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Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish

David L. M. van der Meer,[1] Guido E. E. J. M. van den Thillart,[1] Frans Witte,[1] Merijn A. G. de Bakker,[1] Jaya Besser,[2] Michael K. Richardson,[1] Herman P. Spaink,[2] Jelani T. D. Leito,[1] and Christoph P. Bagowski[1]

[1]*Departments of Integrative Zoology and* [2]*Molecular Cellular Biology, Institute of Biology, University of Leiden, Leiden, The Netherlands*

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van der Meer, David L. M., Guido E. E. J. M. van den Thillart, Frans Witte, Merijn A. G. de Bakker, Jaya Besser, Michael K. Richardson, Herman P. Spaink, Jelani T. D. Leito, and Christoph P. Bagowski. Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. *Am J Physiol Regul Integr Comp Physiol* 289: R1512–R1519, 2005. First published June 30, 2005; doi:10.1152/ajpregu.00089.2005.—Low oxygen levels (hypoxia) play a role in clinical conditions such as stroke, chronic ischemia, and cancer. To better understand these diseases, it is crucial to study the responses of vertebrates to hypoxia. Among vertebrates, some teleosts have developed the ability to adapt to extremely low oxygen levels. We have studied long-term adaptive responses to hypoxia in adult zebrafish. We used zebrafish that survived severe hypoxic conditions for 3 wk and showed adaptive behavioral and phenotypic changes. We used cDNA microarrays to investigate hypoxia-induced changes in expression of 15,532 genes in the respiratory organs (the gills). We have identified 367 differentially expressed genes of which 117 showed hypoxia-induced and 250 hypoxia-reduced expressions. Metabolic depression was indicated by repression of genes in the TCA cycle in the electron transport chain and of genes involved in protein biosynthesis. We observed enhanced expression of the monocarboxylate transporter and of the oxygen transporter myoglobin. The hypoxia-induced group further included the genes for Niemann-Pick C disease and for Wolman disease [lysosomal acid lipase (LAL)]. Both diseases lead to a similar intra- and extracellular accumulation of cholesterol and glycolipids. The Niemann-Pick C protein binds to cholesterol from internal lysosomal membranes and is involved in cholesterol trafficking. LAL is responsible for lysosomal cholesterol degradation. Our data suggest a novel adaptive mechanism to hypoxia, the induction of genes for lysosomal lipid trafficking and degradation. Studying physiological responses to hypoxia in species tolerant for extremely low oxygen levels can help identify novel regulatory genes, which may have important clinical implications.

microarray analysis; Niemann-Pick disease; lysosomal lipase

FOR MOST ORGANISMS, OXYGEN is essential, and oxygen uptake and metabolism are tightly controlled processes. However, changes in the environment, as well as pathophysiological conditions, can lead to reduced oxygen availability. A number of animal species are able to tolerate severe hypoxic stress. Examples are various fish, frog, and turtle species that can often tolerate situations of anoxia and some snakes and insects that can endure severe hypoxia (16). All situations are evocative of ischemia/reperfusion events that are highly damaging to most mammals and birds. In general, hypoxia even for brief periods, can be detrimental or fatal to humans and most mammals. Insufficient blood supply leads to tissue hypoxia in the brain during stroke, in the heart during acute infarction, and to chronic ischemia (reviewed in Ref. 36). Insufficient oxygen supply can lead to severe necrosis and/or apoptosis in tissues of different organs (13, 19).

In mammals and fish, it was shown that hypoxia, to facilitate better oxygen uptake, leads to an increase in ventilation rate (10, 28). For fish species of two different families, it has also been demonstrated that chronic hypoxia leads to morphological changes of the gills bringing about an increase in gill surface area. In several cichlid species, chronic hypoxia caused elongation of gill filaments and increased the size of the secondary lamellae (6, 46).

In the cyprinid, Crucian carp (*Carassius carassius*), which is especially well suited to withstand periods of anoxia, hypoxia leads to dramatic and reversible gross morphological changes (39). The secondary lamellae are the primary site of oxygen uptake in the gills, and in Crucian carp, they are embedded in a cell mass. Hypoxia in these fish reduces the cell mass through a combination of increasing apoptosis and decreasing proliferation, and thereby leads to an increase in the lamella surface exposed to water, and thus to an increase in gill surface area (39). However, in other systems, adaptations to hypoxia have been shown to lead to inhibition of apoptosis and to stimulation of the antioxidant defense and of stress responses (3, 5, and 14). These hypoxia-induced phenotypic changes and adaptations are due to changes in gene expression.

Two earlier studies have shed light on gene expression changes induced by hypoxia in the goby fish (*Gillichthys mirabilis*) and in zebrafish embryos [*Danio (Brachydanino) rerio*], respectively (15, 42). Ton et al. (43) have profiled immediate early gene expression changes of 4,512 genes in zebrafish embryos exposed to extreme hypoxia. In their study, the conditions used were lethal, and zebrafish embryos cannot survive longer than 24 h under these low oxygen conditions (Ton C, personal communication). Thus a struggle for survival was induced in these embryos, which was intended by the investigators, because they were studying the gene-level mechanism of embryonic survival. However, the conditions also led to an arrest of growth and development. It had been shown (30) earlier that zebrafish embryos under hypoxia are developmentally arrested and under anoxic conditions, embryos arrest in S and G2 cell cycle phases. These earlier studies have clearly

Address for reprint requests and other correspondence: C. P. Bagowski, Institute of Biology, Univ. of Leiden, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands (e-mail: bagowski@rulbim.leidenuniv.nl).

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shown how gene expression pattern can change in the zebrafish embryo in the immediate early response to hypoxia. However, in our study, we used adult zebrafish instead of embryos and nonlethal instead of lethal conditions and focused our work on a single organ, the zebrafish gills. Thereby, for the first time, we have studied long-term adaptive responses to severely low oxygen levels.

Understanding changes in gene expression in fish exposed to hypoxia could reveal new mechanisms of hypoxia tolerance and shed light on the evolution of this adaptive response in vertebrates. This can have important clinical implications.

MATERIALS AND METHODS

Animal handling and hypoxia treatment. Adult wild-type zebrafish (*Danio rerio*) were obtained from a local pet store and handled in compliance with local animal care regulations and standard protocols. Our protocol was approved by the review board of Leiden University in accordance with animal protocols of the government of The Netherlands. Fish were kept at 28°C in aquaria with 12:12-h light-dark cycles and were fed twice daily with commercial flake food. Oxygen levels were gradually decreased in 4 days from 80–90% to 60, 40, 20, and the final 10% air saturation. After *day 4* the fish were kept for an additional 21 days at 10% air saturation. (At 100% air saturation and 28°C the O_2 concentration is 8 mg/l). In parallel, a control group was kept at 80–90% air-saturated water. Both groups were kept in identical aquaria of 100 liters. The oxygen level on the hypoxia group was kept constant by a controller (Applikon) connected to an oxygen electrode and solenoid valve inline with an air diffuser. The oxygen level in the tank was kept constant by adding oxygen via the diffuser and thereby compensating the oxygen consumption of the fish.

Gill dissection. The fish were killed with an overdose of anesthetic (MS-222 tricaine methanesulfonate; Argent Chemical Laboratories). Gill covers were removed, and the branchial arches I to IV were dissected from both sides of each fish. For RNA preparation, the filaments of both hemibranches were removed from the bony elements of each gill arch. For scanning electron microscopy, the gill arches of three fish from each group were left intact and fixed immediately in Karnovsky fixative (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) for 4 h at 4°C. After three washes in 0.1 M phosphate buffer, pH 7.2, they were transferred to 70% ethanol.

RNA preparation. After dissection, gills were homogenized in a Dounce homogenizer using 1 ml Trizol solution (GIBCO-BRL, Life Technologies). The whole lamella was used and freed from the pharyngular bone. For each individual sample, a pool of gills from three different animals was used. After Trizol extraction, total RNA was further purified using RNAeasy columns (Quiagen). RNA samples were analyzed for quality control by Lab-on-a-Chip analysis (Agilent) and on agarose gels.

Microarray construction. Microarrays of the MWG Biotech 14k Zebrafish Oligonucleotide Set were produced using the facilities of the Leiden Genome Technology Center (http://www.lgtc.nl). Oligonucleotides were dissolved in 150 mM phosphate buffer (pH 8.5) to a concentration of 20 μM. With Omnigrid 100 (Genemachines), oligonucleotides were spotted on Codelink activated slides (Amersham Biosciences) according to the Amersham Codelink protocol and as described elsewhere (26, 40a). Microarrays were spotted according to the MIAME guidelines with 15,532 unique zebrafish oligonucleotides from the MWG oligonucleotide set. Ambion's array control oligonucleotides (8 sense oligos) and three custom-designed oligonucleotides were spotted together with the MWG Oligonucleotide Set. Spotted Codelink activated slides were treated with blocking solution and washed according to the manufacturer's instructions.

