressed the level of preproET-1 mRNA relative to GAPDH as an internal control. To maximize the effectiveness of GAPDH as an internal control, preproET-1 and GAPDH should be coamplified in the same PCR reaction. However, the suitable cycles of amplification for preproET-1 with a linear correlation between the initial amount of cDNA and the yield of PCR products were different from that for GAPDH, presumably caused by the different levels of initial amount of each cDNA in each sample. Therefore, we performed the amplification of preproET-1 and GAPDH in separate PCR reactions by using same RT reaction.

In summary, we have demonstrated that the expression of preproET-1 mRNA in the kidneys was markedly higher in the exercise rats than in the sedentary control rats, whereas that in the lungs did not differ between the two groups. These findings suggest that the production of ET-1 was increased tissue specifically in the kidneys by exercise. The present study also demonstrated that the plasma norepinephrine concentrations were markedly elevated by exercise and, therefore, that the sympathetic nerve activity was greatly augmented during exercise. Therefore, the present study provides a possibility that the increase in the production of ET-1 in the kidneys may cause vasoconstriction and hence decrease blood flow in the kidneys via the direct vasoconstrictive action of ET-1 and/or an indirect effect of ET-1, enhancement of vasoconstriction to norepinephrine. Further studies as to whether the application of an endothelin-receptor antagonist affects the exercise-induced changes in blood flow are needed as more direct evidence to support this hypothesis, although the present data are consistent with this hypothesis. We propose that endogenous ET-1 participates in the exercise-induced changes in the distribution of blood flow by tissue-specific enhancement of ET-1 production in internal organs, which is helpful in increasing the blood flow to working muscles and to lungs.

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REFERENCES


10. Kuchan, M. J., and J. A. Frangos. Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured