

## Gene-Pharmacological Effects on Exercise-Induced Muscle Gene Expression in Healthy Men

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### Abstract

Angiotensin 2 is a major vasoconstrictor and subject to complex regulation. It is produced by the angiotensin-converting enzyme (ACE), which is itself regulated by blood flow and represents a target for pharmacological (lisinopril) and genetical (ACE-I/D polymorphism) influences. 32 healthy Caucasian men of British descent, including six subjects that were treated with lisinopril, completed standardized one-legged cycling-type endurance exercise to assess ACE-I/D and lisinopril dependent levels alterations of ACE and hypoxia-modulated transcripts in knee extensor muscle. Lisinopril treatment revealed significantly decreased transcript expression at baseline (COX411 -88%; COX412 -67%; HIF-1 $\alpha$  -93%) and 3 hours into recovery (HIF-1 $\alpha$  -78%; ACE -62%). Furthermore, during recovery from cycling-type endurance exercise, COX412 mRNA expression was increased by 281% in ACE-II genotypes ( $p < 0.05$ ) but not in subjects carrying the ACE D-allele. Additionally, ACE-DD genotypes showed a trend for superior expression of ACE mRNA ( $p = 0.07$ ), whereas for carriers of the ACE I-allele a decrease (ACE-ID -35%; ACE-II -67%) was observed. Changes in COX412 and ACE transcript expression were correlated to HIF-1 $\alpha$  protein levels prior to exercise, which was highest in ACE-DD genotypes. The interpretative hypothesis is suggested that exercise-induced COX412 transcript expression is sensitized in dependence of the I-allele, which silences ACE expression. The findings implicate a pronounced influence of anti-hypertensive treatment on muscle gene expression, which is modified by the ACE-I/D genotype. The observed effects of lisinopril and the ACE I-allele suggests a possible role of shear stress and tissue oxygenation on the expression of oxygen-associated transcripts during recovery from the sympatholytic challenge of endurance exercise.

### Keywords:

Exercise; Muscle; Genotype; Vasoconstriction; Mitochondria; Plasticity; Medication

### Introduction

The renin-angiotensin system (RAS) represents an important regulator of blood pressure, fluid volume and vascular response to injury and inflammation whose inappropriate activation results in hypertension, fluid retention and thrombotic/atherogenic effects [1]. The octapeptide angiotensin 2 which is produced through angiotensin converting enzyme (ACE) mediated breakdown of angiotensin 1 is involved in most of the effects of RAS on target tissues. Angiotensin 2 mainly binds on G protein-coupled receptors type 1 (AT1R) resulting in vasoconstriction, whereas the less abundantly binding of angiotensin 2 on AT2R mediates opposing effects such as NO-release and vasodilation. Relating to the cardiovascular system, AT2R expresses in different compartments and the adjustable degree of expression is thought to partly regulate the vasodilatory response [2].

Dysfunction of RAS can be counteracted by blocking the system with angiotensin-converting enzyme inhibitors (ACEIs) or AT2R blockers, resulting in normalization of vascularization and blood pressure. The ACE inhibitor lisinopril (N2-[(S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline; also known as Zestril or Prinivil) acts via an irreversible binding to the active site of ACE thereby preventing proteolytic conversion of angiotensin 1 to the vasopressor angiotensin 2 and degradation of the vasodilator peptide bradykinin. Through this action, treatment with lisinopril is part of a first-line therapy for patients suffer from hypertension, heart failure, myocardial infarction and diabetic nephropathy [3].

Besides a pharmacological regulation, ACE is also affected by expressional regulation through the native ACE insertion (I)/ deletion

(D) polymorphism, first published by Rigat et al. [4]. Subjects with the ACE-I polymorphism carry a 287-bp long insertion (I-allele) within intron 16, which lowers expression and in consequence the activity of the encoded ACE enzyme [4-6]. This does result in changes of blood pressure through the regulation of vasodilatation [7], which represents a potentially important factor for muscle's response to metabolic forms of stress, especially during exercise [8]. Interestingly, during endurance exercise RAS is stimulated [9]. It was therefore suggested that several processes downstream of ACE activity contribute to elevated endurance performance and trainability in subjects carrying the ACE I-allele [10,11]. In this context, we recently identified an ACE I-allele dependent up-regulation of muscle transcripts associated with glucose and lipid metabolism during recovery from endurance exercise [12]. Additionally subjects carrying at least one I-allele, were found to show reduced capillarity at rest, but amplified mitochondrial biogenesis after cycling-type endurance exercise. More recently, we have shown ACE-I/D dependent responses of serum levels of angiotensin 2 and capillary perfusion to intense cycling exercise [8]. These results suggest a critical role of ACE-modulated vascular tone, which is important for the regulation of response to exercise [13,12].

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The literature documents a pronounced influence of the ACE I-allele on muscle capillarity, as well as on their perfusion concomitant with endurance exercise. We therefore wished to assess the interaction of genetical and pharmacological inhibition of ACE on muscle transcript expression post-exercise. We specifically hypothesized that hypoxia-sensitive transcript expression of isoform 1 and 2 of subunit 4 of cytochrome c oxidase, COX4I1 and COX4I2, and HIF-1 $\alpha$  transcript and protein [14-16], would show ACE-dependent level alterations in knee extensor muscle during recovery from cycle-type endurance exercise as this lowers muscle oxygenation in relation to capillary perfusion [17].

