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

Abstract—The effects of physical training on the formation of vasodilating and vasoconstricting compounds, as well as on related proteins important for vascular function, were examined in skeletal muscle of individuals with essential hypertension (n=10). Muscle microdialysis samples were obtained from subjects with hypertension before and after 16 weeks of physical training. Muscle dialysates were analyzed for thromboxane A2, prostacyclin, nucleotides, and nitrite/nitrate. Protein levels of thromboxane synthase, prostacyclin synthase, cyclooxygenase 1 and 2, endothelial nitric oxide synthase (eNOS), cystathionine-γ-lyase, cytochrome P450 4A and 2C9, and the purinergic receptors P2X1 and P2Y2 were determined in skeletal muscle. The protein levels were compared with those of normotensive control subjects (n=12). Resting muscle dialysate thromboxane A2 and prostacyclin concentrations were lower (P<0.05) after training compared with before training. Before training, dialysate thromboxane A2 decreased with acute exercise, whereas after training, no changes were found. Before training, dialysate prostacyclin levels did not increase with acute exercise, whereas after training there was an 82% (P<0.05) increase from rest to exercise. The exercise-induced increase in ATP and ADP was markedly reduced after training (P<0.05). The amount of eNOS protein in the hypertensive subjects was 40% lower (P<0.05) than in the normotensive control subjects, whereas cystathionine-γ-lyase levels were 25% higher (P<0.05), potentially compensating for the lower eNOS level. We conclude that exercise training alters the balance between vasodilating and vasoconstricting compounds as evidenced by a decrease in the level of thromboxane, reduction in the exercise-induced increase in ATP and a greater exercise-induced increase in prostacyclin. (Hypertension. 2011; 58:00-00.)

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

Essential hypertension is associated with impaired vascular function, as evidenced by a reduced vasodilatory response to flow induced vasodilation and acetylcholine.1–3 Training interventions have, furthermore, proven successful in lowering blood pressure in individuals with essential hypertension,4,5 and several studies have shown improved vascular function in skeletal muscle after training in hypertensive individuals.4,5,6 Thus, the general beneficial effects of training on vascular function in this patient group are well documented. A main focus in the literature has been on the role of the endothelial nitric oxide synthase (eNOS) system for vascular function in essential hypertension,1–3 and studies have shown an improved eNOS function with training that could contribute to a reduced vascular tone.5,6 Nonetheless, the effect of hypertension and training on eNOS protein or NO formation in skeletal muscle of individuals with essential hypertension has not been clarified.

The regulation of vascular tone in skeletal muscle is, however, not only dependent on NO but also on several other vasodilating and vasoconstricting compounds, of which many show interactions with NO.7–10 Studies on the effect of training and hypertension on these vasoactive systems are, however, lacking in the literature, and such data provide an important addition to the existing literature.

Several metabolites in the arachidonic acid pathway, eg, thromboxane A2 (TXA2), prostacyclin (PGI2), 11,12-eicosatrienocic acid, and 20-hydoxyeicosatetraenoic acid, have been shown to be of importance for the regulation of vascular tone.11–13 TXA2 and PGI2 are endothelium derived and are produced by the catalytic activity of cyclooxygenase, thromboxane synthase, and prostacyclin synthase.13 20-Hydroxyeicosatetraenoic acid and 11,12-eicosatrienocic acids are formed through hydrolysis of arachidonic acid by the 2 cytochrome P450 isoforms 4A (CYP4A) and 2C9 (CYP2C9).12 The metabolites TXA2 and 20-hydroxyeicosatetraenoic acid are vasoconstrictors, whereas PGI2 and 11,12-eicosatrienocic acids are vasodilators. It has been proposed that 1 of the causes of impaired vascular function in cardio-
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 intracellular 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 (H$_2$S). In endothelial and smooth muscle cells, H$_2$S is produced from cysteine, catalyzed by cystathionine-$\gamma$-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 H$_2$S 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 vasoconstrictor systems. To test this hypothesis, several vasodilator and vasoconstrictor systems were determined in skeletal muscle of individuals with essential hypertension before and after training. Protein levels were also compared with those of normotensive individuals.

**Methods**

**Subjects**

The participants included 10 subjects (6 males, 4 females; age 45±2 years; height 173.7±2.3 cm; body mass index 28.1±1.4; systolic blood pressure 152.1±3.9 mm Hg; diastolic blood pressure 99.7±3.3 mm Hg; mean arterial pressure 117.2±3.4 mm Hg; physical activity index 1.54±0.3) diagnosed with moderate essential hypertension by their physician. In addition, 10 normotensive subjects (5 male, 5 female; age 42.8±2 years; height 174.2±3 cm; body mass index 23.9±0.9; systolic blood pressure 123.8±2.4 mm Hg; diastolic blood pressure 82.9±1.8 mm Hg; mean arterial pressure 95.6±1.6 mm Hg; physical activity index 1.5±0.5) participated in the present study. Physical activity index was determined based on questionnaires regarding weekly habits according to the following 6 categories: 0, sedentary; I, active maximum once per week; II, active 1 to 2 times per week; III, 2 to 3 times per week; IV, 3 to 4 times per week; V, more than 5 times per week. Blood pressure was determined with a DINAMAP PRO (Wipro GE Healthcare) during 3 visits to the laboratory. Mild to moderate essential hypertension was defined as >140 mm Hg systolic pressure or >90 mm Hg diastolic pressure. The criterion for the normotensive control subjects was a blood pressure below 130/85 mm Hg. The subjects did not receive medical treatment, and all subjects were nonsmokers and were not diagnosed with chronic diseases. All participants were informed of the experimental procedure and the potential risks, and were told they could withdraw from the experiment at any time. The subjects gave their informed consent to participate before the start of the experiment. All procedures used conformed to the Declaration of Helsinki, and the study was approved by the Ethical Committee of Copenhagen/Frederiksberg, Denmark.

