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A Causal Role for Endothelin-1 in the Vascular Adaptation
to Skeletal Muscle Deconditioning in Spinal Cord injury

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Objective—Endothelin-1 (ET-1) contributes to the increased peripheral resistance in heart failure and hypertension. Physical inactivity is associated with cardiovascular disease and characterized by increased vascular tone. In this study, we assess the contribution of ET-1 to the increased vascular tone in the extremely deconditioned legs of spinal cord-injured (SCI) individuals before and after exercise training.

Methods and Results—In 8 controls and 8 SCI individuals, bilateral thigh blood flow was measured by plethysmography before and during the administration of an ETa/ETb-receptor blocker into the femoral artery. In SCI, this procedure was performed to determine the role of the ETa-receptors. In controls, dual ET-receptor blockade increased leg blood flow at the infused side (10%, P<0.05), indicating a small contribution of ET-1 to leg vascular tone. In SCI, baseline blood flow was lower compared with controls (P=0.05). This vasodilator response was significantly larger in SCI compared with controls (P<0.001). The response to selective ETa-receptor blockade was similar to the effect of dual blockade. Electro-stimulated training reduced baseline blood flow in SCI and reduced the response to dual ET-receptor blockade in the infused leg (29%, P=0.04).

Conclusion—ET-1 mediates the increased vascular tone of extremely inactive legs of SCI individuals by increased activation of ETα-receptors. Physical training reverses the ET-1-pathway, which normalizes basal leg vascular tone.

Key Words: endothelin receptor ■ endothelium ■ exercise ■ cardiovascular disease ■ paraplegia
TABLE 1. Characteristics of Spinal Cord-Injured Individuals

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (yrs)</th>
<th>LL</th>
<th>TSI (yrs)</th>
<th>Medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M</td>
<td>44</td>
<td>T5</td>
<td>25</td>
<td>metanamine, furadantine</td>
</tr>
<tr>
<td>2 M</td>
<td>52</td>
<td>T7</td>
<td>20</td>
<td>cibutine, antibiotic, sodium-lauryl-sulfoacetate and sodium-citrate-sorbitol (microlax)</td>
</tr>
<tr>
<td>3 F</td>
<td>36</td>
<td>T12</td>
<td>3</td>
<td>bisacodyl, baclofen</td>
</tr>
<tr>
<td>4 M</td>
<td>46</td>
<td>T8</td>
<td>10</td>
<td>methylphenadate (Ritalin), imipramine</td>
</tr>
<tr>
<td>5 M</td>
<td>43</td>
<td>C5</td>
<td>24</td>
<td>dantrium, baclofen, oxybutynine</td>
</tr>
<tr>
<td>6 M</td>
<td>25</td>
<td>T4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>7 M</td>
<td>38</td>
<td>C7</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>8 M</td>
<td>24</td>
<td>T6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>38 ± 3</td>
<td>13 ± 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LL indicates level of lesion, TSI, time since injury

Methods

Subjects

Eight SCI individuals (7 men and 1 woman, age: 38 ± 12 years; Table 1) and 8 healthy, nonsmoking control subjects (7 men and 1 woman, age: 34 ± 12 years) participated in the study. The SCI individuals continued their medication throughout the study (Table 1). Two SCI individuals stopped smoking 2 to 4 weeks before the initial experiment. All SCI individuals except one (incomplete motor lesion from L3: American Spinal Injury Association19; ASIA C) had complete motor and sensor spinal cord lesions of traumatic origin varying from Cervical 5 to Thoracic 12 (ASIA A). The subjects had no history of cardiovascular disease, were normotensive, had no hypercholesterolemia, and used no medication known to interfere with the cardiovascular system. The study was approved by the hospital ethics committee. All subjects gave their written informed consent before participation.