## Materials and Methods

### Experimental design

32 healthy, non-specifically trained Caucasian men of British descent participated in the study (Table 1). All subjects gave written informed consent to the contribution to the study. The protocols, used in the present investigation were approved by the Ethics Committee of the Manchester Metropolitan University and are in line with the Declaration of Helsinki. All subjects were instructed to avoid strenuous exercise for 72 h before the testing procedures.

During the first visit and one week prior to a single bout of cycling-type exercise, subjects performed an incremental cycling exercise to determine aerobic capacity and peak power output (PPO). Additionally, for subsequent genotyping analysis buccal mucosa was collected. In order to assess the interaction of pharmacological ACE inhibition, eight subjects were randomly assigned to an inhibitor group (n=6) that consumed 10 mg · d<sup>-1</sup> of ACE inhibitor lisinopril (Zestril, AstraZeneca, London, UK) for three consecutive days in the morning, including the day of the single bout of cycling-type exercise. During the last visit, subjects completed a body weight-matched one-legged cycling-type endurance exercise to voluntary exhaustion. For further analysis of transcript expression, biopsy samples of the vastus lateralis were collected prior, and 3 hours post-exercise. In subjects that did not consume Lisinopril an additional biopsy was collected 30 minutes post-exercise.

### Endurance-exercise test

Maximal pulmonary oxygen uptake (VO<sub>2max</sub>) and maximal performance (PPO) were determined during an incremental ramp test to volitional fatigue on a cycle ergometer (Excalibur Sport, Lode, Groningen, the Netherlands). All subjects were familiar with the testing procedures. The test protocol started at 40 W, thereafter the power was increased by 30 W every 2 min. Exhaustion was defined as when the subject could not maintain a cadence of >60 rpm. During the whole

test, breath-by-breath respiratory data were collected using a calibrated spirometry system (JAEGER Oxycon alpha, CareFusion, Höchberg, Germany) and heart rate was monitored using a heart rate chest belt (Accurex Plus, Polar Electro Oy, Kempele, Finland).

### One-legged exercise test

In order to reduce the effects of ACE activity on systemic perfusion that might have an influence on exercise performance and to maximize the metabolic stimulus within recruited muscle groups, all subjects performed a one-legged endurance exercise test on a cycle ergometer (Excalibur Sport, Lode) while the non-exercising leg rested on a fixed chair. The protocol started with a 3-minute warming up at 50 W, followed by 25 min at an intensity corresponding to 1.2-times body weight (kg) in Watts and ended with an incremental increase of performance (10 W · min<sup>-1</sup>) until voluntary exhaustion. Exhaustion was defined as when the cadence dipped below 60 rpm.

### Biopsy sampling

Baseline samples were obtained from the midportion of the vastus lateralis of the non-exercising leg between the spina iliaca anterior superior and the lateral part of the patella immediately prior to exercise. Biopsies were gathered 2.5 cm below the fascia using the percutaneous needle biopsy technique [18]. Samples were then freed from visible blood, mounted in embedding medium (Tissue-Tek O. C. T. Compound, Sakura Finetek, VWR International, Leicestershire, UK), frozen in liquid nitrogen-cooled isopentane and stored at -80°C until further analysis. 30 minutes or 3 hours post-exercise, biopsies were collected from the exercised leg according to the same protocol.

### Genotyping

Genomic DNA was isolated from buccal mucosa cells using the DNeasy Blood & Tissue Kit (Cat. No 69504, Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. ACE I/D genotyping was performed using PCR with subsequent high-resolution melt (HRM) analysis. The primers used for the detection of the 66 bp amplicon specific for the presence of the I-allele were: ACE1 (forward, 5'-TGGGATTACAGGCGTGATACAG-3') and ACE1/2 (reverse, 5'-AATTTTCAGAGCTGGAATAAAAATT-3'). ACE2 (forward, 5'-CATCCTTCTCCCATTCTC-3') and ACE1/2 primers were used to detect the 84 bp amplicon specific to the presence of the D-allele (for details see GenBank No X62855.1). PCR reactions were run in duplicate with a mix of the three primers using a KAPA HRM FAST PCR Kit (Ref# KK4201, LABGENE Scientific, Châtel-St-Denis, Switzerland) according to the manufacturer's protocol on an Eco Real-Time PCR system (LABGENE Scientific). Reaction specificity was validated by DNA gel electrophoresis followed by band isolation and DNA sequencing (Microsynth, Balgach, Switzerland). The presence of the I/D specific amplicons was then identified by HRM analysis (Tm I-allele = 73.5°C; Tm D-allele = 75.5°C).