Gene expression analysis. cDNA was synthesized from 2 μg total RNA and then amplified using Ambion's Message Amp kit by ServiceXS (Leiden, the Netherlands). During in vitro transcription from the cDNA template, 5-(3-aminoallyl)-UTP (Ambion) was incorporated. Subsequently, 5 μg of cRNA was used for coupling of Amersham's monoreactive Cye 3 or Cye 5 dyes and 1.5 μg of the fluorescent-labeled cRNA was used for the hybridizations as previously described (40a). The Ambion array control RNA spikes (amount per spike varying from 0–200 pg) were added during the labeling reaction. Labeled cRNA was purified over RNAeasy columns (Quiagen), and the quality was checked by Agilent's Lab-on-a-Chip total RNA nanobiosizing assay. Prehybridization, hybridization (55°C), and scanning were carried out as previously described (26, 40a).

Data analysis. The local background was subtracted from the fluorescent value of each spot. Feature intensities were extracted from scanned microarray images using GenePix Pro 5.1. (Axon Instruments). Images were visually inspected, and spots from low-quality areas of the array were flagged and excluded from further analysis. Spots were also excluded from analysis if both the combined fluorescent intensity for both channels was < 1.4 times that of the local background and the pixel-by-pixel correlation coefficient of the spot was < 0.4. Fluorescence ratios were normalized as previously described (44). A hierarchical clustering algorithm (11) was applied to those genes that fulfilled all of the following conditions: *1*) they were not flagged, *2*) they were differentially expressed (equal or higher than 1.5-fold induced or equal or $<$ 2-fold repressed), *3*) they had P values ≤ 0.02; and they were consistent on at least four of the five arrays. In total, 367 genes matched all of these criteria. The average linkage clustered data were visualized using Treeview (11). Data were submitted to GenBank (NCBI Gene Expression Omnibus Series Accession No. GSE2069; Sample Accession Nos. GSM37552, GSM37577, GSM37578, GSM37579, GSM37580; Platform Accession No. GPL1743; http://www.ncbi.nlm.nih.gov/projects/geo).

Real-time quantitative RT-PCR. For verification of gene expression, we used quantitative real-time RT-PCR. The Roche Master SYBR Green kit was used for the RT-PCR reactions. The annealing and synthesis temperature was 55°C alternating with 96°C for 45 cycles. Dissociation protocols were used to measure melting curves and control for unspecific signals from the primers. We used 100 ng of total RNA per reaction. A standard curve for β-actin using 1, 5, 10, 100, and 500 ng of total RNA was used for normalization. Samples were measured in the Roche LightCycler. The Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design primers for short amplicons between 50 and 100 bases. The primers used are shown in Table 1.

Scanning electron microscopy. Gills stored in 70% ethanol were dehydrated to prepare them for scanning electron microscopy. Gills were dehydrated through an ascending acetone series until reaching 100%. Acetone in the tissue was replaced with liquid CO_2 cooled to 10°C in a Critical Point Dryer (Baltec CPD 030). The temperature was raised to 40°C after several rinses with CO_2 to saturate the gills completely with CO_2. During this temperature rise, the critical point was reached and the CO_2 liquid was instantaneously converted to gas (35). Silver glue was used to attach the gills to stubs, and they were then sputter coated (Polaron Equipment, scanning electron microscopy coating unit E5100) with gold for 3 min in a low vacuum/argon environment. In the scanning electron microscopy (Jeol JSM-6400) it is possible to tilt and turn the specimen and inspect and photograph the gills from every angle (35). The acceleration voltage was set to 80 kV and the working distance was between 19 and 22 mm. Pictures were taken of the whole gill arch and the primary and secondary lamellae.