Experimental Design

Control subjects and SCI individuals were studied to quantify the vasodilator response to blockade of ET-receptors in the leg. Subsequently, at least two weeks thereafter, SCI individuals started with a 6 weeks functional electro-stimulated training of the paralyzed legs to assess whether the observed alterations were reversible on training. Exactly the same experimental protocol was repeated after the final training session. In SCI individuals, the role of the ETA- and ETB-receptor antagonist (BQ-123 and BQ-788, respectively, ClinAlfa, Calbiochem-Novabiochem AG) was started to block the ET-receptors of the right leg. BQ-123 and BQ-788 were infused at 10 and 1 nmol/min/L leg volume, respectively, and the infusion was continued for 75 minutes.2,22 Total leg-volume was determined by anthropometry as described and validated by Jones et al.21 Previous studies showed that a 60-minute coinfusion of BQ-123 and BQ-788 (at, respectively, 10 and 1 nmol/min/L tissue into the brachial artery) resulted in maximal vasodilation in the forearm of healthy subjects without triggering any systemic blood pressure effects.22,24

Selective ET-A-Receptor Blockade

To determine the contribution of the ET-A-receptor in the observed effect of dual ETA/B-receptor blockade in SCI individuals, we infused the selective ET-A-receptor antagonist BQ-123 (10 nmol/min/L leg volume), while the syringe with BQ-788 was replaced by saline. These experiments were performed at least 21 months after cessation of the FES-cycling, which is sufficient to exclude any possible chronic training effect.25 The combined ETA/B-blockade was compared with the selective ETA-receptor blockade to explore the role of the ETA-receptor in the observed effects in SCI individuals.

Drugs and Solutions

BQ-123 (10 nmol/min/L leg volume) and BQ-788 (1 nmol/min/L leg volume) were dissolved in saline at the beginning of the experiment. During the whole protocol, infusion rate was kept constant at 0.1 mL/min/L tissue.

Hybrid Exercise-Training

A stationary computer-controlled functional electro-stimulation (FES)-ergometer (BerkelBike BV) was used for hybrid FES-cycling exercise; including stimulated leg-cycling and voluntary arm-cranking. The FES-ergometer provides stimulation via surface electrodes (5 × 8 cm, Farmadomo) to the hamstring, gluteal, and quadriceps muscles. Details regarding this training are described...
ET-receptor blockade reached its maximal effect (Figure 1) and blood flow ratio were analyzed over the last 10 minutes, where regulation of baseline vascular tone. Blood flow, vascular resistance, and blood volume were lower in SCI individuals (n = 8) both before (SCI, A) and after the training procedure (SCI-training, B). Each symbol represents the change in blood flow ratio over 15 minutes, with the error bars representing the SE. The black square indicates the maximal effect during the final 10 minutes of the ET-receptor blockade.

This effect was used for statistical analysis (see Figure 2).

 SCI individuals trained twice a week for 6 weeks. Compliance with the training sessions was 100%.

Data Analysis

Plethysmographic data of both legs were digitalized with a sample frequency of 100 Hz (MIDAC, Instrumentation Department, Radboud University Nijmegen Medical Centre) and analyzed off-line by a customized computer program (Matlab, Mathworks Inc). Leg blood flow (in ml/min per dl of leg tissue) was calculated as the slope of the volume change curve over a 4-second interval, starting directly after the inflation-induced cuff artifact (2 seconds). Thigh vascular resistance was calculated as the mean arterial pressure (mm Hg) divided by thigh blood flow (in ml/min/dL) and expressed in arbitrary units (AU; mm Hg/ml/min/dL).

Because of the differences in baseline vascular characteristics between SCI and control subjects, analysis of absolute values could affect the comparison between both groups. Therefore, data were expressed as percentage changes from baseline. Baseline blood flow and vascular resistance were calculated by averaging registrations over a 10-minute interval before the infusion of the ET-receptor blockers. In addition, leg blood flow was expressed as the ratio between the infused and noninfused leg to correct for possible random systemic changes.

An increase of the flow ratio during ET-receptor blockade indicates vasodilation in the leg vascular bed, suggesting a relevant role for endogenous ET-1 in the regulation of baseline vascular tone. Blood flow, vascular resistance, and blood flow ratio were analyzed over the last 10 minutes, where ET-receptor blockade reached its maximal effect (Figure 1).

Statistics

The primary end point of this study is the vasodilator response to ET-receptor blockade in the leg. We decided that with an estimated SD of 20% of the baseline value, a mean relevant effect of the ET-receptor blockade should be at least 25% (for the control versus SCI comparison) or 17.5% (for the before versus after training comparison) and calculated that with an alpha of 0.05, 8 subjects per group would be needed to achieve a power of 80%. Kolmogorov-Smirnov test indicated a normal (Gaussian) distribution of data. Results are expressed as mean ± SE. To assess differences between SCI individuals and controls (unpaired) and between the pre- and post-FES-training measurement (paired), the maximal effect of ET-receptor blockade (as defined as the average of the final 10 minutes of infusion) was compared using a Student t test. Differences were considered to be statistically significant at a 2-sided probability value of <0.05.