### Transcript expression

Total RNA was isolated from 25  $\mu$ m cryosections of the muscle biopsies using the RNeasy Mini Kit (Cat. No 74104, Qiagen) following manufacturer's instructions, quantified, and 600 ng were reverse transcribed using the Omniscript RT Kit (Cat. No 205111, Qiagen) that uses random hexamers (Cat. No 79236, Qiagen). RT-qPCR was performed for cytochrome c oxidase subunit IV isoform 1 (COX4I1), cytochrome c oxidase subunit IV isoform 2 (COX4I2), hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), Angiotensin I-converting enzyme (ACE), and 28S rRNA. Volumes corresponding to 600 ng cDNA (60 ng

Variable	I-allele (n=19)	No I-allele (n=13)	Inhibitor (n=8)	No inhibitor (n=24)
age [y]	30.0 ± 1.8	26.1 ± 1.9	34.1 ± 3.5	26.8 ± 1.3*
weight [kg]	79.4 ± 2.7	73.2 ± 2.8	76.8 ± 5.0	76.9 ± 2.2
height [cm]	180.8 ± 1.6	179.2 ± 2.5	176.5 ± 3.8	181.4 ± 1.3
BMI [kg · m <sup>-2</sup> ]	24.3 ± 0.8	22.7 ± 0.7	24.5 ± 1.3	23.4 ± 0.6
PPO [W]	192.1 ± 9.8	190.8 ± 11.8	177.5 ± 10.0	196.5 ± 9.3
relative VO <sub>2max</sub> [ml · min <sup>-1</sup> · kg <sup>-1</sup> ]	45.4 ± 2.1	44.9 ± 1.7	45.4 ± 4.1	43.8 ± 2.2

Additionally subject parameters between the inhibitor and no inhibitor group are given. Data represented as mean ± SE. \*p < 0.05 vs. lisinopril intake.

**Table 1:** Anthropometrical and physiological characteristics between subjects split ACE-I/D genotypes and ACE inhibitor intake.

for 28S rRNA) were subjected to RT-qPCR with specific primers and reaction was run in duplicate using the KAPA SYBR FAST Universal Kit (Ref# KK4601, LABGENE Scientific) according to manufacturer's instructions. Relative transcript amounts were calculated using the comparative CT method, taking the efficiency of amplification for each template into account. For each sample, transcript signals were standardized to 28S rRNA. The following primers were used: COX4I1 (forward, 5'-GCCATGTTCTTCATCGGTTTC-3'; reverse, 5'-GGCCGTACACATAGTGCTTCTG-3'), COX4I2 (QuantiTect Primer Assay, Cat. No QT00044939, Qiagen), HIF-1 $\alpha$  (forward, 5'-TAGTGAACAGAATGGAATGGAGCAA-3'; reverse, 5'-GAGCCACCAGTGTCCAAAAA-3'), ACE (forward, 5'-TCACTACGGGGCCAGCACA-3'; reverse, 5'-TGCGCCACATGTTCCCCAG-3'), 28S rRNA (forward, 5'-ATATCCGCAGCAGGTCTCCAA-3'; reverse, 5'-GAGCCAATCCTTATCCCGAAG-3').

### HIF-1 $\alpha$ protein expression

Biopsy material corresponding to an estimated volume of 1 mm<sup>3</sup> was sectioned at 20  $\mu$ m, pooled, and homogenized in ice-cold PBS/inhibitor-cocktail [1 ml PBS + 9 ml dH<sub>2</sub>O + 1 complete Mini, EDTA-free tablet (Sigma Aldrich, Buchs, Switzerland)] in a 1.5 ml Eppendorf tube by using a steel pistil (Behrens-Labortechnik, Germany). Protein content was estimated using the Pierce BCA Protein Assay Kit (Thermo SCIENTIFIC, Town, USA). 10  $\mu$ g of total protein homogenate from pre/post sample pairs in Laemmli buffer (Biorad Laboratories AG, Cressier, Switzerland) and 2% mercapto-ethanol were denatured and separated by 7.5% SDS-PAGE using precast gels (Biorad Mini-protean TGX Stain-free). Proteins were blotted onto a nitrocellulose membrane with a Trans-blot-Turbo Transfer System (Biorad), stained with Ponceau S to record protein loading and subjected to immunodetection with specific monoclonal HIF-1 $\alpha$  antibody GR218993 (Sigma-Aldrich Chemie AG, Buchs Switzerland) and horseradish peroxidase-conjugated secondary antibody in 5%-milk/1% BSA -TTBS (20 mM Tris-base, 150 mM NaCl, 0.05% Tween-20, pH 7.6). Signal was recorded with enhanced chemoluminescence (Supersignal West Femto, Thermo Scientific) using a PXi System (Syngene). Pixel values for HIF-1 $\alpha$  protein were related to those of the respective actin band on the Ponceau S-stained membrane.

### Statistics

Physiological and anthropometric data were assessed with a Student's t test (Statistica, StatSoft, Tulsa, USA). A one-way ANOVA with Fisher post-hoc test for standardized transcript values was performed to evaluate the effect of lisinopril intake (yes, no) on transcript expression at baseline and during recovery from cycling-type endurance exercise. The same analysis was performed to determine differences in the exercise response for all subjects combined. Relative changes vs. baseline values were assessed from values that arose from the normalization to mean pre-values for each genotype to reveal mean normalization units (MNE units). Then a one-way ANOVA with Fisher post-hoc test for post vs. pre changes was calculated in order to assess increases/decreases of the exercise response. Furthermore, a MANOVA with post-hoc test of Fisher was carried out for factors ACE-I/D genotype (II, ID, DD) x time (pre, post) and in addition lisinopril intake (yes, no). Data are presented as mean  $\pm$  SE. Significance was declared at  $p < 0.05$ . A  $p$  value between 0.05 and 0.1 was considered to represent a trend. Linear relationships were assessed based on Pearson correlations.