RESULTS AND DISCUSSION

Cellular and organismal responses to hypoxia come in two kinds, those that help to survive short hypoxia exposures and those that are necessary to continue living under hypoxia. While the first kind is based on slowing down or even com-

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Table 1. *Primers used for short amplicons between 50 and 100 bases*

Gene Name	Primer Sequences	Accession No.
β-Actin (ACTIN)	Forward: 5′-TCCTGGGTATGGAATCTTGC-3′ Reverse: 5′-GCACTGTGTTGGCATACAGG-3′	CK739369
Chaperonin-containing tcp-1 delta (CCTD)	Forward: 5′-ATTGCTGGAGCTCTTCTGGA-3′ Reverse: 5′-CAGCGGTGTACTGATGGATG-3′	CD015665
Melanoma differentiation-associated protein 9 (MDA9)	Forward: 5′-CATCAGGAGGGCAGAGATTC-3′ Reverse: 5′-CAGCTGCACAAACACTCCAT-3′	BQ449764
Membrane type 1 matrix metalloproteinase (MT1MMP)	Forward: 5′-CAAGGAGGAAGGATCTGGAA-3′ Reverse: 5′-TGCATGCAGACCTGTAAAGC-3′	BI883810
Death-associated protein 3 (DAP3)	Forward: 5′-CGGGTTACAACGAGAAGGAG-3′ Reverse: 5′-TCTTCAGACCGACTGTGTGG-3′	BM155684
Progesterone receptor-binding protein (PRBP)	Forward: 5′-AATGGTTCCTCCAGTGCAAG-3′ Reverse: 5′-TCTCCTCCTCTGCAATTGGT-3′	BM958174
Cytochrome c oxidase subunit iv (COX)	Forward: 5′-TCCCTGAACAGGGTTGATTC-3′ Reverse: 5′-GCAGAAGGAGATCCAGAGGA-3′	BU709821
Nadh dehydrogenase ubiquinone 42 kDa subunit (NADHDH)	Forward: 5′-GTTTCAATGGCCTTCTGCTC-3′ Reverse: 5′-GAGCCCTATACAACCCGTGA-3′	CK738951
Nieman-pick disease gene C (NP)	Forward: 5′-GTTTGTGGGAATGCTGTCCT-3′ Reverse: 5′-GATAGCGTCTGCTCCAGTCC-3′	BG799923
Lysosomal acid lipase (LAL)	Forward: 5′-AAACCTGCCCAACAACAGTC-3′ Reverse: 5′-CGACTGTTCCCGATCCATAC-3′	BQ419125

Abbreviations for gene names are in parentheses.

pletely shutting down metabolic processes and also involves induction of cell stress responses (29), the second kind provides the key adaptive mechanisms of long-term survival. Among vertebrates, some teleost fish have developed the ability to adapt to extreme low oxygen levels. Earlier studies have measured the survival time during lethal hypoxia in adult zebrafish (34). It was shown that zebrafish can survive a P_{O_2} of 15 Torr (10% air saturation; 0.8 mg/l) for 2 days, whereas immediate exposure to P_{O_2} of 8 Torr (5% air saturation; 0.4 mg/l) was lethal within 12 h (34). It was further shown that preconditioning to hypoxia lowered the mortality rate (34).

For our study, we have used adult zebrafish and exposed them to hypoxia. We were interested in the adaptive response to hypoxia and gradually lowered the P_{O_2} to a nonlethal dose of 10% air saturation (0.8 mg/l). At this oxygen level, zebrafish are still able to grow and gain weight and they can survive these conditions for longer than 6 mo (data not shown). We observed a decrease in activities, a complete lack of rapid turns and movements, and an increased ventilation rate (Supplemental data for this article may be found at http://ajpregu.physiology.org/cgi/content/full/00089.2005/DCI); shown are control fish (normoxia) or zebrafish, which have been under hypoxic conditions for 14 days (hypoxia). We have focused our initial studies on the respiratory organs, the zebrafish gills.

We were particularly interested in gene expression changes associated with the adaptive response to hypoxia in vertebrates. We used cDNA microarrays to profile gene expression changes in response to hypoxia in gills of adult zebrafish. We have identified 367 genes that were differentially expressed under hypoxic conditions. The majority of differentially regulated genes showed a decrease in gene expression (68.1% or 250 genes in total) compared with genes that showed increased expression levels (31.9% or 117 genes in total). Criteria used for differential expression were $\geq$ 1.5 induced and twofold reduced. If criteria for both cases were twofold induced, 286 genes showed differential expression, including 36 induced genes. In our study, we used the same criteria employed in earlier studies on zebrafish embryos (43). All relevant data are submitted (NCBI Gene Expression Omnibus).

