HIF signaling and overall gene expression changes during hypoxia and prolonged exercise differ considerably

Renate Kopp, Louise Köblitz, Margit Egg, and Bernd Pelster
Institut für Zoologie and Center for Molecular Biosciences, Universität Innsbruck, Innsbruck, Austria
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Kopp R, Köblitz L, Egg M, Pelster B. HIF signaling and overall gene expression changes during hypoxia and prolonged exercise differ considerably. Physiol Genomics 43: 506–516, 2011. First published February 22, 2011; doi:10.1152/physiolgenomics.00250.2010.—Exercise as well as hypoxia cause an increase in angiogenesis, changes in mitochondrial density and alterations in metabolism, but it is still under debate whether the hypoxia inducible factor (HIF) is active during both situations. In this study gene expression analysis of zebrafish larvae that were raised under normoxic, hypoxic, or training conditions were compared, using microarray analysis, quantitative real-time PCR and protein data. Although HIF expression is posttranslationally regulated, mRNA expression levels of all three isoforms (HIF-1α, HIF-2α, and HIF-3α) differed in each of the experimental groups, but the changes observed in hypoxic animals were much smaller than in trained larvae. Prominent changes were seen for HIF-2α expression, which significantly increased after the first day of exercise and then decreased down to values significantly below control values. HIF-3α mRNA expression in turn increased significantly, and at the end of the training period (9–15 days postfertilization) it was elevated three times. At the protein level a transient increase in HIF-1α was observed in hypoxic larvae, whereas in the exercise group the amount of HIF-1α protein even decreased below the level of control animals. The analyzed transcriptome was more affected in hypoxic zebrafish larvae, and hardly any genes were similarly altered by both treatments. These results clearly showed that HIF proteins played different roles in trained and hypoxic zebrafish larvae and that the exercise-induced transition to a more aerobic phenotype was not achieved by persistent activation of the hypoxic signaling pathway.

ontogeny; hypoxic signaling; environmental adaptation; muscle tissue; metabolism; zebrafish; hypoxia inducible factor

SKELETAL MUSCLE OF ADULT VERTEBRATES represents a highly specialized, but also a highly adaptive tissue as well. Endurance training, for example, is known to induce characteristic changes in muscle size, in red, white, and intermediate fiber type composition, in key metabolic enzyme activities and in mitochondrial density (23, 24, 50, 51, 65). Concomitant with these changes in muscle cellularity the performance of the cardiovascular system is modified to match metabolic oxygen requirement and oxygen delivery to the tissue. Thus, exercise can stimulate arteriogenesis by remodeling of adjacent arteries and/or increase muscle capillarity via angiogenesis, as already demonstrated in humans, guinea pigs and rats (11, 56).

These muscle training-induced changes are best documented for mammals, but several studies also demonstrated that swim training leads to similar physiological adaptations in fish, for example, causing an increase in growth rate and food conversion efficiency in salmonids (9, 10, 15, 62–64). In fact, significant parallels do exist in the physiological response of fish and of mammals to various environmental stimuli like exercise or the lack of oxygen (44, 73). In previous experiments we could demonstrate that prolonged swimming activity causes an increase in muscle tissue capillarization and in mitochondrial density in zebrafish larvae (49).

It is obvious that these adaptations at the cellular, tissue, as well as at the organismic level must have a molecular basis, and various gene expression changes have been indeed been demonstrated (5). Nevertheless, many of the mechanisms underlying the adaptation of skeletal muscle to exercise still remain to be discovered (20). Timmons et al. (72) stated that, even for humans, very little is known about the mechanisms that connect muscle activity to gene expression.

An intriguing facet in this context is the question whether hypoxic signaling, which is pivotally regulated by the hypoxia inducible factor (HIF), also contributes to the control of gene expression changes during exercise. Three isoforms of HIF have been described (HIF-1α, HIF-2α, HIF-3α), which together with HIF-1β (ARNT) form an active, heterodimeric transcription factor. Regulation, best described for the HIF-1α isoform, is achieved by oxygen-dependent degradation of HIF-1α protein, initiated by the activity of oxygen-dependent prolyl hydroxylases (PHD). Hydroxylation of HIF-1α finally results in proteosomal degradation. Under hypoxic conditions PHD activity is inhibited so that HIF-1α is no longer degraded and together with HIF-1β initiates hypoxic signaling (29, 69). The significantly increased oxygen demand during exercise has been reported to cause very low oxygen tensions in muscle tissue (25, 60, 61), which may of course decrease the activity of PHD and thus stabilize HIF proteins (26, 69). Many training-induced responses are indeed connected to the delivery and utilization of oxygen to the working muscle, which fostered the idea that HIF may play a central role in these processes (14, 34). The increase in HIF-1α mRNA and protein in exercising humans (19) and the stabilization of HIF protein following acute exercise (3) reinforced this idea.

