Exercise and menstrual cycle dependent expression of a truncated alternative splice variant of HIF1α in leukocytes

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ABSTRACT

HIF1α is a subunit of the transcription factor HIF1 and a key regulator of angiogenesis, glucose metabolism and innate immune response. Recently, it has been shown that increases in free DNA in the plasma induce expression of HIF1α. Large increases of free DNA in plasma are typically found after exercise. HIF1α is therefore a potential candidate to orchestrate gene expression changes that initiate adaptations of the innate immune response after exercise. Although differential gene expression of HIF1α in response to exercise has been studied extensively and increases in expression are described for the skeletal muscle, no significant changes have been reported in leukocytes so far.

Here we show a highly significant increase in expression of the alternative splice variant HIF1α-2 coding for a truncated 736 amino acid protein in peripheral blood derived mononuclear cells following a 1h aerobic exercise. In contrast, expression of the regular full length HIF1α mRNA remained unchanged. Initial increase of HIF1α-2 mRNA expression and subsequent return to baseline levels was not different between twelve men and nine women. The latter were studied in the follicular and luteal phases of their menstrual cycle. Baseline expression of HIF1α-2 but not HIF1α-1 was significantly higher in the follicular phase than in the luteal phase. So far studies on the effects of HIF1 routinely measure only HIF1α-1. Further studies are needed to elucidate the hitherto unknown influence of HIF1α-2 on regulation of the innate immune response.

Key words: HIF1, alternative splicing, innate immune response, aerobic exercise, menstrual cycle

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INTRODUCTION

The heterodimeric transcription factor HIF1 is a key regulator of the innate immune response (1, 3), cell survival (4), angiogenesis (5) and glucose metabolism (6). Low O_2-tension within cells is the main driving force to increasing activity of HIF1 by both stabilization and increased transcription of its controllable subunit HIF1α (7,8). Principally, the effects, together with its regulation by O_2-tension, have formed the idea that HIF1α is an ideal candidate to orchestrate gene expression changes that finally lead to adaptations induced by exercise (9).
So far many studies have investigated involvement of HIF1α in response to exercise in the skeletal muscle *in vivo* (9-13) and *in vitro* (14-16). Most *in vivo* studies did not find significant changes in HIF1α mRNA or HIF1α protein expression in the exercising muscle under normoxic conditions (9-11, 17-19), although a physiological drop in pO_2 in the working muscle cell can be expected to be high enough to stabilize HIF1α (7). Until now, only two studies reported increases in HIF1α protein (12) and mRNA (13) after exercise under normoxic conditions and one study only after additional application of hypoxia (11). The common factor in these three studies was that the effect of a single exercise session on HIF1α expression had been investigated. The study by Ameln and co-workers applied a 45min one leg knee extension training session and verified the previous findings of their group indicating that there was no increase in mRNA expression (9) but a significant increase of protein (12). This result is in line with the hypothesis that HIF1α protein expression is achieved by post-transcriptional stabilization of HIF1α mRNA and does not require *de novo* expression of HIF1α mRNA (8). This finding is in contrast with the results of the study of Drummond and co-workers reporting increases of HIF1α mRNA after resistance training at only 20% of the 1RM (13). Also the study by Vogt and co-workers reported increased expression of HIF1α mRNA following 6 weeks of aerobic exercise under hypoxic but not under normoxic conditions (11).
The results obtained *in vivo* on induction of HIF1α expression due to exercise are rather disappointing and, in parts, even contradictory. However, recent *in vitro* studies have once more strengthened the concept that expression of HIF1α mRNA and protein is an important first step leading to metabolic (14,15) and vascular (16) adaptations of the working muscle.

In leukocytes no changes in expression of HIF1α have been reported so far and two recent studies that investigated HIF1α mRNA expression in leukocytes failed to demonstrate significant effects (20, 21). Microarray studies that focused on gene expression changes in leukocytes did not report changes in HIF1α mRNA expression due to exercise (22-24). One study that claimed to find increases in HIF1α upon exercise did, in fact, find significant changes of a downstream target of HIF1α but not of HIF1α itself (23, and personal communication). Lack of solid and conclusive evidence of HIF1α expression due to exercise in muscle and in leukocytes is surprising. As far as the muscle is concerned it remains to be shown that additional hypoxia does not reduce pO_2 in the working muscle enough to justify the assumption that only hypoxia in combination with exercise allows stabilization or transcription of HIF1α. In leukocytes it can be argued that the intracellular drop in pO_2 during short term passage through the capillaries of the

