Exercise Training Alters the Balance Between Vasoactive Compounds in Skeletal Muscle of Individuals With Essential Hypertension

Ane H. Hansen, Michael Nyberg, Jens Bangsbo, Bengt Saltin, Ylva Hellsten

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Key Words: thromboxane ■ prostacyclin ■ microdialysis ■ vascular function ■ endothelial nitric oxide synthase

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vascular disease is an imbalance between vasoconstrictive and vasodilating compounds, including prostanoids. In spontaneously hypertensive rats, the metabolism of arachidonic acid has been found to be altered and has been proposed to contribute significantly to the enhanced peripheral resistance. Moreover, overexpression of vascular CYP4A in rats has been found to cause hypertension and endothelial dysfunction. Thus, 1 of the potential effects of physical training could be an alteration in the metabolism of arachidonic acid, leading to elevated formation of vasodilators or a reduction in the formation of vasoconstrictors.

Nucleotides such as ATP, ADP, and adenosine are known to increase in the muscle interstitium during exercise, where they are likely to contribute to blood flow regulation. ATP has been shown to have opposing effects on vascular tone in the circulation versus the interstitium; circulating ATP can bind to purinergic P2Y receptors on the endothelium and mediate vasodilation by formation of NO, prostacyclin, and endothelium-derived hyperpolarizing factors, whereas interstitial ATP can bind to purinergic receptors, probably P2X1, on the smooth muscle cells and mediate vasoconstriction. Adenosine, the degradation product of ATP, can induce vasodilation either by directly acting on the smooth muscle cells or by acting on endothelial cells, leading to formation of prostacyclin and nitric oxide (NO).

Another potent vasorelaxative substance that may establish itself alongside NO is hydrogen sulfide (H₂S). In endothelial and smooth muscle cells, H₂S is produced from cysteine, catalyzed by cystathionine-γ-lyase (CSE), and it has been shown that mice lacking this enzyme display increased blood pressure similar to that observed in mice lacking eNOS. It has been proposed that H₂S may be particularly important when endothelial function is impaired, suggesting that the CSE system may be altered in essential hypertension. The level of CSE in human skeletal muscle has not been reported.

The main hypothesis of the present study was that exercise training of individuals with essential hypertension elevates the formation of vasodilating compounds and the protein levels of corresponding vasodilator systems and reduces the formation of vasoconstrictors and the corresponding vasoconstrictor systems. To test this hypothesis, several vasodilator training of individuals with essential hypertension by their physician. In addition, 10 normotensive control subjects at rest.

Performance Tests

The subjects performed a maximum workload test on a 1-leg knee extensor model and a cycle ergometer test for determination of maximal oxygen uptake (VO₂max) before and after the training period. The incremental test was initiated by a 5-minute warm-up period at a power output of 6 W, and resistance was then increased by 6 W per minute until a kiccing frequency of 60 revolutions per min could no longer be maintained. The highest workload at which the subject could maintain for 1 minute was set as maximal workload (WLmax). VO₂max was estimated by the Åstrand-Ryhming 6-minute cycle test. Heart rate was monitored while subjects pedaled at 60 rpm for 6 minutes. Heart rate was kept between 130 to 160 bpm by adjusting the workload. The Åstrand-Ryhming nomogram was used to estimate VO₂max.

Experimental Protocol

The subjects were told not to exercise or ingest caffeine 24 hours before the 2 experimental days. The microdialysis procedure in human skeletal muscle has been described previously. In short, 5 microdialysis probes with a cut-off of 5 kDa were inserted into the m. vastus lateralis of the subjects after local anesthesia. The probes were perfused with Ringer acetate buffer, and microdialysate was collected for 30 minutes at rest. The subjects then performed 60 minutes of 1-leg knee extensor exercise at a power output of 12 W, followed by 60 minutes of exercise at 40% of each subject’s individual maximal workload (40% WLmax). Muscle dialysate was collected for 30 minutes at rest, throughout the exercise protocol, and for the 60 minutes after exercise. The same experimental design was used before and after the training period. Muscle biopsies were obtained from the middle portion of the m. vastus lateralis at rest, before training, and after training, by the use of the percutaneous needle biopsy technique. It was not possible to obtain muscle biopsies or microdialysate from 2 of the subjects after training.