Results

Baseline Characteristics

Because of muscle spasms in one SCI subject and of technical problems in one control subject, the data of the noninfused leg were not available for analysis in these 2 subjects. Baseline leg blood flow was significantly lower in SCI individuals as compared with controls (Table 2; t test: P = 0.05). The training procedure increased baseline leg blood flow in SCI individuals (Table 2). Leg volume of SCI individuals was lower as compared with control subjects, but increased in response to the training procedure (Table 2). Baseline mean arterial pressure, heart rate, and plasma ET-1 levels were not different between SCI individuals and controls, and did not change by training in SCI individuals (Table 2).

Dual ET-Receptor Blockade in Controls and SCI Individuals

Controls

Blockade of the ET-receptors did not affect mean arterial pressure, heart rate, or blood flow and vascular resistance in the noninfused leg in the control subjects (Table 2). The ET-receptor blockade induced a small but significant change in blood flow (10 ± 4%) and vascular resistance (−10 ± 4%) of the infused leg. As such, the blood flow ratio between both legs increased by 9 ± 4% (Figures 1 and 2).

SCI Individuals

During the ET-receptor blockade in SCI individuals, heart rate did not change, whereas mean arterial pressure significantly decreased (Table 2). After ET-receptor blockade, blood flow clearly increased in the infused leg of the SCI individuals (41 ± 5%, Figure 2). In the noninfused leg, a slight but significant increase in blood flow was observed (17 ± 6%, Table 2). Blockade of the ET-receptors induced a significant increase in the blood flow ratio (22 ± 5%, Figures 1 and 2).

Controls Versus SCI Individuals

In contrast to controls, blockade of the ET-receptors induced a significant decrease in mean arterial pressure in the SCI individuals (Table 2). The change in blood flow and vascular resistance of the infused leg during ET-receptor blockade in SCI was larger than in the controls. Likewise, the increase in blood flow ratio in response to the ET-receptor blockade was significantly larger in SCI individuals (Figures 1 and 2).

Dual ET-Receptor Blockade in SCI Individuals After FES-Cycling

SCI Individuals After FES-Cycling

ET-receptor blockade did not change heart rate but decreased mean arterial pressure. After training, blood flow of the infused and noninfused leg both increased during the infusion protocol (29 ± 3 and 24 ± 6%, respectively, Table 2). Vascular resistance of both legs decreased during ET-receptor blockade (−31 ± 2 and −29 ± 5%, respectively, Table 2). The blood flow ratio did not change during blockade of the ET-receptors (Figures 1 and 2).
we examined a subset of the SCI individuals (n = 8) and controls (C, n = 8), both before (SCI) and after the training procedure (SCI-training). *Significant relative increase from baseline.

**Pre-Training Versus Post-Training**

The response of heart rate and mean arterial pressure to ET-receptor blockade did not differ between pre- and post-training measurements. The increase in leg blood flow in the infused leg as well as the flow ratio during ET-receptor blockade were significantly lower after training than before (Figure 2), whereas the difference between the pre- and post-training fall in vascular resistance did not reach statistical significance (Table 2).

**Selective ETα-Receptor Blockade**

Because of medical problems (n = 2) and withdrawal (n = 2), we examined a subset of the SCI individuals (n = 4, male, 36±12 years). At least 21 months after cessation of the exercise training, baseline characteristics did not differ between both situations (Table 3). During selective ETα-receptor blockade in SCI individuals, heart rate did not change. Mean arterial pressure showed a decrease, but did not reach statistical significance (Table 3). After ETα-receptor blockade, blood flow increased in the infused leg (Figure 3), but did not change significantly in the noninfused leg (Table 3). Blockade of the ETα-receptor induced an increase in the blood flow ratio, but did not reach statistical significance (Figure 3). The hemodynamic responses did not differ between selective ETα- and combined ETαβ-receptor blockade (Table 3, Figure 3).