Additional support for this conclusion is obtained when looking at mitochondrial function and density, which is increased in endurance-trained muscle tissues. In mammals mitochondrial density correlates with oxygen consumption (77, 79). Therefore, trained animals with an increased number of mitochondria should have a higher rate of oxygen consumption. A higher oxygen requirement of the mitochondria in turn decreases oxygen availability for the prolyl hydroxylases, resulting in a stabilization of HIF protein and subsequent HIF signaling (45). In later developmental stages of trained zebrafish larvae a higher rate of oxygen consumption actually was observed (4).

On the other hand, regular exercise training has been shown to reduce HIF-1α and HIF-2α expression (34). Furthermore, if...
skeletal muscle HIF-1α was knocked out (HIF-1α null mice) an adaptive response akin to endurance training was induced, but training of these mice did not stimulate any further improvement with respect to oxygen transport and utilization (38). These results suggest that HIF signaling may be switched off during endurance training and therefore are in contrast to the hypothesis that local oxygen deficiency might induce HIF signaling in endurance-trained muscle.

Previous studies demonstrated that zebrafish larvae can be trained in a swim tunnel and are a good model for exercise experiments (4, 49, 76). Furthermore, they have also been successfully used for hypoxic incubations (27, 67, 73). The present study therefore was designed to address hypoxic signaling and gene expression changes in endurance-trained zebrafish larvae and to compare the results with that of larvae that were raised under hypoxic conditions. The findings demonstrated accumulation of HIF-1α protein and therefore HIF signaling in hypoxic larvae, but in trained larvae HIF-1α protein concentration decreased. Overall expression changes observed in trained larvae largely differed from expression changes detected in hypoxic larvae, supporting the conclusion that HIF signaling is not responsible for the improvements in oxygen transport and utilization observed in trained zebrafish larvae.

MATERIALS AND METHODS

Animals

The experiments were performed with zebrafish larvae (Danio rerio), which were obtained from our breeding colony. Larvae were kept at the experimental temperature of 25°C. Adult stocks were continuously reconditioned by new fish from a local supplier, which ensured control fish with a naturally evolved genotype. Animal experiments were performed according to animal ethics permission GZ.6.008/4-B Rutgers of the Austrian Federal Ministry for Education, Arts and Culture.

Experimental Design

The training apparatus was a gravity-fed system consisting of eight separate treatment tubes with an inside diameter of 1.4 cm and a length of 28 cm long. The desired water velocity was set by measuring and adjusting the flow (ml/min). Following established protocols (4, 49) larvae were placed into the training tubes with a water flow of 5 BL/s at 25°C. About 100 larvae were placed in each of the tubes with a water flow of 5 BL/s at 25°C. Control fish were raised in tanks with almost no water flow. Larvae were trained daily starting at 8:00 AM and ending at 8:00 PM, with a 15 min feeding break at 2:00 PM. PO2 was maintained at normoxic levels by aeration of the reservoir tanks.

In a second set of experiments larvae of the same age were incubated under hypoxic conditions. At 9 dpf larvae were transferred to a hypoxic chamber and raised until 15 dpf. Oxygen tension was measured by an oxygen probe (PreSense, Regensburg, Germany) and adjusted by the controlled inflow of an air-nitrogen gas mixture (PreSense, Regensburg, Germany). PO2 was adjusted to either 10 kPa or to 5 kPa at 25°C. Control larvae were kept in normoxic water for the same time period.

RNA Sampling, Isolation, and cDNA Synthesis

Total RNA was extracted from 10 zebrafish larvae according to the procedure described by (8) using TRizol reagent (Live Technologies, Gibco, BRL, Karlsruhe, Germany). Pelleted RNA was resolved in 20 μl of DEPC water and treated with DNaseI (deoxyribonuclease I, Fermentas International, Canada) to remove residual DNA. The quality of extracted RNA was determined by electrophoretic separation on a 1% agarose gel in TBE buffer and ethidium bromide staining. Total RNA concentrations were measured fluorometrically in triplicates with a maximum fluorescence plate reader (Molecular Devices, Sunnyvale, CA) using Quant-iT Ribogreen (Invitrogen, Carlsbad, CA). For each sampling point, 450 ng of total RNA was taken for first-strand cDNA synthesis using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H minus point mutant; Fermentas International, Burlington, Ontario, Canada) according to manufacturer’s specifications in volumes of 50 μl.

Primer Design and Quantitative Real-time PCR

Primers were designed using Primer Express Software 3.0 (Applied Biosystems, Foster City, CA). Accession numbers and primer sequences for the respective genes are listed in Table 1. The vegf-a gene comprises several isoforms and vegf121 and vegf165 are the dominant isoforms in zebrafish. Based on the information regarding vegf-a exon - intron junctions (17) primers allowing for a separation of the two isoforms were designed previously (31).