For comparison, muscle biopsies were also obtained in untrained normotensive control subjects at rest.

Training

Each training session consisted of a 10-minute warm-up period of light (30% to 40% of VO₂max) work on a cycle ergometer. The warm-up was followed by a 50-minute period consisting of moderate (60% of VO₂max)-intensity exercise, varying between cycle ergometer exercise and fast walking/running, combined with a limited...
amount of upper and lower body strength training (8–10-repetition maximum). The training was performed 3 times per week, and each session lasted ~1 hour and was under supervision of physiotherapists. Compliance with the training program was >90%.

### Thromboxane and Prostacyclin
PGI₂ and TXA₂ in the dialysate fluid were detected by the presence of their stable metabolites, 6-keto-PGF₁α, and TXB₂, respectively, using commercially available ELISA kits (Cayman Chemical Co) according to the manufacturer’s protocol.

### ATP, ADP, and Adenosine
ATP, ADP, and adenosine concentrations in the dialysate fluid were detected by the presence of high-performance liquid chromatography without previous treatment of the samples, as previously described.²⁴

### Potassium
The potassium concentration of the muscle dialysate fluid samples was measured using a flame photometer (FLM3, Radiometer), using lithium as the internal standard.

### Nitrite and Nitrate
The stable NO metabolites nitrite and nitrate (NOₓ) in the muscle dialysate samples were analyzed using a fluorometric kit (Cayman Chemical Co).

### Western Blot Analysis
Protein levels were determined on freeze-dried skeletal muscle samples by Western blot procedure as previously described.²⁵ The antibodies used and the detection procedure are presented in the supplemental material, available online at http://hyper.ahajournals.org.

### Statistical Analysis
All data are expressed as mean±SEM. One- and 2-way repeated-measures ANOVA was used to evaluate the effect of time and exercise training on physiological characteristics and protein and dialysate concentrations. The Student-Newman-Keuls method for multiple comparisons was used as a post hoc test. A nonpaired Student 𝜋 test was used to compare protein levels between normotensive and hypertensive individuals. 𝑃<0.05 was accepted as statistically significant. As stated in the Methods, it was not possible to obtain complete sets of muscle and microdialysis data from 2 hypertensive subjects. Nevertheless, there was adequate statistical power for the most important variables. However, for the purinergic receptors and the enzymes of arachidonic acid metabolism, the power was below the desired level, and therefore a lack of significant difference between groups due to a type II error cannot be excluded.

### Results

#### Physical Characteristics
The mean arterial pressure of the hypertensive subjects was 250±60 and 400±280 mm Hg during exercise. After training, the potassium concentrations at rest (3.1±0.3 mmol/L) and during exercise (5.7±1.1 mmol/L) were similar to those before training. None of the included probes had abnormally high levels of potassium indicating damage.²⁶

#### Dialysate NOₓ and Potassium
Dialysate concentrations of NOₓ were 13.5±1.7 μmol/L at rest and 16.0±4.2 μmol/L during exercise. Values were similar before and after the training program (Figure 2).

#### Dialysate ATP, ADP, and Adenosine
Before training, the dialysate concentrations of ATP and ADP at rest were 250±60 and 400±280 mmol/L, respectively, and the levels were not changed with training (Figure 3). Before training, acute exercise induced a 20- and 9-fold increase in ATP and ADP above rest, respectively, whereas after training, the exercise-induced changes were markedly reduced (𝑃<0.05). The resting dialysate adenosine concentration was 0.17±0.04 μmol/L before training, and acute exercise induced a 2-fold increase. Training did not alter the adenosine levels (Figure 3).

#### Protein Levels of Enzymes in Muscle Tissue
The eNOS protein level in the muscle biopsy samples was unaltered by training (𝑛=8). Compared with the normotensive subjects (𝑛=12), the eNOS protein level was ≈40% lower (𝑃<0.05) in the hypertensive subjects.