skeletal muscle is not profound enough to induce changes in HIF1α protein or mRNA. Nevertheless, microarray and gene expression studies reported alterations of many genes that are under the control of HIF1α (9, 10, 22-24). Typical effects of exercise such as systemic inflammatory response (25), redistribution of cell populations of the peripheral blood (26), induction of the expression of inflammatory cytokines and response to free radicals (27) are HIF1α related processes.
A very recent study showed that free DNA is a strong trigger of HIF1α expression that leads to the subsequent activation of the innate immune response (28). Different kinds of exercise have been shown to increase the levels of free DNA in plasma or serum more than five-fold (29-31). Recently, we have shown for EGR1 and SGK1 that transcriptional diversity of these two genes with key regulator function can be the cause for contradictory results in gene expression analysis (32, 33). The quantification strategy in gene expression studies should therefore take the transcriptional diversity of such key regulator genes into account. HIF1α, EGR1 and SGK1 have in common that they are enrolled in processes that are crucial for the induction of physiological processes essential for the adaptation to physical exercise (34). Each of the three candidates is also supposed to bear key regulator functions for several distinct physiological pathways. It is therefore tempting to speculate that their transcriptional diversity might be causal for their functional diversity.

Given the high likelihood of a central involvement of HIF1α in the processes initiated in leukocytes upon exercise and given the clear responsiveness of HIF1α to free DNA, we hypothesize that solid and reproducible increases in expression of HIF1α can be found in leukocytes upon exercise, if the transcriptional diversity of HIF1α is taken into account. There are two well described, functional and highly expressed splice variants of HIF1α: HIF1α-1 coding for the full length 826 AA protein and HIF1α-2 coding for a truncated 736 AA version (35). Both may react differently in leukocytes upon exercise. Of the above mentioned studies only Vogt et al. (11) measured the expression of both HIF1α splice variants and showed differential expression of both candidates upon exercise in the skeletal muscle.
In a recent microarray analysis focussing on inflammatory genes we revealed a gender and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise, but failed to show involvement of HIF1α. Here we analyzed the mRNA samples using a splice variant specific, quantitative real-time RT-PCR approach.

MATERIAL AND METHODS

Subjects and experimental procedures
A detailed description of the study subjects and the exercise protocols applied is provided in the original study by Northoff et al. (24). The most important information is given below in brief.
Twelve female and twelve male experienced athletes gave informed consent to participate in the study. The investigation was approved by the University Ethics Committee. The females included in the study (n=9) had regular menstrual cycles as verified by monitoring the cyclic hormonal status measuring oestrogen,

progesterone and LH using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics, Fernwald, Germany).
The exercise protocol comprised continuous runs (CR) on the treadmill with duration of 60 min and a running velocity corresponding to 93% of the velocity at their individual anaerobic threshold (IAT). IAT was determined in a pre-test several days before the experiment by the method of Roecker et al. (36). The exercise protocol started at 09:00 a.m after a standadized meal at 08:00. The females performed the identical CR twice: once in the follicular phase (F) of their cycle at day 10 and once in the luteal phase (L) of their cycle at day 25. Venous blood samples were drawn before (t0) immediately after the end of the CR (t1) 3h (t2) and 24h after the start of exercise (t3).

Splice variant specific real-time RT-PCR analysis of HIF1α expression
Peripheral blood derived mononuclear cells were isolated and total RNA was extracted as described previously (24) and the integrity of extracted RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California, USA).
500 ng total RNA were reversely transcribed into cDNA using the GeneAmp ® RNA PCR Core kit from Perkin Elmer according to the manufacturer's recommendations.
Quantitative real-time PCR was subsequently performed using Sybr Green as a fluorophore as described previously (33) using the primers for HIF1α-1 and HIF1α-2 shown in Table 1 and Fig. 1. GAPDH served as reference gene and Mean Normalized Expression (MNE) values for HIF1α-1 and HIF1α-2 were computed using the procedures described previously (33, 37).