## Results

### Subjects

Subject characteristics split up between genotypes are given in Table 1. Additionally characteristics of the inhibitor and non-inhibitor group are shown. At baseline there were no differences between ACE-II/ ID and ACE-DD genotypes in any assessed variable, except age. The same result was shown for the inhibitor vs. non-inhibitor group.

### Transcript expression with acute exercise

Baseline values of the measured transcripts in vastus lateralis muscle for both the control and inhibitor group are shown in Table 2. The expression of ACE, COX4I1, COX4I2 and HIF-1 $\alpha$  transcripts in vastus lateralis muscle was not affected by one-legged cycling-type exercise over the entire group of subjects (data not shown). Also, for the subjects that did not consume lisinopril post vs. pre changes were not significant for COX4I1 ( $p = 0.7$ ), COX4I2 ( $p = 0.5$ ), HIF-1 $\alpha$  ( $p = 0.6$ ) and the ACE transcript ( $p = 0.4$ ).

### ACE-I/D genotype and muscle transcript expression post-exercise

Expression of gene transcripts in vastus lateralis muscle 3 hours after exercise, but not at rest, depended on the ACE I/D genotype (Table 3). In ACE-II genotypes, but not in subjects carrying the ACE D-allele (i.e. ACE-ID/ACE-DD), COX4I2 was 281% increased after exercise (Figure 1).

For the ACE transcript we found a trend for an increase 3 hours post-exercise in homozygous carriers for the D-allele. In addition, our results revealed a higher increase for the ACE transcript in DD

		control	lisinopril intake
at rest	COX4I1	12.1 $\pm$ 3	1.5 $\pm$ 0.3*
	COX4I2	430.1 $\pm$ 52	141.9 $\pm$ 40.8*
	HIF-1 $\alpha$	8952.8 $\pm$ 2181.6	665.4 $\pm$ 158.5#
	ACE	76.9 $\pm$ 13.1	34.8 $\pm$ 5.4*
post vs. pre	COX4I1	0.9 $\pm$ 0.2	2.0 $\pm$ 0.7*
	COX4I2	1.2 $\pm$ 0.3	1.4 $\pm$ 0.5
	HIF-1 $\alpha$	0.8 $\pm$ 0.3	1.5 $\pm$ 0.4
	ACE	1.3 $\pm$ 0.4	1.0 $\pm$ 0.2

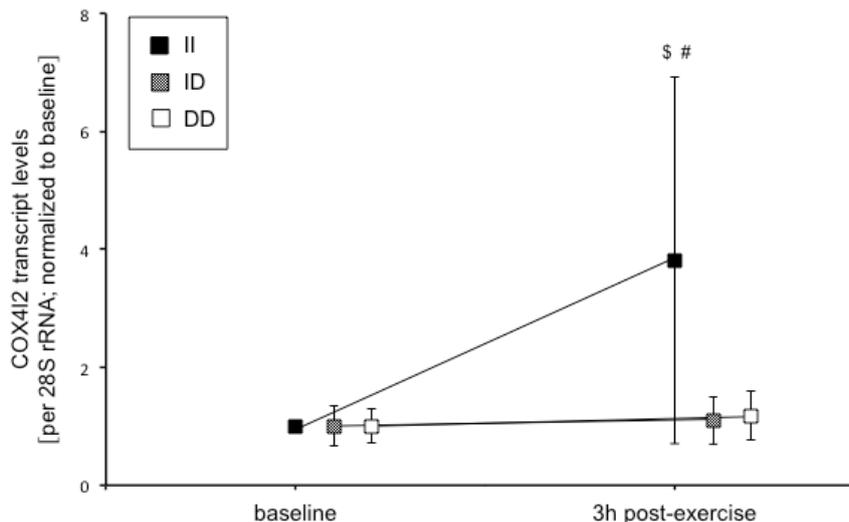
Values represent mean  $\pm$  SE of transcript levels per 28S rRNA (Mean Normalization Units (MNE units)). \* $p < 0.01$ ; # $p \leq 0.05$ ; \* $0.05 \leq p < 0.10$  for fold differences between the lisinopril intake vs. control (no lisinopril intake) group.

**Table 2:** COX4I1, COX4I2, HIF-1 $\alpha$  and ACE transcript expression in vastus lateralis muscle of the control (no lisinopril intake) and inhibitor (lisinopril intake) group at rest and fold changes with exercise.