Amplification of the samples was performed with an ABI PRISM 7500 Sequence Detector (Applied Biosystems). Nonspecific products such as primer dimer formations were checked by dissociation curves and optimal primer concentrations were determined by primer matrices of different primer concentrations. PCR was carried out in triplicates, using 20 μl volumes of a cocktail comprising 1x Power SYBR Green PCR Master Mix (Applied Biosystems), 3 μM forward and 3 μM reverse isoform specific primer, 0.5 μg nonacetylated bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 2 μl of cDNA. For sdha, pdk1, cox1, pdk2, a primer concentration of 9 μM was used. No-RT controls (RNA incubated with random hexamers but without reverse transcriptase) were routinely included. Data acquisition and

<table>
<thead>
<tr>
<th>Table 1. Sequences of forward and reverse primer sets used for PCR amplification of the mRNA of the various genes quantified by real-time</th>
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<th>Reverse</th>
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<td>5’-cga gga ggg taa ggg ttt g-3’</td>
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<tr>
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<td>5’-cga cct cgg tgg gtt tta-3’</td>
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<tr>
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<td>5’-gtc tta ctc gac agt tga atg-3’</td>
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<tr>
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<td>5’-gca tgc gta agc cgt act gg-3’</td>
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<tr>
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<tr>
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analysis were performed with the SDS 1.9.1 software package (Applied Biosystems). Serial dilutions of gene specific cloned and quantified zebrafish cDNA were performed to determine quantitative real-time PCR (qRT-PCR) efficiency. By plotting the CT values versus the log10 of the initial copy numbers, we calculated absolute copy numbers (absolute quantification method). Samples were normalized to 10 ng of total RNA.

Microarray Analyses

RNA preparation. For total RNA isolation phenol-chloroform extraction with TRIzol reagent (Invitrogen) was performed (see above). Therefore, 40 zebrafish larvae were lysed according to Ref. 8. An additional purification step was achieved by using the RNaseasy kit (Qiagen, Venlo, The Netherlands). Quality and concentration were checked by an electrophorogram [2100 expert (B.02.03.S1307) ca. 2003–2006 Agilent Technologies, Santa Clara, CA]. For further analysis only high-quality RNA (RNA integrity number: 10) was used.

Target labeling and hybridization. For Affymetrix GeneChip analyses 1.5 μg total RNA was processed to generate a biotinylated hybridization target using “One Cycle cDNA Synthesis” and “One Cycle Target Labeling” kits from Affymetrix (Affymetrix, Santa Clara, CA). All procedures were performed according to the manufacturer’s protocol (Gene Expression Analysis, Technical Manual, revision #4; Eukaryotic Target Preparation, revision #3). Briefly, total RNA was reversely transcribed into cDNA using an anchored oligo-dT-T7-primer, converted into double-stranded cDNA, and purified with the “Affymetrix Sample Cleanup Kit” according to the manufacturer’s protocol. Thereafter, cRNA was generated by a T7 in vitro transcription step including a modified nucleotide for subsequent biotinylation. Following RNA purification, 20 μg of cRNA were fragmented at 95°C using the Affymetrix fragmentation buffer, mixed with 200 μl hybridization buffer containing hybridization controls and hybridized to U133 Plus 2.0 microarrays. The arrays were stained and washed in an Affymetrix fluidic station 450 following the EukGE- ws2v4 protocol. Fluorescence signals were recorded by an Affymetrix scanner 3000 and image analysis was performed with the GCOS software (version 1.2).

Data processing and statistics. GeneChip raw expression values were normalized and summarized using the CARMAweb analysis program (57). The Affymetrix GeneChip analysis was done by calculating a background correction, normalization (gcma), probe-specific background correction, and summarizing of the probe set values. For the test statistics a moderated t-statistics (limma) was applied. Multiple testing corrections by adjusted BH values for the Benjamini and Hochberg set-up false discovery rate controlling procedure (independent and positive regression-dependent test statistics) were done. Regulation values (M values) for upregulation and downregulation were calculated with the formula M = log2 (R/G), where R and G are the expression values from a sample and the according control sample, respectively. A P_MHT value of <0.05 marked significant differences. For comparing control and treatment values additionally a P_anno of <0.05 plus M > 1.5 (more than threefold induction) or M < −1.5 (less than one-third expression) was marked as significant. Gene Ontology (GO) analyses were also performed using the CARMAweb analysis program (57).

Plasmids, Antibodies, Fusion Protein Expression/Purification, and Immunoblotting

Full-length zebrafish HIF-1α was amplified from larval zebrafish cDNA using the following primers: forward primer 5′-gatactgacatggtgtc-3′; reverse targetgcagttgtgtatgtcagacagc. The PCR product was cloned into the EcoRV/HindIII sites of a pET29b vector (Novagen, Merck KGaA, Darmstadt, Germany), and the fusion protein was then expressed in BL21(DE3) cells (Invitrogen) as described previously (2, 66). Because HIF-1α was mainly expressed in inclusion bodies it was necessary to take the pellet fraction. The pellet was solubilized by 8 M urea buffer (pH 8). The lysate was incubated with Ni-NTA sepharose beads (Qiagen), eluted with 8 M urea buffer (pH 5.9 and 4.5), and dialyzed against phosphate-buffered saline (PBS). Protein concentrations were determined by DC Protein Assay kit (Bio-Rad, Hercules, CA).