**Discussion**

The present study provides three interesting and clinically important new findings: (1) endogenous ET-1 hardly contributes to baseline vascular tone in the leg of healthy control subjects; (2) in contrast, in SCI individuals endogenous ET-1 has a prominent role in the regulation of vascular tone in the deconditioned leg; and (3) exercise training in SCI individuals reverses the ET-1-pathway, which normalizes basal leg vascular tone. Based on the fact that ET-1 plasma levels did not change, our findings in SCI may be explained by an upregulation of the ET-receptor sensitivity or signaling. Thus, our results indicate that intensive long-term inactivity in humans results in a significant, though reversible, ET-1–mediated vasoconstrictor state in the skeletal muscle vascular bed.

In contrast to previous studies, using the perfused forearm model, we only observed a slight vasodilator effect of ET-1 blockade (9% increase in blood flow) in the leg of healthy subjects, whereas forearm blood flow increased by 35% to
TABLE 3. Dual and Selective ETA-Receptor Blockade in SCI (n=4)

<table>
<thead>
<tr>
<th></th>
<th>Dual ET-Receptor Blockade</th>
<th>Selective ETα-Receptor Blockade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>End-Infusion</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>58±3</td>
<td>59±4</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>90±2</td>
<td>81±3*</td>
</tr>
<tr>
<td>Infused leg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BF, ml/min/dl</td>
<td>2.3±0.3</td>
<td>3.3±0.5*</td>
</tr>
<tr>
<td>BF, %</td>
<td>100±0</td>
<td>143±7*</td>
</tr>
<tr>
<td>VR, AU</td>
<td>42±7</td>
<td>27±5*</td>
</tr>
<tr>
<td>VR, %</td>
<td>100±0</td>
<td>64±3*</td>
</tr>
<tr>
<td>Non-infused leg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BF, ml/min/dl</td>
<td>2.0±0.2</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>BF, %</td>
<td>100±0</td>
<td>119±8</td>
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<tr>
<td>VR, AU</td>
<td>46±5</td>
<td>36±7*</td>
</tr>
<tr>
<td>VR, %</td>
<td>100±0</td>
<td>76±8*</td>
</tr>
<tr>
<td>Infused and non-infused leg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow ratio, %</td>
<td>100±0</td>
<td>117±8</td>
</tr>
</tbody>
</table>

Vascular characteristics are presented for a subset of spinal cord-injured (SCI) individuals (n=4), before the start of the infusion (Baseline) and at the end of the infusion of the dual ET_{A/B} and selective ET_{α}-receptor antagonists (last 10 minutes, End-infusion). Data are presented as mean±SE.

* P<0.05 from Baseline (f test)

60% during infusion of the ET-receptor antagonists BQ-123 and BQ-788. The difference in response to ET-receptor blockade between forearm and leg vascular bed suggests that ET-1 has a different physiological effect on the lower limbs than on the upper limbs in healthy subjects. Recently, Newcomer et al. reported significantly different responses for human forearm and leg vascular beds to endothelium-dependent (acetylcholine and substance P) and -independent (sodium nitroprusside) stimuli. Moreover, infusion of ET-1 in the rat hindquarters skeletal muscle bed results in a significantly lower vasoconstrictor response as compared with the mesenteric bed. It may be hypothesized that the level of activity may partly explain the differences in contribution of ET-1 to vascular tone between both limbs. The average level of skeletal muscle activity varies markedly between the upper and lower extremities. As bipeds, the legs are far more active during daily life (ie, locomotion, standing) and during sports activities (ie, running, cycling) than the human forearm.

The larger vasodilator response to ET-receptor blockade in SCI individuals compared with controls indicates that ET-1 importantly contributes to the elevated baseline leg vascular tone in SCI. Moreover, because ET-receptor blockade eliminates the difference in leg blood flow between SCI and control subjects, ET-1 may even be primarily responsible for the increased basal leg vascular tone in SCI. This agrees with previous studies from our department which excluded a role for nitric oxide or for α-adrenergic receptor-mediated effects in the elevated leg vascular tone in SCI individuals. Apart from the sympathetic denervation, the main difference between SCI individuals and controls is the paralysis of the legs, resulting in an extreme inactivity of the legs. This suggests a functional link between the contribution of ET-1 to vascular tone and inactivity, which is supported by the second part of our study demonstrating that exercise training in SCI individuals attenuates the contribution of ET-1 to leg vascular tone. This observation is in line with recent animal studies that report exercise training in healthy animals to result in a downregulation of the ET pathway. For example, a decrease in receptor sensitivity to ET-1 in pig coronary arteries and rat aortic and cerebellar vessels is reported after exercise training. However, the reversal of the contribution of ET-1 to leg vascular tone in our study was not complete, suggesting that ET-1 may not be the only factor in vascular adaptation to SCI or, alternatively, that the training period did not last long enough.