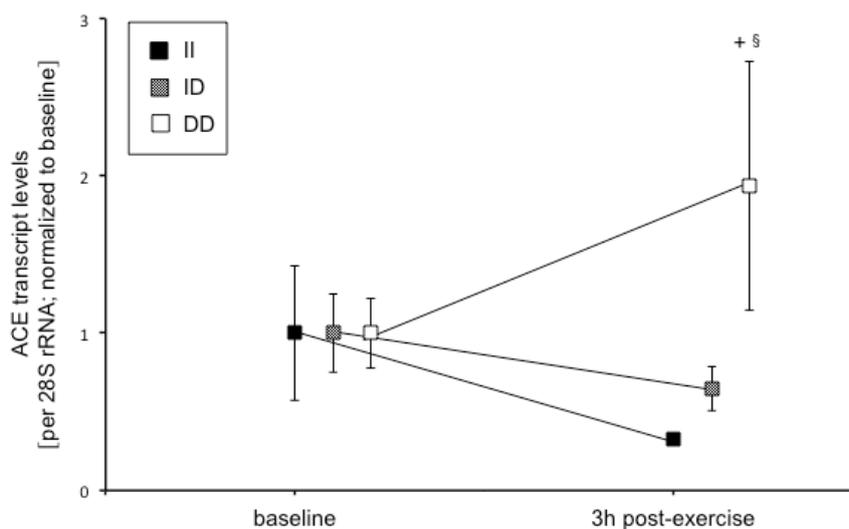
		ACE-II	ACE-ID	ACE-DD
at rest	COX4I1	2.2 $\pm$ 1.1	14 $\pm$ 3.1	9.3 $\pm$ 4.6
	COX4I2	169.6 $\pm$ 62.8*	401.7 $\pm$ 76.5	335.0 $\pm$ 69.1
	HIF-1 $\alpha$	3470.9 $\pm$ 2880.2	5924.2 $\pm$ 2034.4	8222.9 $\pm$ 3433.2
	ACE	70.8 $\pm$ 30.2	73.3 $\pm$ 18.2	53.2 $\pm$ 11.8
post vs. pre	COX4I1	1.0 $\pm$ 0.5	2.2 $\pm$ 0.9	0.6 $\pm$ 0.3*
	COX4I2	3.8 $\pm$ 3.1#	1.1 $\pm$ 0.4	1.2 $\pm$ 0.4
	HIF-1 $\alpha$	0.9 $\pm$ 0.2	1.1 $\pm$ 0.6	0.7 $\pm$ 0.3
	ACE	0.3 $\pm$ 0.0	0.6 $\pm$ 0.1	1.9 $\pm$ 0.8 <sup>##</sup>

Values represent mean  $\pm$  SE of transcript levels per 28S rRNA (MNE units). \* $p < 0.05 \leq p < 0.10$  vs. ID; # $p < 0.05$  vs. baseline; # $p < 0.05$  vs. ID and DD; # $p < 0.05$  vs. ID; \*\* $p < 0.05 \leq p < 0.10$  vs. baseline.

**Table 3:** COX4I1, COX4I2, HIF-1 $\alpha$  and ACE transcript expression in vastus lateralis muscle of the three ACE-I/D genotypes at rest and fold changes with exercise.



**Figure 1:** COX4I2 expression in ACE-I/D genotype after exercise. (Graph represents relative changes of COX4I2 mRNA expression prior and 3 hours post-exercise. Transcript levels  $\pm$  SE are expressed vs. 28S rRNA and normalized to mean pre-values for each genotype (MNE units).  $^{\$}p < 0.05$  vs. ID and DD at the same time-point;  $^{\#}p < 0.05$  vs. baseline).



**Figure 2:** ACE expression in ACE-I/D genotype after exercise. (Graph represents relative changes of ACE mRNA expression prior and 3 hours post-exercise. Transcript levels  $\pm$  SE are expressed vs. 28S rRNA and normalized to mean pre-values for each genotype (MNE units).  $^{\#}p < 0.05 \leq p < 0.10$  vs. baseline;  $^{\$}p < 0.05$  vs. ID at the same time-point).

compared to ID genotypes following exercise (Figure 2). For COX4I1 a trend for reduced transcript expression was found 3 hours after exercise for the ACE-DD genotype, which distinguished to the increased COX4I1 transcript levels in ACE-ID genotypes (Table 3). The HIF-1 $\alpha$  transcript was not affected by the ACE-I/D genotype.