Table 1: forward and reverse primer sequences

Gene	forward	reverse
GAPDH	5`- TCA ACA GCG ACA CCC ACT CC -3`	5`- TGA GGT CCA CCA CCC TGT TG -3`
HIF1α-1 (including exon 14)	5`-AAC ATT ATT ACA GCA GCC AGA CG-3`	5`-ACT GGT CAG CTG TGG TAA TCC A-3`
HIF1α-2 (without exon 14)	5`-AGC AGT AGG AAT TAT TTA GCA TGT AGA C-3`	5`-ACT GGT CAG CTG TGG TAA TCC A-3`

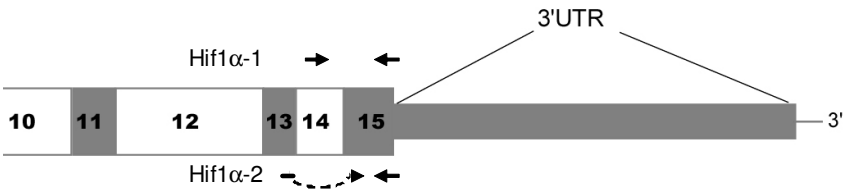

Fig. 1: Localization of the forward and reverse primer sequences for HIF1α-1 (upper arrows) and HIF1α-2 (lower arrows) with regard to the 3'end of the HIF1α mRNA starting with exon 10. For HIF1α-1 the forward primer is located in exon 14 and the reverse primer in exon 15. For HIF1α-2 the same reverse primer was used, but the forward primer was chosen to span the exon 14 and therefore composed of a part located upstream of the 5'end and a part located downstream of the 3'end of exon 14, respectively.

RESULTS

In the study population the average training distance in km/week was 60.8 (95% CI: 53.9 – 67.7) for men and 38.9 (95% CI: 28.6 – 49.2) for women. For all three groups (men, women in the follicular and the luteal phases) exercise intensity at 93% of their IAT did not lead to increases of blood lactate significantly different to the lactate levels at IAT, verifying that the exercise could be regarded as intense

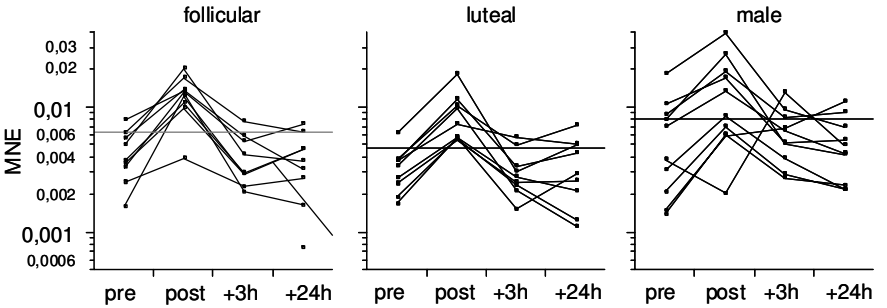

Fig.2: The mean normalized expression (MNE) values of HIF1α-2 (normalized to GAPDH as housekeeping gene) are presented on a logarithmic scale for the follicular (left; n = 9) and the luteal (middle, n = 9) phase of the menstrual cycle as well as for 12 male participants (right) before (pre) and immediately after (post) an aerobic exercise as well as 3h (+3h) and 24h (+24h) after the start of the exercise. All three graphs share the same y-axis. The horizontal line represents the total mean within the respective group. Note that just one male showed a delayed increase of HIF1α-2 after exercise.

Table 2: Fold-changes of HIF1α-2 expression

group / point in time	fold-change	95 %-CI	p-value
follicular / t0 – t1	3.0	2.0-4.4	$1.5*10^{-6}$
follicular / t0 – t2	1.0	0.6-1.4	n.s.
follicular / t0 – t3	0.8	0.6-1.2	n.s.
luteal / t0 – t1	2.7	1.8-4.0	$2.0*10^{-5}$
luteal / t0 – t2	1.0	0.7-1.4	n.s.
luteal / t0 – t3	1.0	0.7-1.4	n.s.
male / t0 – t1	2.3	1.6-3.3	$1.6*10^{-4}$
male / t0 – t2	1.2	0.8-1.8	n.s.
male / t0 – t3	1.0	0.7-1.4	n.s.

The table summarizes the fold-changes of HIF1α-2 expression, their respective 95%-confidence interval (95 %-CI) and the significance (p-value). Within each group (follicular, luteal or male) the point in time before exercise (t0) is compared to immediately after exercise (t1), 3h (t2) and 24h (t3) after beginning of exercise. For all three groups a highly significant increase in expression was found for the comparison of t0 with t1, only and after 3 hours expression values returned to baseline levels (see Fig.2).