A polyclonal antibody was generated in rabbits by using the purified, bacterially expressed 6× His-tagged full-length zebrafish HIF-1α (amino acids 1–777) as antigen. The fourth immune bleed (70 days after initial immunization) was taken for affinity purification against purified bacterial expressed 6× His-tagged full-length zebrafish HIF-1α. Affinity purification was performed by Eurogentec (Seraing, Belgium).

To assess the amount of Hif-3α protein an antiserum originally generated for grass carp Hif-4α (32) was used. This antiserum was a generous gift from Dr. Richard Kog (City University Hong Kong). According to our sequence analysis grass carp Hif-4α shows the highest homology to zebrafish Hif-3α. We successfully purified the antisem on protein A/G sepharose columns and used this antibody for Western blot analysis.

For Western blotting zebrafish embryos were lysed and homogenized by Ultraturrax T25 (IKA Labortechnik, Staufen, Germany) on ice in Lysis buffer containing glycerol (25%), NaCl (420 mmol/l), MgCl₂ (1.5 mmol/l), EDTA (0.2 mmol/l), HEPES (20 mmol/l), DTT (125 μmol/l), and protease inhibitor cocktail (including aprotinin, leupeptin, pepstatin, sodium vanadate, and phenylmethane sulfonyl-fluoride). Lysates were centrifuged, and the supernatant was used for Western blotting using standard procedures as described previously (66). Protein concentrations were measured fluorometrically in triplicates with a maximum fluorescence plate reader (Molecular Devices) using a DC Protein Assay kit (Bio-Rad). We took 40 μg for each lane. After electrophoretic transfer of proteins to a polyvinylidene fluoride membrane, the membrane was Coomassie R250 (Sigma-Aldrich) stained as described by Naryžný (43) for control of equal loading (Supplemental Figs. S1 and S2).1 The membrane was destained with methanol. Unspecific protein binding sites were blocked by preincubating the membranes in Tris-buffered saline containing 5% skim milk powder and 0.1% Tween 20 (blocking buffer) for 45 min at room temperature. Primary polyclonal HIF-1α antibody was diluted in blocking buffer 1:500. Incubation with primary antibody was performed overnight at 4°C. Binding of the primary antibody was detected with a secondary antibody conjugated to horseradish peroxidase (Abcam, Cambridge, MA; UK) diluted 1:30,000 in blocking buffer for 1 h at room temperature by enhanced chemiluminescence detection (Immun-Star WesternC kit, Bio-Rad).

In zebrafish development as well as during hypoxia, mRNA expression variations are induced (41). To our knowledge no gene has been described yet that may serve as a reliable housekeeping gene in developing zebrafish larvae, especially if development is combined with environmental stress conditions. Testing the expression level of actin and GAPDH expectedly revealed increasing actin levels in certain developmental stages and also GAPDH was not stable in developing hypoxic or trained larvae. Therefore, protein concentration was accurately determined in triplicates (see above), and in addition Western blot loading was checked via Coomassie staining of the loaded total protein (36). Comparing overall intensity of protein staining of the different lanes demonstrated equal loading of all samples.

Statistics

Data are presented as mean ± SE. To compare differences between two means, Student’s t-tests (two tails) were performed. To compare more than two means, we analyzed data by two-way ANOVA fol-

1 The online version of this article contains supplemental material.
RESULTS

At 9 dpf, prior to the start of the training regime or the hypoxic incubation, HIF-2α expression levels by far exceeded the expression of HIF-1α, whereas HIF-3α expression was almost 10 times lower than HIF-1α expression (Fig. 1, A–C, left). Between 9 and 15 dpf prolonged swim training of zebrafish larvae at a flow rate of 5 BL/s, which has been shown to elicit typical endurance training-related modifications in muscle tissue (49), affected HIF-1 mRNA expression in an isoform-specific manner. After the first training bouts, HIF-1α expression was not different from controls, but toward the end of the training period it increased significantly. HIF-2α in turn was almost doubled after 1 day of swimming activity but then continuously decreased, and at 15 dpf mRNA concentration was <50% of control level. HIF-3α expression increased between 10 and 15 dpf (except 11 dpf) but still was three to four times lower than the expression of HIF-1α. To address the changes in HIF expression evoked by environmental hypoxia a second group zebrafish larvae of the same age were incubated at a PO2 of ~10 kPa or of ~5 kPa. As shown in Fig. 1, A–C right, a PO2 of 10 kPa did not elicit obvious changes in HIF-1α and HIF-2α expression between 9 and 15 dpf. At a PO2 of 5 kPa, however, Hif-1α mRNA levels were significantly elevated at 10, 14, and 15 dpf, while Hif-2α was only elevated at 14 dpf. HIF-3α expression was transiently increased at 11 and 12 dpf at a PO2 of 10 kPa, while at a PO2 of 5 kPa expression was significantly reduced at 11 and 12 dpf. Thus, hypoxia at least slightly enhanced the expression of all three isoforms, while swimming exercise reduced the expression of HIF-2α but increased the expression of HIF-1α and HIF-3α, thereby even exceeding the stimulation of expression observed under hypoxia.