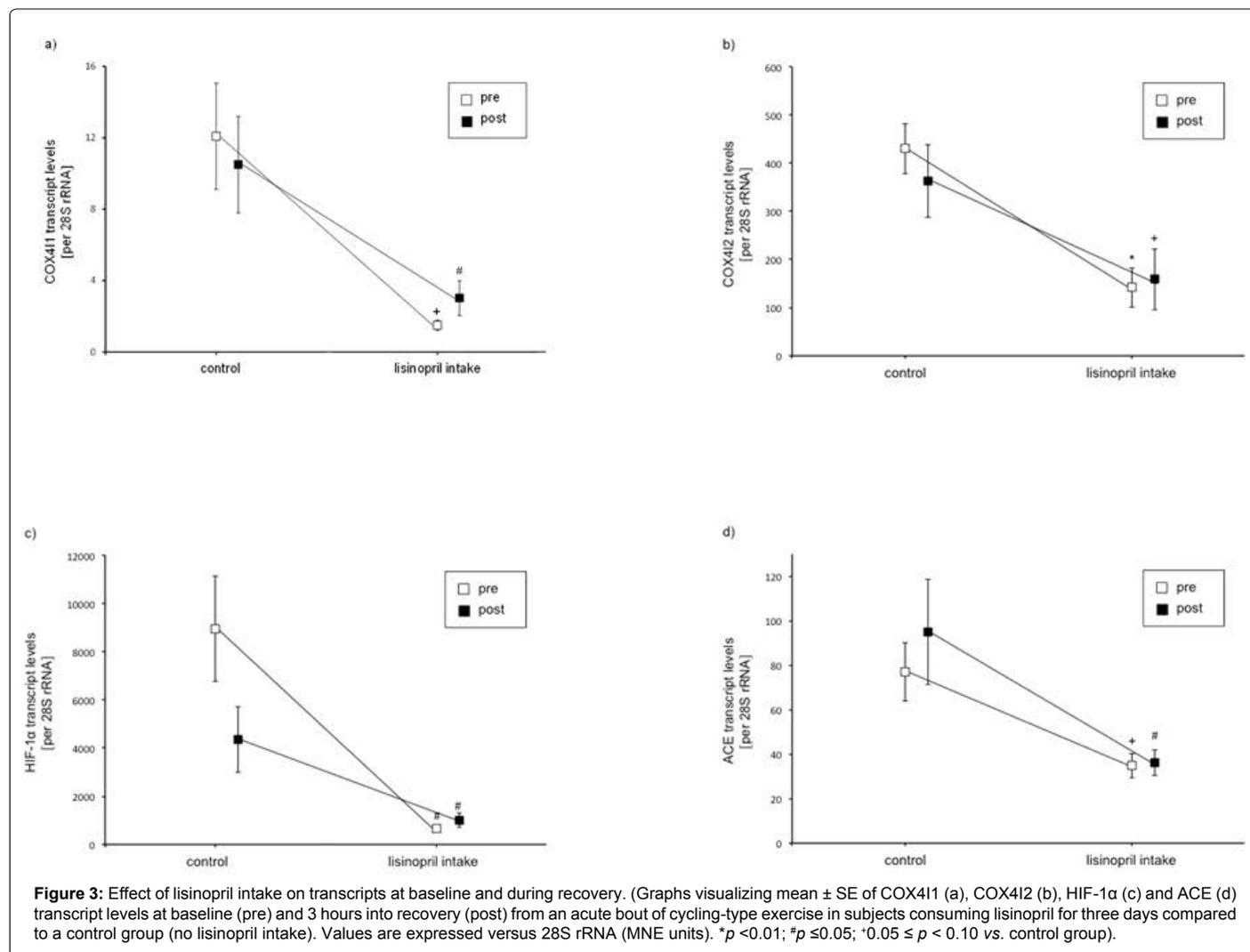
### Effect of lisinopril on muscle transcript expression pre and post-exercise

The effect of ACE inhibitor intake on muscle transcript expression at rest and after exercise is shown in Table 2. Compared to control subjects the three days of lisinopril intake decreased COX4I2 (-67%) and HIF-1 $\alpha$  (-93%) transcript levels in skeletal muscle at rest. Lisinopril treatment tended to decrease ACE transcript levels by 55% and COX4I1 by -88%.

Lisinopril maintained its effect on oxygen-dependent gene transcripts post-exercise. 3 hours after cycling-type endurance exercise the HIF-1 $\alpha$  (-78%), ACE (-62%), COX4I1 (-88%,  $p = 0.04$ ) and COX4I2 (-56%,  $p = 0.07$ ) transcript levels all remained lower in the lisinopril-treated group compared to control subjects (Figures 3a-d).

### Influence of ACE-I/D genotype on muscle transcript expression under lisinopril treatment

The ACE-ID genotype did not demonstrate a general influence on the effect of the ACE inhibitor on transcript levels post-exercise. The  $p$ -values of the ANOVA amounted to 0.96 for ACE, 0.72 for COX4I2, and 0.72 for HIF-1 $\alpha$ . However, lisinopril was only effective in ACE-ID to lower COX4I2 transcript expression in muscle at rest and 3 hours after exercise (Figure 4).



### HIF-1 $\alpha$ protein expression

HIF-1 $\alpha$  protein levels in vastus lateralis muscle depended on the ACE-I/D genotype. At rest protein levels were highest in ACE-DD genotypes (Figure 5). Over all subjects HIF-1 $\alpha$  levels were reduced 30 minutes after the bout of single leg exercise ( $p = 0.03$ ). The decrease reached the level of significance for the ACE-DD genotype. Lisinopril did not affect HIF-1 $\alpha$  protein levels before exercise ( $p = 0.13$ ).