1h aerobic exercise. Women in the follicular and in the luteal phase, as well as men, showed robust and immediate increases in HIF1α-2 expression following exercise (figure 2 and table 2). In contrast to this, HIF1α-1 expression showed a very high inter- and intra-individual variability without a common or significant response pattern over time. HIF1α-2 expression levels returned to baseline values just 3h after the start of the run and remained stable thereafter compared to the 24h values on the day after exercise.

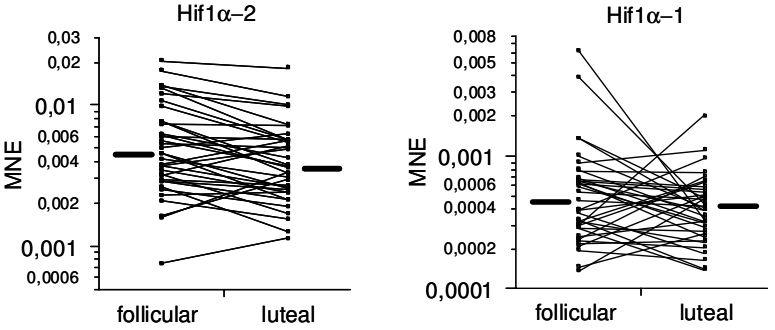

Fig. 3: The mean normalized expression (MNE) values (normalized to GAPDH as housekeeping gene) of HIF1α-2 (left) and HIF1α-1 (right) are presented on a logarithmic scale for a paired comparison between the follicular phase (left; n = 36) and the luteal (middle, n = 36) phase of the menstrual cycle. Comparisons are made in a paired fashion connecting the expression value of a certain individual at a distinct point in time during the course of the experiment (t0 – t3) in the follicular phase with its related value in the luteal phase. Note that even though there was a strong induction of HIF1α-2 expression due to the aerobic exercise, there is a highly significant difference that can be found between the two different phases of the menstrual cycle due to a very robust and consistent response pattern for a particular individual (p = 0.0018). The thick horizontal lines show the respective group medians.

In order to assess the influence of the menstrual cycle on the expression of HIF1α-2 and HIF1α-1, we pooled all different points in time for the different female individuals into a luteal phase group and a follicular phase group and made a pairwise comparison of the expression of HIF1α-2 and HIF1α-1 of a particular individual at a distinct point in time within the course of the experiment. Interestingly, it appeared that the phase within the menstrual cycle had a very constant and consistent effect on the expression of HIF1α-2 with significantly 1.3-fold (95 % CI: 1.1-1.5-fold) higher values in the follicular than in the luteal phase (p = 0.0018). Again a very high inter- and intra-individual variability was found for HIF1α-1.

DISCUSSION

HIF1α is a key regulator for different physiological processes, for example: glucose metabolism, innate immune response and angiogenesis, among many

others (2). Here we show for the first time that increases of a HIF1α splice variant (HIF1α-2) can be found in leucocytes following exercise. Moreover, the expression of HIF1α-2 is higher in the follicular than in the luteal phase of the menstrual cycle. Interestingly, we have previously shown that IL6, as the typical down-stream effector of HIF1α in leukocytes, is increased in response to exercise in the follicular phase, when compared to exercise in the luteal phase (24). Along with increased expression of IL6 in the follicular phase there was a significant decrease of inflammatory cytokines, but a significant increase of anti-inflammatory cytokines, suggesting that innate immune response may indeed be altered differently in response to intense aerobic exercise, depending on the phase of the menstrual cycle. The proposed role of HIF1α-2 with regard to this difference as outlined here, clearly needs to be elucidated in more detail, since interaction and physiological function of alternative splice variants are often rather complex as discussed below.

There is a rapidly growing body of literature that proposes, suggests and shows involvement of HIF1α in different diseases. In this regard HIF1α is by no means unique. It shares its supposed versatility with other key regulators and transcription factors such as EGR1 (32).