Earlier studies (6, 46) have shown that hypoxia causes rapid phenotypic changes in gills of species of two other fish families leading to an increase in surface area. Using scanning electron microscopy, we found different phenotypic changes in zebrafish gills, which could potentially have a similar effect. We observed an increase in cell number and a decrease in cell size in epithelial cells of the secondary lamellae (Fig. 1). The observed cells also appeared to be more ruffled under hypoxic conditions compared with gills isolated from the normoxic control group (Fig. 1). This could lead to an increase in effective surface area of the gills and potentially increase the oxygen extraction capacity at low P_{O_2}.

The observed increase in cell number in the secondary lamellae in the gills could be explained by changes in the gene expression we observed for genes important for cell growth and cell division (Fig. 2*G*). The protooncogene neuroblastoma-ras is a membrane-bound small G protein often coupled to growth factor receptors. Ras serves as a molecular switch, converting signals from the cell membrane to the nucleus leading to protein synthesis and cell proliferation (1). Hypoxia not only induces neuroblastoma-ras gene expression in the gills (Fig. 2*G*), it also leads to a decrease of expression of the ras inhibitor p70 ste5 (Ref. 25; Fig. 2*G*). Furthermore, hypoxia increases expression of cyclin ania-6a (cyclin L; Fig. 2*G*), which plays a role in promoting cell cycle entry and is commonly overexpressed in primary tumors (44a). These changes in gene expression indicate cell proliferation in the zebrafish gills under long-term hypoxia.

Fish respond to low oxygen levels by activation of anaerobic glycolysis and, in most cases, by metabolic depression and energy conservation (7, 41, 44b, 48). This is reflected by gene expression changes observed earlier in zebrafish embryos exposed to hypoxia (43). We observed similar gene expression changes in the gills of the adult fish. Genes of the TCA cycle and the electron transfer chain that are involved in mitochondrial energy production were found to be repressed. No genes involved in these processes were found to be induced (Fig. 2*A*). In contrast, genes involved in glycolysis, like glycogen phosphorylase and aldolase, showed increased expression under

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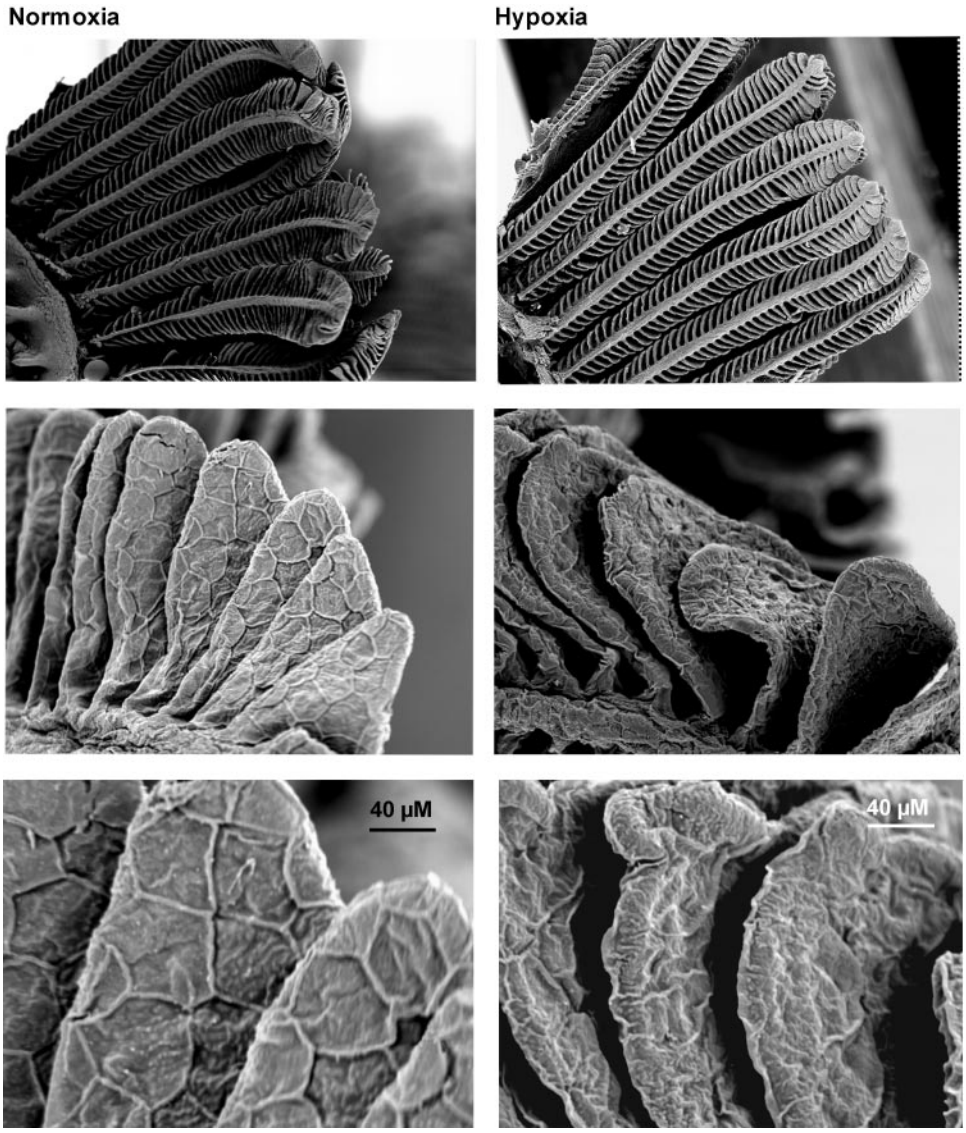