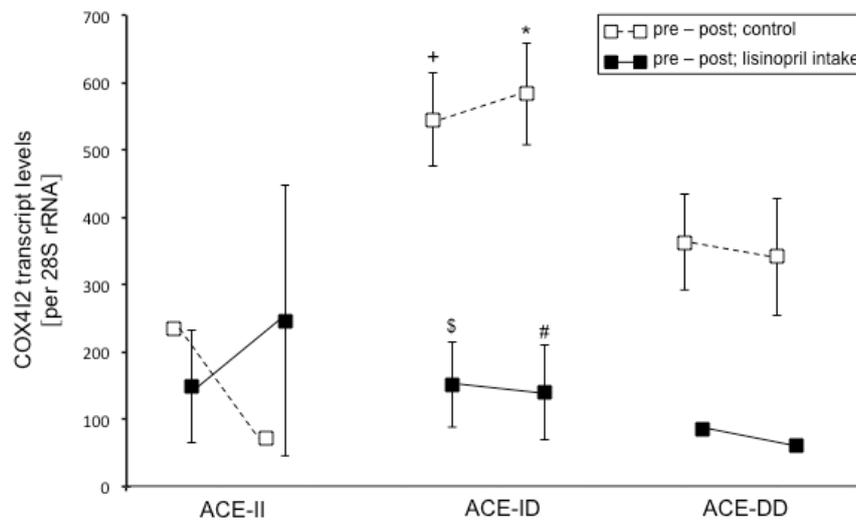
Correlations were identified between transcript level alterations and HIF-1 $\alpha$  protein. Fold changes in COX4I2 and HIF-1 $\alpha$  protein levels prior to exercise were correlated ( $r = 0.81$ ). Fold changes in COX4I1 transcript levels were correlated to HIF-1 $\alpha$  protein levels 30 minutes post-exercise ( $r = 0.62$ ). HIF-1 $\alpha$  protein pre ( $r = -0.75$ ) and 30 min post-exercise ( $r = -0.85$ ) was correlated to fold changes in ACE transcript levels with exercise.

### Discussion

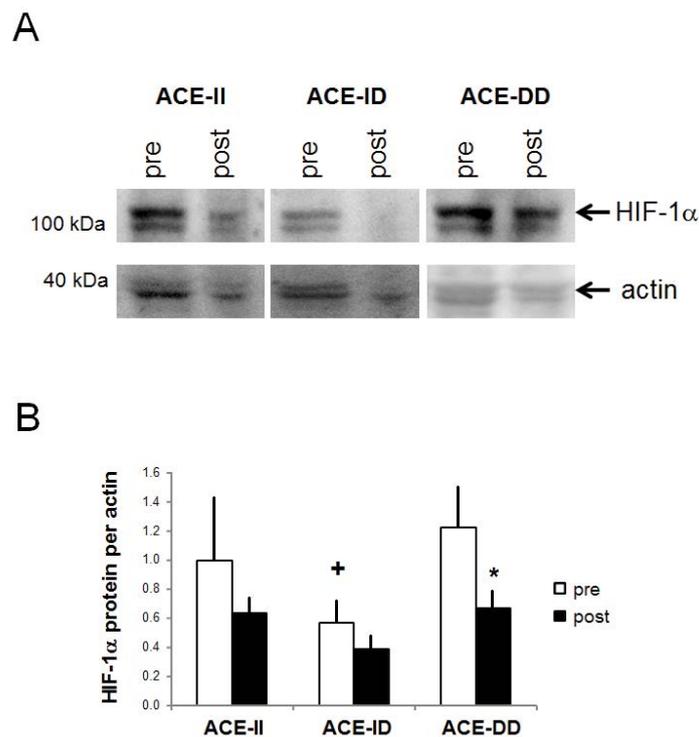
The present study investigated the effects of pharmacological and genetical ACE inhibition on hypoxia-modulated muscle gene transcript expression as well as on ACE transcription itself. Based on published experimentation we assumed that a time point 3 hours after cycle-type exercise would be suitable to detect exercise induced transcript

regulation in the knee extensor muscle, m. vastus lateralis [19]. In order to reduce the influence of central (i.e. cardiac) limitations on muscle performance, which may interact with the systemic effects of ACE activity and perfusion [20] we chose a one-leg endurance intervention to maximize the metabolic stimulus for recruited muscle groups. Under this approach, we find that the ACE-I/D gene polymorphism exerted a distinct influence on expression of the selected gene transcripts in knee extensor muscle 3 hours into recovery from the one-legged endurance stimulus. It thereby appeared that opposing adjustments between the ACE I/D genotype cancelled the expected effect of exercise on muscle levels of the ACE gene transcripts.

Our findings also identified that 3 days of lisinopril intake lowered the expression of all assessed transcripts. Based on the absent interaction effects it thereby appeared that the pharmacological intervention (i.e. lisinopril) exerted a generally larger influence than the ACE-I/D genotype on muscle transcript levels due to an effect on muscle at rest, already (Table 2). Due to its inhibitory effect on the ACE enzyme, lisinopril treatment is expected to increase vasodilatation dependent perfusion [21]. In our current investigation we identified lower levels of HIF-1 $\alpha$ , and COX4I2 expression levels in vastus lateralis muscle with lisinopril consumption, before and 3 hours into recovery from aerobic cycling-type endurance exercise to exhaustion.