In most animals and cells HIF expression is regulated at the protein level. HIF-1α is the most studied isoform, and we therefore generated a specific antibody against the zfHIF-1α protein to assess the influence of prolonged exercise and of hypoxia on the HIF-1α protein. As shown in Fig. 2A hypoxic conditions with a PO2 of 5 kPa caused an increase in the HIF protein level after 24 h, but the protein concentration then

![Graphs showing HIF expression levels](graphs.png)

Fig. 1. Number of hypoxia inducible factor (HIF)-1α (A), HIF-2α (B), HIF-3α (C) mRNA copies per 10 ng of total RNA in zebrafish larvae raised under control conditions (PO2 = 20 kPa), raised at a PO2 of either 5 or 10 kPa or exposed to a water current of 5 BL/s for 12 h a day between 9 and 15 days postfertilization (dpf). Values are represented as means ± SE, n = 3 (pooled samples). *Significance with P < 0.05, tested by ANOVA between control and trained or control and hypoxic (PO2 = 5 kPa) larvae. °Significant differences between control and hypoxic larvae (PO2 = 10 kPa).
decreased again and, compared with control conditions, was no longer elevated at 15 dpf. Trained larvae, however, never showed an elevated Hif-1α protein content; the Hif-1α protein content even decreased with the proceeding training protocol. At 15 dpf Hif-1α had almost totally disappeared in trained fish.

Hif-3α protein could successfully be detected by the Western blot analysis with the grass carp Hif-4α antibody (32). Under control conditions a clear band with the molecular weight of 70 kDa was detected, corresponding to the molecular weight calculated based on the amino acid composition of zebrafish Hif-3α (= 70.2 kDa) and revealing the presence of the protein under normoxic conditions (Fig. 2B). This band was also detected at 10 and 15 dpf in control larvae, while at 12 dpf the band was faint, suggesting an influence of development. Compared with the control situation, prolonged hypoxia and even more so prolonged swim training caused a reduction in the Hif-3α protein level (Fig. 2B).

HIF signaling was also assessed by the expression of downstream genes, i.e., erythropoietin (epo) and vascular endothelial growth factor (vegf). Epo mRNA expression increased following the first training bout and except for day 15 remained elevated (Fig. 3A). Changes in epo expression under hypoxia were also remarkable. While a Po2 of 10 kPa stimulated epo expression at 12 dpf and at 15 dpf, a Po2 of 5 kPa elevated epo expression only for a short time (24 h of hypoxia). After the first day, epo expression decreased even below control levels until 15 dpf.

Vegf is mainly expressed in two isoforms, vegf121 and vegf165. While vegf121 expression was not affected by swimming activity (Fig. 3C), expression of vegf165, the dominant isoform in larval zebrafish (17), was significantly elevated at 13, 14, and 15 dpf (Fig. 3B). Under hypoxic conditions expression of both vegf isoforms did not show obvious alterations. There was some variation in the expression level, but only at 15 dpf the value was significantly different from controls. So no clear pattern emerged (Fig. 3, B and C).

Given the obvious HIF signaling differences and changes in oxygen-regulated gene expression in trained and hypoxic animals we tested the hypothesis that general expression analysis using Affymetrix expression arrays would not show similar expression pattern changes but identify additional functional groups that were differently affected by exercise and by hypoxia. As illustrated in Fig. 4, the overall trend in the expression changes of the two experimental groups was largely different and therefore confirmed the data obtained by the expression analysis of oxygen-related genes. Compared with untrained control larvae 26 genes were differentially expressed in trained larvae at 15 dpf, and only six of these genes were upregulated. By contrast, in larvae raised at a Po2 of 10 kPa 20 genes were significantly different from controls, with 19 genes upregulated, while at a Po2 of 5 kPa 73 genes were differentially expressed, out of which 53 were upregulated (Fig. 4). The different effects of exercise and of hypoxia became even more obvious when the gene expression patterns were identified and directly compared. Only two of the affected genes were expressed similarly in trained larvae and in larvae raised at a Po2 of 10 kPa, namely growth arrest and DNA damage-inducible beta and Krüppel-like factor 2a, both of which were downregulated. Comparing larvae raised at a Po2 of 5 kPa and trained larvae at 15 dpf again only two of the genes were differently expressed in both treatment groups. Heat shock cognate 70 kDa protein was upregulated in both groups, and vang like protein was downregulated (Table 2).