##### Discussion
The major novel findings in the present study was that a period of exercise training lowered basal thromboxane levels, reduced the exercise-induced increase in ATP, and enhanced the exercise-induced increase in prostacyclin, indicating a shift in the formation of vasodilators versus vasoconstrictors. Individuals with essential hypertension furthermore had markedly lower eNOS levels than the normotensive individuals and higher CSE levels, potentially reflecting a compensatory mechanism for the reduced eNOS levels. Nevertheless, these 2 systems, as well as several of the other proteins and compounds determined, remained unaltered with training, suggesting that not all vasoactive systems implicated in the regulation of vascular tone in skeletal muscle respond to physical training.
Dialysate TXB₂ and 6-Keto-PGF₁α Concentrations

Hypertension has been proposed to be associated with an imbalance between vasodilating prostacyclin and vasoconstricting thromboxane; however, the levels of prostanoids or the corresponding enzyme systems have not previously been reported in muscle of hypertensive individuals. In this study, training was found to lower the resting values of TXB₂ in the muscle dialysate, which could be a contributing factor to the lowering of resting blood pressure. Moreover, before training, the dialysate levels of TXB₂ decreased almost 3-fold from rest, which is in accordance with findings in young, untrained subjects, where an approximate 1.5-fold decrease in TXB₂ was observed during bicycle exercise. After training, when resting TXB₂ levels were lower, the levels remained unaltered with exercise. Of note is that the protein level of TXA₂ synthase was similar before and after training, whereas cyclooxygenase 1 and 2 protein levels both tended to increase, suggesting a lack of relationship between these enzyme activities and the formation of TXB₂. Instead, TXB₂ formation may be regulated by substrate levels or the degree of enzyme activation.

The resting dialysate concentration of PGI₂ was lower after training compared with before, but whereas before training, the PGI₂ concentration was unaffected by exercise, after training, there was an exercise-induced increase in PGI₂ concentration. An increase in muscle dialysate PGI₂ concentration with acute exercise, as was observed after training, is in accordance with previous studies, in which muscle dialysate PGI₂ levels have been determined during moderate intensity exercise in healthy, young subjects. These data suggest that the activation of PGI₂ synthesis by exercise is impaired in hypertensive subjects and that exercise training can improve this function. However, it should be noted that although training led to an increase in PGI₂ synthesis during exercise, the actual vasodilatory response to PGI₂ may have been reduced, in part because of its dependence on an impaired NO system, as previously observed in aging.

Figure 1. Concentrations of dialysate 6-keto-PGF₁α (n=7) (A) and TXB₂ (n=6) (B) in muscle dialysate during an acute exercise bout before and after 16 weeks of training. Protein levels of enzymes of the arachidonic pathway before and after training (C) and compared with control (D). W indicates Watt; Wmax, maximal workload. §P<0.05 before vs after training, *P<0.05 vs rest.
The hypothesis of the current study was that exercise training would increase the expression of CYP2C9 and reduce the levels of CYP4A, thereby improving the balance between vasodilating and vasoconstricting compounds in the muscle. However, there were no alterations in the protein levels of either CYP2C9 or CYP4A. The respective metabolites 11,12-eicosatrienolic acid and 20-hydroxyeicosatetraenoic acid were found to be below the level of detection in the muscle dialysate. Our data therefore cannot support the hypothesis of an effect of training on these enzyme systems.

CYP2C9 and CYP4A
The hypothesis of the current study was that exercise training would increase the expression of CYP2C9 and reduce the levels of CYP4A, thereby improving the balance between vasodilating and vasoconstricting compounds in the muscle. However, there were no alterations in the protein levels of either CYP2C9 or CYP4A. The respective metabolites 11,12-eicosatrienolic acid and 20-hydroxyeicosatetraenoic acid were found to be below the level of detection in the muscle dialysate. Our data therefore cannot support the hypothesis of an effect of training on these enzyme systems.