**Figure 4:** Interaction effect between ACE-I/D genotype and ACE inhibitor on the exercise response of COX4I2. Graphs visualizing mean  $\pm$  SE of COX4I2 transcript levels for ACE-II/ -ID and -DD genotypes at baseline (pre) and 3 hours into recovery (post) from an acute bout of cycling-type exercise in subjects consuming lisinopril for three days compared to a control group (no lisinopril intake). Values are expressed versus 28S rRNA (MNE units). \* $0.05 \leq p < 0.10$  vs. ACE-DD pre control; \* $p < 0.05$  vs. ACE-II post control; § $p < 0.01$  vs. ACE-ID pre control; # $p < 0.05$  vs. ACE-ID post-control.



**Figure 5:** HIF-1 $\alpha$  protein expression. (A) Representative immunoblots showing the detection of HIF-1 $\alpha$  and actin loading controls in ACE-II, -ID and -DD genotypes (pre) and 30 minutes into recovery (post) from the acute bout of cycling-type exercise. The position of the respective protein is indicated with an arrow. (B) Bar graph visualizing mean  $\pm$  SE of HIF-1 $\alpha$  protein levels for the three genotypes pre and post-exercise. Values represent pixel volumes for HIF-1 $\alpha$  per actin protein normalized to the mean of pre-values in ACE-II genotypes. \* and +, respectively, denote  $p < 0.05$  and  $0.05 \leq p < 0.10$  vs. ACE-DD pre).

In consequence of the vasodilatory effect of lisinopril, we suggest that the down-regulating effect of lisinopril on oxygen-modulated transcripts is related to muscle perfusion and associated alterations in oxygen metabolism. The expression of HIF-1 $\alpha$  and COX4I2 transcripts and HIF-1 $\alpha$  protein is thought to represent markers of tissue hypoxia

[22]. We reported before that their expression in vastus lateralis muscle after endurance type bicycle exercise is markedly affected by ambient hypoxia [16], which is known to produce a further lowering of tissue oxygenation [17]. This finding compares to a higher gene expression for components of mitochondrial respiration and Krebs cycle with the

lowering of levels in metabolically active yeast [23]. Our observation on reduced transcript expression of hypoxia markers under lisinopril consumption is thus consistent with the expectation of an improved muscle oxygenation with vasodilatation.

The notion of a role of muscle oxygenation for explaining ACE-related variability in transcript expression was supported by the higher protein levels of the hypoxia-sensitive transcription factor HIF-1 $\alpha$  in ACE-DD genotypes before exercise, which is expected to show increased vasoconstriction [7,24]. In support of hypoxia-related transcript alterations post-exercise we identified a correlation between HIF-1 $\alpha$  protein levels with transcript expression of the hypoxia marker COX4I2. Lisinopril did, however, not affect HIF-1 $\alpha$  protein levels prior to exercise. Due to preset limitations in biopsy collection no samples were available for assessing the lisinopril's effect 30 minutes after exercise. Intriguingly, HIF-1 $\alpha$  protein was decreased 30 minutes after the one-legged exercise but when it has been shown to increase after 45 minutes of one-legged knee-extension exercise [25]. We do not know how to explain the difference. Possibly differences in exercise-induced hyperemia are involved. Our results therefore suggest that the relationship between tissue perfusion and oxygenation require a further detailed analysis to disentangle their relationship to the observed lisinopril and ACE-I/D genotype modulated expression of respiratory factors during recovery from aerobic exercise.

ACE inhibitor dependent down-regulation was also observed for the ACE transcript itself. ACE expression in skeletal muscle is thought to primarily localize to the endothelium of blood vessels [26]. ACE gene expression and activity *in vitro* and as well as *in vivo* in the rat aorta has been shown to down-regulated by shear stress, and this may involve nitric oxide synthase activity [27,28]. Additionally the authors provided evidence that two mechano-sensitive, alternative cis-acting elements (Barbie and GAGA-boxes) play a key role in mediating ACE down-regulation by shear stress [29]. It remains to be elucidated whether shear stress exerts similar effects on ACE transcription in the complex human-biological system. Because pharmacological treatment with lisinopril is expected to increase shear stress of the capillary endothelium due to vasodilatation, mechanical stress to the capillary endothelium is a possibly important factor in the observed down-regulation of ACE transcription levels during recovery from exercise in the inhibitor group. This contention is supported by our observation that changes in ACE transcript levels 3 hours after exercise differed according to the ACE-I/D genotype. Our results revealed lower ACE transcript levels in ACE-ID genotypes compared to ACE-DD. As well our data showed that exercise elevated expression of the hypoxia sensitive COX4I2 gene transcript in homozygous carriers of the I-allele compared to subjects carrying the D-allele. Interestingly, exercise-induced hypotension due to elevated muscle perfusion appears to be confined to carriers of the ACE I-allele [30], thus further supporting a role of capillary perfusion and associated increases in shear stress in the regulation of ACE transcription expression in skeletal muscle.

## Conclusion

Besides a regulation of the ACE protein, lisinopril exerts effect on oxygen-modulated gene transcripts (COX4I1, COX4I2 and HIF-1 $\alpha$ ) and the ACE transcript itself in skeletal muscle. The response of ACE and COX4I2 transcripts post-exercise depends on the ACE-I/D genotype. Despite the pronounced effects, caution applies when extending conclusions from our data to the general population because possibly many confounding variables interfere, which in our investigation were controlled by having subjects undergo a dominant one-legged exercise stimulus under standardized conditions.

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