Comparing all sample groups and performing GO analyses revealed that several genes of different gene clusters were affected, but the different treatments did not significantly affect a single GO term. This statistical spread might be due to the high number of altered transcripts that were not related to certain GO terms. Supplemental Table S1 summarizes all differently expressed genes in trained larvae at 15 dpf. After completion of the 6-day swim training program, several genes related to energy metabolism were affected, including glucokinase isoform 2, glycogen synthase kinase binding protein, and protein phosphatase 1 regulatory subunit 3C like. In addition, expression changes of several transcription factors including BMP2b and various members of the nuclear receptor subfamily and of cell cycle-related genes indicated a downregulation of cell proliferation and growth.

Hypoxia in turn stimulated an upregulation of various members of the cytochrome P450 family at 15 dpf (Supplemental Tables S2 and S3). A lower Po2 of only 5 kPa in the younger larvae caused expression changes in many more genes than the higher Po2. Again erythrocyte and hemoglobin synthesis-related genes were significantly upregulated, but again a large number of yet unidentified transcripts were significantly affected in their transcription rate.

To verify array data 27 genes represented in the array were also analyzed by qRT-PCR. The comparison of microarray data and qRT-PCR data showed that ~90% of upregulated or downregulated genes detected by microarray analysis were also detected by qRT-PCR measurements. Actually, qRT-PCR provided a higher number of differently expressed genes. Thus, microarray analysis might rather underestimate the actual expression changes. In this context four genes related to energy
metabolism were analyzed in exercised and in hypoxia-incubated larvae (Fig. 5). In trained larvae pyruvate dehydrogenase kinase isoenzyme 2 (pdk2) was significantly upregulated, while it was unaffected by hypoxia. Furthermore, succinate dehydrogenase (sdh) and pyruvate dehydrogenase (pdh) were downregulated in the hypoxic group.

**DISCUSSION**

For the first time this study reports mRNA expression changes of all three HIF-H9251 isoforms and also protein expression changes for HIF-1/H9251 and Hif-3/H9251 in endurance-trained zebrafish larvae. Larvae at 9-15 dpf responded to the exercise protocol with isoform-specific alterations. This suggests that the three HIF-α isoforms may be involved in exercise adaptation and that in zebrafish HIF expression is not only regulated at the protein level.

**Influence of Exercise on Hif-α Transcription and Hif Signaling**

Looking at the individual HIF-α isoforms our study showed that under resting, normoxic conditions expression of HIF-2α mRNA by far exceeded the expression of HIF-1α and especially of HIF-3α. The expression level of HIF-3α in turn was at least 10 times lower than the expression of the other two isoforms. This was also seen in various rat tissues under resting conditions (22). In the training group HIF-2α was elevated only after the first day of exercise but then significantly decreased during the following days, and this downregulation of HIF-2α was the most prominent change in the transcription of the HIF isoforms. In contrast to HIF-1α and HIF-2α, HIF-3α mRNA was consistently overexpressed in trained larvae. An influence of exercise on HIF transcription has also been reported for human muscle (34). In untrained persons an increase in HIF-1α and HIF-2α mRNA was observed during recovery from prolonged exercise, while in trained persons this response was blunted. The latter observation is in line with a study reporting that an intensive training regime lasting 3 mo had no effect on HIF-1α mRNA levels (46). In untrained persons an increase in HIF-1α and HIF-2α mRNA was observed during recovery from prolonged exercise, while in trained persons this response was blunted. The latter observation is in line with a study reporting that an intensive training regime lasting 3 mo had no effect on HIF-1α mRNA levels (46). Thus, in the initial phase of an exercise program, transcription of HIF-α mRNA appeared to be affected in humans as well as in young zebrafish larvae. A number of factors have indeed been reported to modify HIF-α mRNA expression, including interleukin-1β, insulin like growth factor (IGF), insulin, tumor necrosis
factor-α, nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), angiotensin-2, mitogen-activated kinase pathways, and nitric oxide (NO) (6, 68, 69). In particular IGF-1 and NO are known to be highly responsive to exercise (12, 28) and therefore could be crucial for HIF transcription activation. A recent study also indicated that in rat skeletal muscle HIF induction may be related to stretch during exercise, possibly involving PI3K and ERK1/2 activity (42).

Our results thus suggested that the HIF-α isoforms are affected by exercise in an isoform-specific manner. In recent studies more and more differential activities of HIF-1α and HIF-2α could be identified (7, 33, 48). Epo, for example, appears to be under the control of HIF-2α (58, 78). In our study the initial bout in HIF-2α mRNA after the first training day therefore may have contributed to an increase in HIF-2α protein and triggered the increase in epo expression. In a previous study, however, an increase in the number of red blood cells was not detected after the exercise protocol was completed (49). Recent studies revealed that epo may also stimulate ventilation under hypoxic conditions (16), which may explain the elevated epo level in our experiments. The additionally detected increase in vegf165 during the last training days in younger larvae, however, was in line with the observed increase in tissue vascularization (49).