Expression of these important transcription factors is strictly controlled by complex feedback loops. It is tempting to speculate that versatility in function is concomitant with versatility in mRNA and protein sequence accompanied by a complex control of the expression patterns of the related splice variants and isoforms. Isoforms derived from alternative splicing can have opposing effects on downstream signalling. In such a setting splice variants control the activity of their respective counterparts: the resulting physiological effect of gene expression depends not only on the amount of mRNA in total but, rather, on the ratio of expression of the respective splice variants as shown, for instance, for the gene IRAK2 (38). The ratio of isoform expression will determine whether expression of one and the same gene acts inhibitory or excitatory on downstream signalling. A physician or exercise physiologist, who wants to get a first impression whether a gene like IRAK2 is a “good” or “bad” guy in a certain pathophysiological process leading to disease, will get the answer by virtue of choosing the sequence part of the IRAK2 mRNA or protein that is measured. If predesigned microarrays or antibodies from a company are used, the company will decide whether IRAK2 is a good or a bad guy. If sequence parts are chosen by chance, one could save the money and throw a coin instead.

Unfortunately, very little is known about the amount of alternative splicing within our genome. When it comes to “key regulators” like HIF1α most studies describe expression of “the HIF1α" by measuring one particular, very small sequence part that has been chosen by chance or based on technical decisions. It is well known that “the HIF1α" does not actually exist.

Apart from the two variants HIF1α-1 and HIF1α-2, there is supposed to be additional sequence variation in the 5’-region of HIF1α (39–42). However, these variants have not been studied by different groups and are mostly of unknown physiological relevance. HIF1α-2 described by Gothié et al. (35) is able to dimerize with ARNT, which is HIF1α–1’s counterpart to form the heterodimer and transcription factor HIF1. Just as with the regular HIF1, the dimer HIF1α-2/ARNT has been shown to be translocated to the nucleus, where it is able to induce expression of

VEGF as a typical gene under transcriptional control of HIF1. However, the dimer HIF1α-2/ARNT is about a 3-fold less potent enhancer for VEGF than the regular dimer HIF1α-1/ARNT. Over-expression of HIF1α-2 can therefore suppress or enhance expression of VEGF, depending on the ratio of HIF1α-1/HIF1α-2, as well as the absolute expression values of both candidates competing for ARNT binding (35). One could therefore conclude that it is misleadingly simplistic to state that HIF1α was an enhancer for VEGF. Scepticism is the more appropriate the more there is increasing evidence that angiogenesis is strongly induced via the HIF1 and VEGF-independent pathway of PGC1α expression (43).
However, if we can not take the assumption for granted that "the" HIF1α expression is inducing expression of VEGF, the general assumptions with regard to the effects of HIF1α expression on the innate immune response (1-3) are even more uncertain. To put it bluntly, all that is apparently known so far, is that artificial over-expression of HIF1α-1 is a key regulator of the innate immune response. In leukocytes induction of VEGF and of the key proteins of glycolysis and glycogenolysis are of minor relevance compared to the effects of HIF1α on the innate immune response. Here we show, for the first time, that exercise induces expression of HIF1α following aerobic exercise training on a treadmill. We confirm the results of other studies that found an uncoordinated expression of the splice variant HIF1α-1 with very high inter- and intra-individual variability (20, 21). In contrast to this, the expression of HIF1α-2 only showed a high inter-individual variability, while the intra-individual variability found in this study was highly significantly related to an immediate increase in expression upon exercise as well as an increased expression during the follicular phase in women. While variability of inter-individual expression might be due to sequence variations in HIF1α that have been shown to be related to a certain exercise phenotype (44,45), almost nothing about the physiological meaning of the coordinated changes in expression of HIF1α-2 upon exercise is know. Given the menstrual cycle and exercise dependent effects on gene expression we recently reported (24), we propose here that HIF1α-2, but not its more famous relation HIF1α-1, is a key regulator of the innate immune response. Further studies in immunology and exercise physiology should therefore take differential transcriptional regulation of HIF1α splice variants into account.

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ABBREVIATIONS

3'End	End of a messenger RNA
5'End	Start of a messenger RNA
5'region	Sequence region at the start of an mRNA

AA	Amino acids
ARNT	Aryl hydrocarbon receptor nuclear translocator
CI	Confidence interval
CR	Continuous run
EGR1	Early growth response gene 1
F	Group in the follicular phase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HIF1	Hypoxia inducible factor 1
IRAK2	Interleukin-1 receptor-associated kinase 2
L	Group in the luteal phase
LH	Luteinizing hormone
M	Group of male study subjects
MNE	Mean normalized expression
RT-PCR	Reverse transcription polymerase chain reaction
SGK1	Serum glucocorticoid inducible kinase 1
VEGF	Vascular endothelial growth factor
W	Group of women

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