The mechanism behind the adaptation of the ET pathway to (in)activity remains to be solved. In the present study, ET-1 plasma levels were not different before and after training in SCI individuals, nor between controls and SCI individuals. This indicates that different ET-1 plasma levels cannot explain the changes in the contribution of ET-1 to leg vascular tone with (in)activity. As such, changes in sensitivity (and/or density) of the ET_{α}- and ET_{β}-receptors or changes in endothelin receptor signaling are more likely to explain the inactivity-induced upregulation of the ET pathway. The magnitude of the response to selective ET_{α}-receptor blockade in the subset of SCI individuals is similar to the vascular response observed during dual blockade of the ET_{A/B}-receptors. In combination with the marked increase in blood flow during ET-blockade in the SCI subjects, these results indicate that the ET_{α}-receptor mediates the increased contribution of ET-1 to leg vascular tone in SCI subjects. A strong argument against a role of the ET_{β}-receptor is related to the fact that these receptors mediate vasodilation by endothelial generation of nitric oxide (NO). We recently demonstrated that the contribution of NO to baseline leg vascular tone is preserved in SCI subjects as examined with infusion of L-NMMA in the femoral artery. Because the vasodilator properties induced by agonism of the ET_{β}-receptors are mediated by NO, the ET_{β}-receptor cannot account for the increased ET-1-mediated vasoconstriction observed in the legs of SCI subjects.
Because both groups were sex-matched, each population consisted of 7 males and 1 female. Interestingly, there was no gender difference noted in any of the measured parameters. Excluding the female subjects did not change the magnitude of the data outcome measures.

Clinical Relevance
SCI individuals are prone to develop decubitus and have poor wound healing, which may be caused by the increased leg vascular tone. Based on the constrictor action of ET-1 in SCI, pharmacological interventions that block the ET-1 activity may improve or prevent these pathologic conditions. Prolonged administration may even improve general cardiovascular function, but at present, no data are available. Increasing evidence supports a pathophysiological role for ET-1 in the modulation of vascular tone in cardiovascular disease. Based on our findings one should realize that the increased contribution of ET-1 in basal vascular tone in cardiovascular disease result from the reduced level of inactivity. Therefore, inactivity, rather than the pathology of these specific cardiovascular diseases, is emerging as a strong candidate to explain the ET-1-mediated elevated vascular tone in cardiovascular disease.

Limitations
In this study we used local infusions of drugs into the femoral artery. Because the leg represents an ~8-fold larger vascular bed as compared with the forearm, higher dosages were necessary, and therefore systemic spill-over effects may have occurred. Control subjects, however, showed no change in mean arterial pressure or heart rate during ET-receptor blockade. In SCI individuals, although the same protocol was used, a significant decrease in mean arterial pressure was found. This change in arterial pressure is most likely mediated through the ET-receptor blockade-induced vasodilation, without the concomitant contra-regulation via baroreflex-mediated sympathetic vasconstriction in SCI. The non-infused leg showed a vasodilator response, indicating a systemic vascular effect, which may be caused by a minor spillover of the ET-antagonists. Differences in body composition between controls and SCI individuals (eg, less fat-free mass in SCI) may have contributed to the spillover. As a consequence of the vasodilation in the control limb in SCI, flow ratios underestimate unilateral vascular changes in the infused leg. The contribution of ET-1 to leg vascular tone in SCI may, therefore, be even more pronounced than indicated by the flow ratio. Although in this study design it was not possible to avoid systemic actions of the ET-receptor antagonists, these issues will not alter the major outcome of the study.

In conclusion, compared with the minor effect of ET-1 on leg vascular tone in healthy subjects, ET-1 is a key mediator in the increased leg vascular tone in SCI individuals. In addition, data of this study indicate that exercise training can reverse the contribution of ET-1 to vascular tone in SCI. These adaptations in the ET pathway may be the result of changes in ET receptor sensitivity or signaling, rather than changes in plasma levels of ET-1. Thus, inactivity appears to upregulate the ET pathway in the human skeletal muscle vascular bed.

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Disclosures
None.

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