HIF isoforms not only show a certain level of target specificity, they also appear to be expressed in a muscle type-specific manner. In mouse white muscle HIF-1α mRNA and protein levels were significantly higher than in red soleus muscle, and in rat a fiber type-specific response in the HIF-1α mRNA expression was caused by activity restriction (52). In zebrafish red muscle, although already present in larval stages (49), makes up only a very small portion of total muscle tissue. Therefore, the HIF expression changes in our trained zebrafish group should not be related to an alteration in muscle type composition.

**HIF Protein Expression and the Role of Hypoxia in Trained Zebrafish Larvae**

Typically HIF expression is seen in relation to hypoxia. The downregulation of HIF-2α mRNA after an initial increase and especially the long-term disappearance of HIF-1α and Hif-3α protein, however, were in glaring contradiction with the results of the hypoxic group and indicated that the exercise regimen used in our study did not turn on prolonged HIF signaling in zebrafish larvae. This conclusion was in line with the observation that muscle-specific HIF-1α knockout mice did not show reduced endurance performance. The oxidative metabolic capacity of the muscle tissue was not impaired, indicating that endurance exercise-related improvements in the oxygen transport capacity and oxidative metabolic activity were HIF-1α independent (37, 38). In fact, the phenotype seen in the HIF-1α knockout mice appeared to be preadapted to endurance exercise, i.e., the lack of HIF-1α resulted in an improvement in oxygen transport capacity in muscle tissue and in an improvement of the aerobic metabolic capacity in this tissue.

Interestingly, in trained animals HIF-1α and in both experimental groups HIF-3α protein expression level was below control level toward the end of the experimental protocol. Thus, either the PHD-dependent degradation was enhanced under these conditions, or additional pathways came into play influencing the stability of HIF protein. Binding to HSP90, the RACK1 protein, sumoylation, or perhaps a change in the production rate of reactive oxygen species (ROS) may play a role. Endurance exercise may indeed result in a decrease in ROS (1), and ROS has been shown to inhibit hydroxylases and thus HIF degradation (69, 70). A decrease in ROS and the concomitant reduction in the stability of HIF proteins may therefore explain the decrease in HIF protein concentration with prolonged exercise. Very little is known about the physiological importance of the HIF-3α isoform, which lacks the COOH-terminal transactivation domain and therefore may act

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**Table 2. List of genes similarly affected by either hypoxic incubation or swimming exercise**

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<thead>
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as a negative regulator of Hif-1α (39, 71). In our experiments HIF-3α protein expression reached its maximum under normoxic control conditions and demonstrated the highest variability, strongly indicating a physiological function during normoxia.

**Hif Signaling in Hypoxia**

The hypoxic conditions selected in our study were based on previous experience (27, 67) and avoided developmental retardation, which was encountered in other studies looking at the effect of hypoxia on general expression changes in developing zebrafish (73). Nevertheless, the oxygen partial pressure selected in our study affected transcriptional expression of all three HIF isoforms, but not as much as the training protocol. HIF-1α mRNA was elevated initially and toward the end of the hypoxic incubation. HIF-3α was transiently enhanced at 11 and 12 dpf at a PO2 of 10 kPa. Similarly, in hypoxic rat HIF-3α mRNA was elevated in all tissues examined, while the other two isoforms showed no response to hypoxia at the transcriptional level (22). In line with previous reports (30, 59, 70), HIF-1α protein was significantly increased in hypoxic larvae, indicating that HIF signaling has indeed been switched on at least for the first days of hypoxia. Thus, in contrast to trained larvae HIF-1α signaling was clearly initiated in hypoxic animals. In hypoxic larvae HIF-3α was altered complementarily to HIF-1α. As HIF-3α competes with HIF-1α for DNA binding sites it might be a negative regulator of the other two HIF proteins (39) or at least exert negative effects on the expression of some HIF-1α-dependent genes (71). Concomitant with an increase in HIF-1α protein the expression of the two well-known downstream genes of Hif signaling *epo* and *vegf* was stimulated in the young group. These changes thus were comparable to results previously obtained in hypoxic zebrafish or zebrafish tissues (35, 73, 75).

**Interacting Signaling Pathways and Regulation of Hif Signaling**

Interestingly, the HIF-1α and HIF-3α mRNA levels were elevated at the end of the training regime, while the protein contents were reduced and hardly detectable. Inconsistencies between mRNA concentration and protein content have repeatedly been observed and discussed (18, 47). It most likely is attributable to posttranscriptional and/or posttranslational regulation. Transcription and translation are sequential processes, thus causing a temporal delay between mRNA and protein regulation. Because promoters might stochastically change their stage of activation, bursts of mRNA production may occur (13), and also changes in mRNA stability may cause changes in the amount of mRNA available for the translation. Translational activity may be variable, and especially for Hif proteins posttranslational control of protein stability is well documented. It appears quite possible that a reduced ROS level in endurance-exercised larvae caused a reduced stability of the protein and contributed to the discrepancy in mRNA and protein concentrations. In prolonged hypoxia high HIF-1α protein levels have been shown to suppress mRNA expression (74). Recently it has also been shown that mRNA concentrations may feed back on the level of transcription (55). Thus, several mechanisms may impair a direct correlation between mRNA and protein levels. Nevertheless, the changes in the mRNA level of all three Hif isoforms induced by exercise training indicate a regulation of transcriptional activity in zebrafish larvae, and the functional significance of this phenomenon remains to be shown.