**Dialysate ATP, ADP, and Adenosine Concentrations**
In the muscle interstitium ATP can act directly on the smooth muscle cells, causing vasoconstriction; thus an elevated level of ATP in the interstitium is indicative of an elevated signaling for vasoconstriction. The combined mean level of muscle dialysate ATP and ADP in the hypertensive subjects before training was ≈450 nmol/L, which is higher than the resting levels of ATP and ADP (≈200 nmol/L) that we have observed in healthy, young subjects. Before training, acute exercise enhanced the dialysate ATP and ADP levels about 20-fold and 9-fold, respectively, whereas after training the increase in ATP was markedly reduced, and no significant increase in ADP was observed. Interstitial ATP has been shown to stimulate interstitial norepinephrine concentrations in rats, leading to increased vasoconstriction. Thus, the observed reduction in interstitial ATP and ADP after training in individuals with essential hypertension may have reduced the level of local vasoconstrictors.

![Figure 2. A, Concentration of nitrate/nitrite (NOx) in muscle dialysate during an acute exercise bout performed before and after training (n=7). B, Protein levels of endothelial nitric oxide synthase (eNOS) and cystathionine-γ-lyase before and after training (n=8) and compared with control (n=10). W indicates Watt; Wmax, maximal workload. *P<0.05 vs control.](http://hyper.ahajournals.org/)

![Figure 3. Concentrations of ATP (A), ADP (B), and adenosine (C) in muscle dialysate during an acute exercise bout before and after 16 weeks of training (n=7). W indicates Watt; Wmax, maximal workload. §P<0.05 before vs after training, *P<0.05 vs rest, †P<0.05 vs recovery.](http://hyper.ahajournals.org/)
at the level of ATP formation and not by altered expression of nucleotide receptors in skeletal muscle.

Dialysate NOx and eNOS Protein

In this study, the eNOS protein levels in muscle were found to be unaltered by training. However, improved NO availability could occur without an enhancement in eNOS protein levels. Important findings have been made regarding alterations in NO function by factors such as eNOS uncoupling, eNOS phosphorylation, and circulating levels of the endogenous NO synthase inhibitor ADMA.2,16,29 Nevertheless, as the measurements of NOx levels at rest and during exercise were similar before and after training, an improvement in eNOS function with training could not be supported. In comparing the basal eNOS protein level with normotensive controls, we found that the eNOS level of the hypertensive subjects was 40% lower than in the normotensive subjects. This finding is in contrast to an observation in arteries of aged men showing that aging was associated with similar or even higher levels of arterial eNOS protein.30 This discrepancy indicates that the lower eNOS protein content observed herein is specific for borderline essential hypertension and that with time and severity of hypertension, eNOS protein levels will be upregulated to compensate for the poor vascular function.30 Alternatively, essential hypertension may be associated with an impaired ability to upregulate the expression of eNOS protein, as indicated by the lack of effect of training on eNOS protein levels.

CSE

The protein content of CSE was found to be higher in the hypertensive subjects compared with the normotensive subjects. As CSE knockout mice display reductions in H2S levels and elevations in blood pressure,21 a lower enzymatic content associated with hypertension could have been expected. However, the higher CSE level may reflect a compensatory adaptation to the lower eNOS also observed, as an increased level of H2S potentially could counteract part of the deleterious effect of lowered levels of NO on vascular resistance. Accordingly, formation of NO from eNOS is predominantly dependent on L-arginine, and a lower level of eNOS does suggest a higher level of this substrate. L-Arginine has been shown to upregulate CSE expression and H2S synthesis,31 indicating that the higher content of CSE observed in the current study may be a direct result of alterations in the NO system. The lack of training effect on CSE suggest that this system is not responsible for the training-induced reduction in blood pressure.

Methodological Considerations

The levels of proteins of relevance for vascular function were determined in skeletal muscle biopsies obtained from the thigh muscle. Most of the proteins measured in our study are known to be endothelial cell specific,17,22,24,28,32 and it is likely that the protein levels assessed in the muscle primarily reflected levels in microvascular endothelial cells, but with a potential contribution from other cells associated with the interstitium, including skeletal muscle cells. As our intention was to determine protein levels of vasoactive systems in cells that can contribute to the muscle interstitial levels of vasoactive compounds, determination in muscle biopsies seemed to be the most appropriate.