**Experimental Design**

**Hypertensive Individuals**

The full experimental period for the hypertensive subjects was approximately 17 weeks, of which 16 weeks consisted of a training period. Before and after the training period, the subjects performed a microdialysis experiment.

**Normotensive Control Individuals**

Blood pressure, body weight, and height were determined. After 60 minutes of rest, a muscle biopsy was obtained from the m. vastus lateralis.

**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 (VO$_{2_{max}}$) 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 kicking frequency of 60 revolutions per min could no longer be maintained. The highest workload that each subject could maintain for 1 minute was set as maximal workload (WL$_{max}$). VO$_{2_{max}}$ was estimated by the Astrand 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 Astrand-Ryhming nomogram was used to estimate VO$_{2_{max}}$.

**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% WL$_{max}$). 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 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 VO$_{2_{max}}$) work on a cycle ergometer. The warm-up was followed by a 50-minute period consisting of moderate (60% of VO$_{2_{max}}$)-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 analyzed with high-performance liquid chromatography without previous treatment of the samples, as previously described.

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. 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 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 t 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 7±2 mm Hg lower (P<0.01) after training than before. WLa(max) was unaltered, but VO₂b(max) was higher by 17% (P<0.01), and body mass index was 3% lower (P<0.05) after training.

Dialysate TXB₂ and 6-Keto-PGF₁α
The resting concentration of TXB₂ in the muscle dialysate was ~50% lower (P<0.05) after compared with before training. Before training, the concentration of TXB₂ was markedly reduced (P<0.05) with acute exercise, whereas after training, the concentration of TXB₂ remained unaltered with acute exercise (Figure 1).

The resting concentration of 6-keto-PGF₁α in the muscle dialysate was ~50% lower (P<0.05) after training compared with before training. Before training, acute exercise did not alter the 6-keto-PGF₁α dialysate concentration, whereas after training, the concentration increased (P<0.05) with acute exercise (Figure 1).

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).

The dialysate concentration of potassium before training was 3.1±0.4 mmol/L at rest, and the level increased (P<0.05) to 5.1±0.3 mmol/L 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.

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.

Compared with the normotensive subjects, the hypertensive subjects had a ~25% lower (P<0.05) protein content of CSE, but levels were unaltered by training (Figure 2). The muscle protein levels of TXA₂ synthase, PGI₂ synthase, cyclooxygenases 1 and 2, CYP2C9, and CYP4A (Figure 1) and the purinergic receptors P2Y1 and P2Y2 (n=8, Supplemental Figure 1) in the hypertensive individuals were not changed by training. The levels were all similar to those of normotensive 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.
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 TXB$_2$ in the muscle dialysate, which could be a contributing factor to the lowering of resting blood pressure. Moreover, before training, the dialysate levels of TXB$_2$ decreased almost 3-fold from rest, which is in accordance with findings in young, untrained subjects, where an approximate 1.5-fold decrease in TXB$_2$ was observed during bicycle exercise. After training, when resting TXB$_2$ levels were lower, the levels remained unaltered with exercise. Of note is that the protein level of TXA$_2$ 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 TXB$_2$. Instead, TXB$_2$ formation may be regulated by substrate levels or the degree of enzyme activation.

The resting dialysate concentration of PGI$_2$ was lower after training compared with before, but whereas before training, the PGI$_2$ concentration was unaffected by exercise, after training, there was an exercise-induced increase in PGI$_2$ concentration. An increase in muscle dialysate PGI$_2$ concentration with acute exercise, as was observed after training, is in accordance with previous studies, in which muscle dialysate PGI$_2$ levels have been determined during moderate intensity exercise in healthy, young subjects. These data suggest that the activation of PGI$_2$ 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 PGI$_2$ synthesis during exercise, the actual vasodilatory response to PGI$_2$ may have been reduced, in part because of its dependence on an impaired NO system, as previously observed in aging.
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-eicosatrienonic 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. ATP induces its effect by binding to purinergic receptors, where, in skeletal muscle, P2X1 and P2Y2 have been found to be present; however, both P2X1 and P2Y2 remained unaltered with training, and the levels were similar in normotensive and hypertensive individuals. Combined, these findings therefore suggest that the role of exercise training on the nucleotide system may be
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. 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. This discrepancy suggests 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. Alternatively, essential hypertension may be associated with an impaired ability to upregulate the expression of eNOS protein, as indicted 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, 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 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, 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, 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 prostanoids 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. published online September 6, 2011;
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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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-\(\gamma\)-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

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.