Our data show that persistent HIF signaling was not responsible for the remodeling of muscle following endurance exercise. However, a transient activation of HIF transcription following exercise or during recovery or even the transient accumulation of the HIF-1α protein after a single bout of exercise (3) has repeatedly been observed. The strong initial
increase in HIF-2α mRNA seen in our study suggested initial HIF-signaling following the onset of the exercise protocol. These observations, which at first glance are puzzling, may be the result of feedback loops in the signaling involved and the interaction of different signaling pathways, possibly even depending on intensity and/or length of the exercise protocol. For example, endurance exercise is well known to stimulate PGC-1α expression, and expression of this transcriptional cofactor is required for mitochondrial biogenesis. PGC-1α, however, together with the increase in mitochondrial density, is coupled to HIF signaling because under conditions of reduced oxygen availability, such as in endurance-exercising muscle, the increased oxygen demand of the mitochondria drains the oxygen from the PHDs and thus stimulates HIF signaling (45). HIF in turn suppresses mitochondrial biogenesis and increases PDK expression, a negative regulator of PDH.

Overall Expression Changes

Microarray analyses indicated that swimming exercise as well as hypoxia caused a stress response in zebrafish larvae. Both groups had an increased expression of heat shock cognate 70 kDa protein, and under hypoxia additionally several cytochrome P450 family members were upregulated. The most expression differences between exercised and hypoxic larvae were detected in genes related to energy metabolism. While in hypoxic larvae fatty acid biosynthesis was stimulated (upregulated fatty acid desaturase 2 expression) cholesterol synthesis was significantly lower during exercise (lower expression level of the insulin-induced gene). The only metabolic gene that was more highly expressed than in hypoxic zebrafish was the solute carrier family 25, member 25, a mitochondrial phosphate carrier required for ATP transfer through the inner mitochondrial membrane. The expression of additional transcripts of citric acid cycle enzymes and the respiratory chain (SDH and cytochrome oxidase) was reduced in the hypoxic group, confirming that hypoxia causes a reduction in aerobic metabolic activity in hypoxic larval zebrafish (73). In exercised larvae transcription of these genes was unaltered, but the expression of PDK, an inhibitor of pyruvate dehydrogenase, was increased. If this is translated to the protein level it suggests that the entry of glucose-derived metabolites into the citric acid cycle was inhibited, while fatty acid oxidation continued. This conclusion is in line with previous observations that after 10 wk of exercise training axial muscle of zebrafish shifted toward a slow aerobic phenotype (76). In muscle-specific HIF-1α knockout mice a lower level of endurance exercise also significantly stimulated fatty acid oxidation, while glucose oxidation was inhibited (37, 38). Zebrafish are capable of very high swimming speeds (53, 54) suggesting that 5 BL/s as used in our study may be a speed that is fuelled by lipid metabolism and does not yet enforce the transition to glucose oxidation. The stimulation of PDK expression combined with an unchanged or even enhanced expression of enzymes involved in mitochondrial ATP production may therefore indicate that the training protocol stimulated lipid metabolism in zebrafish larvae.

Conclusion

Our results showed almost no overlap in either exercise- or hypoxia-induced expression changes in zebrafish larvae. In fact, the greatest differences by far in the expression patterns were observed when the two experimental groups were compared directly. Accordingly, exercise-induced expression changes could not be related to hypoxic conditions. In fish adaptation to low temperature, hypoxia, and endurance exercise is known to result in a remodeling of muscle tissue, favoring a more aerobic phenotype with a better blood supply and a higher density of mitochondria and of enzymes of the aerobic metabolic pathways, but the signaling behind these adaptations of the muscle tissue appears to be different in each case. Some overlap in the gene expression changes and the changes in enzyme activities between cold-adapted fish and exercised fish has been detected, but there were also clear stimulus-dependent responses (40). Our study clearly revealed that there are stimulus-dependent adaptations when hypoxia and exercise are compared and that exercise-induced adaptations are not caused by oxygen-related HIF signaling. An important candidate for exercise-related increase in the aerobic capacity of muscle tissue appears to be adenosine monophosphate-activated protein kinase (AMPK) (38). AMPK has been identified as one of the major regulators in cell metabolism (21), and a change in AMP concentration during exercise will change the AMP:ATP ratio, and this will activate AMPK.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

HIF SIGNALING DURING EXERCISE