Perspectives

This study provides the first direct proof that exercise training alters the level of prostacyclin, thromboxane, and ATP in the skeletal muscle interstitium, whereas several other systems, such as the NO system and CYP2C9 and CYP4A, appear to be unaffected (for overview, see Supplemental Figure II). On the basis of these findings, we propose that prostanoids and ATP were primary contributors to the observed training-induced lowering of blood pressure in hypertensive individuals. The finding also indicates that prostanooids and nucleotides may be important players in essential hypertension, as a primary cause of the development of the disease is physical inactivity. Moreover, the finding that the eNOS levels were markedly reduced and CSE levels higher in the hypertensive subjects, compared with the normotensive subjects, suggests that increased CSE may be a compensatory mechanism during conditions of impaired vascular function. Future studies addressing the cystathionine system, including measurements of H2S, in cardiovascular disease will be important to provide further information regarding the specific role of this system in the regulation of vascular tone.

Acknowledgments

Karina Olsen and Dorte Jessing Agerby Hanskov are gratefully acknowledged for technical assistance.

Sources of Funding

This study was funded by the Danish Council for Independent Research, Medical Sciences, and the Lundbeck Foundation.

Disclosures

None.

References


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Hypertension. 2011;58:943-949; originally published online September 6, 2011;
doi: 10.1161/HYPERTENSIONAHA.111.176529
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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http://hyper.ahajournals.org/content/58/5/943

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EXERCISE TRAINING ALTERS THE BALANCE BETWEEN VASOACTIVE COMPOUNDS IN SKELETAL MUSCLE OF INDIVIDUALS WITH ESSENTIAL HYPERTENSION

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**Short title:** Training and vasoactive systems in hypertension

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Supplemental methods

Antibodies and Western Blot procedure

Protein levels were determined on freeze-dried skeletal muscle samples by western blot procedure as previously described\(^1\). Antibodies used were: PGI\(_2\) synthase (sc-20933, Santa Cruz Biotechnology, CA), anti-eNOS/NOS Type III(BD Transduction Laboratories, US), cytochrome P450 2C9 (458209, Gentest, BD, USA), P2Y2 and P2X1; (APR-010 and AAPR-001, Alomone Laboratories, Israel), TXA\(_2\) synthase (ab39362), Cystathionine-γ-lyase (ab5457), cytochrome P450 4A (ab48566), cyclooxygenase 1 (ab53766) and cyclooxygenase 2 (ab52237), all from Abcam, Cambridge, MA). Secondary goat-anti-rabbit or -mouse HRP-conjugated antibodies (P-0448 and P-0447; DakoCytomation, Denmark) and standard Western-blotting procedures were used throughout the experiments. Following detection (Kodak Image Station, 2000MM) and quantification (Kodak Molecular Imaging software), the protein content was expressed in arbitrary units using total protein measured by MemCode (Pierce, US) staining as internal standard.

Supplemental references

Supplemental figures

Figure S1. Protein levels of the purinergic receptors P2Y2 and P2X1 before and after 16 weeks of training (n=7).
Figure S2. Physical training in individuals with essential hypertension leads to a shift in the level of vasodilators to vasoconstrictors in the human muscle interstitium as evidenced by an alteration in the level of prostacyclin (PGI$_2$), thromboxane A$_2$ (TXA$_2$) and ATP. In contrast, several other vasoactive systems do not appear to respond to training. We propose that prostanoids and ATP are important for the training induced lowering of blood pressure. Abbreviations: COX, Cyclooxygenase; CYP4A, Cytochrome P-450 4A; eNOS, endothelial nitric oxide; nNOS, neuronal nitric oxide; NO, nitric oxide; CES, cystathionine-\(\gamma\)-lyase; H$_2$S, dihydrogen sulphide; HETEs, hydroxyeicosatetraenoic acid; K$^+$, potassium; P2X and P2Y, purinergic receptors; TP, thromboxane receptor; IP, prostacyclin receptor.