Fig. 1. Scanning electron microscopy pictures of gills from normoxia control zebrafish (*left*) and hypoxia-treated fish (*right*). Magnification for the 2 pictures on *top*, for pictures in the *middle*, and for pictures on the *bottom* was comparable. The scale indicated is only valid for the two *bottom* pictures. The top pictures show the primary lamellae, whereas higher magnifications only show secondary lamellae (*middle* and *bottom*). Under hypoxic conditions, cells seen on the surface of the secondary lamellae appear smaller and the surface appears to be more ruffled. Similar data were seen in gills of 3 independent preparations. Several gill arches were investigated for each preparation.

hypoxic conditions (Fig. 2*A*). These findings are not novel, but indicate that some gene expression changes are comparable between the long-term hypoxia response in adult zebrafish gills and the immediate response in embryos.

We discovered in the gills that hypoxic conditions reduced gene expression of components of fat metabolism, uptake, and transport. Examples are acyl-CoA dehydrogenase, intestinal fatty acid binding protein, and oocyte-type fatty acid binding protein, respectively (Fig. 2*A*). β-oxidation consumes a lot of oxygen, and decreasing β-oxidation could help to save oxygen. It is well known that ischemia, due to the accumulation of intermediates of the β-oxidation, causes major cell damage and leads to heart failure (27).

One of the most energy-consuming cellular processes is protein biosynthesis. In the goby fish (*Gillichthys mirabilis*), and in zebrafish embryos, hypoxia leads to repression of genes encoding components of the protein translation machinery (15, 43). Likewise, in our studies, ribosomal proteins and their subunits are the largest group of hypoxia-repressed genes. No ribosomal protein was found to be induced by hypoxia (Fig. 2*B*). Conversely, we found that hypoxia stimulated gene expression of two translational elongation factors, eef-1a and eef-2 (Fig. 2*B*). Roles other than translational control have been assigned to eef-1a, and it is possible that this is true for eef-2 as well (23). We further found that hypoxia decreased gene expression of the eukaryotic initiation factor-2α subunit kinase (PEK) (Fig. 2*B*). In response to environmental stresses, PEK has been shown to reduce protein synthesis by phosphorylation of the α-subunit of eukaryotic translation initiation factor-2α (37). It is possible that PEK is not directly involved in hypoxia-induced repression of translation. PEK had also been shown to be involved in the cellular stress response (40). Another possible explanation for this observation is that the reduction in PEK expression does not correlate with a decrease in PEK activity.

An organism uses many different strategies to adjust to hypoxic conditions. We found several novel hypoxia-dependent gene expression changes that point to physiological adaptations to low oxygen levels previously not described. An example is the monocarboxylate transporter (mct4), which transports metabolites like pyruvate and lactate. In our study, we found that hypoxia increased gene expression of mct4 (Fig.

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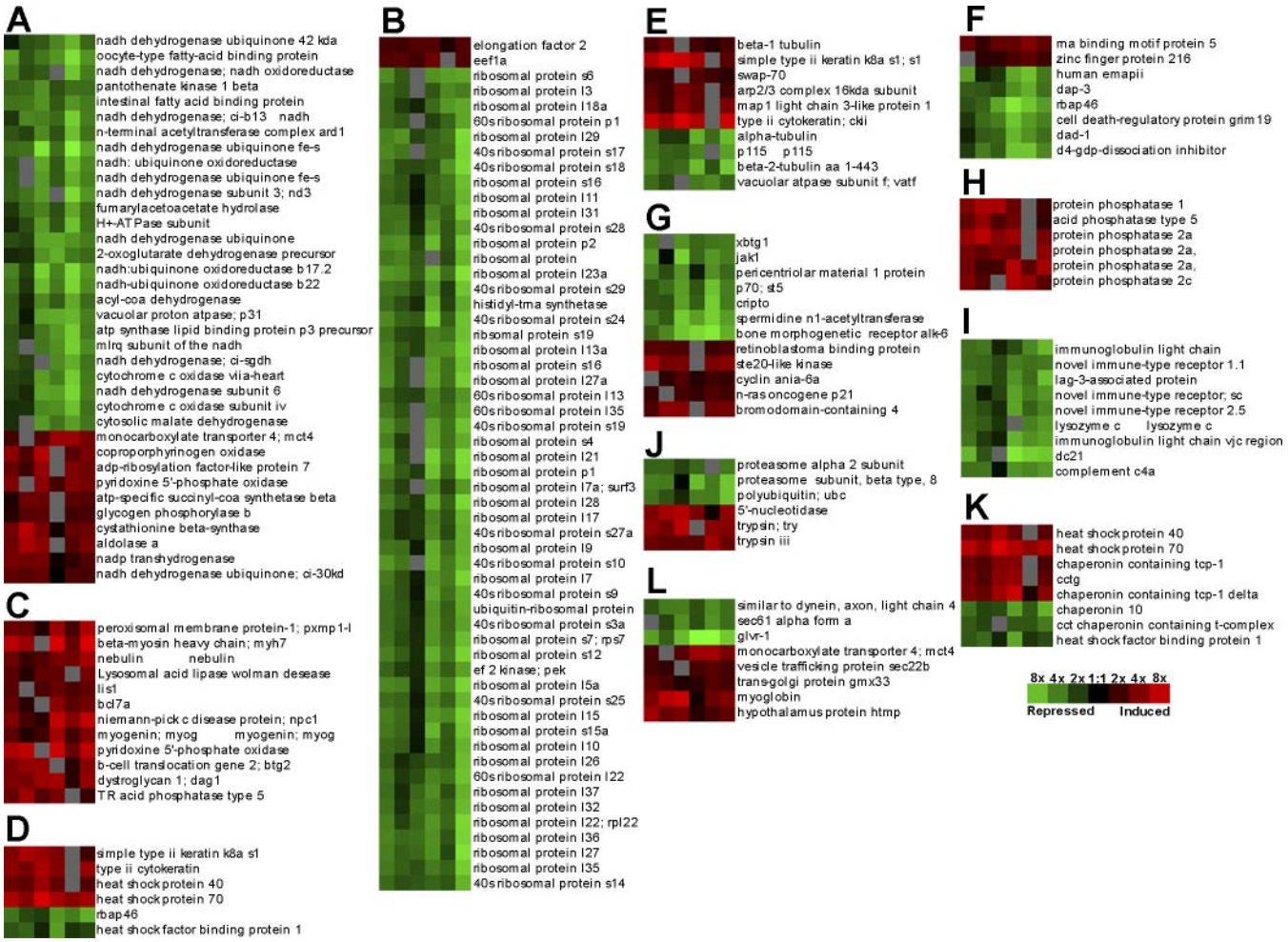

Fig. 2. Selected genes from a total of 367 genes found to be differentially expressed under hypoxic conditions were grouped into categories based on their biological function or their biological properties. The cluster analysis of the following categories is shown: metabolism (*A*), translation (*B*), disease related (*C*), stress response (*D*), cytoskeleton and cell architecture (*E*), apoptosis (*F*), growth regulation (*G*), phosphatases (*H*), immune response (*I*), Degradation (*J*), chaperones (*K*), and transport (*L*). Quantitative changes in gene expression are induced genes (red), and repressed genes (green). Results of the individual slides are in vertical *columns 1-5* (starting at the *left* of each cluster). Vertical *column 6* (far *right* column of the cluster) represents the mean. Gene expression changes had to be consistent on at least 4 of 5 arrays. Each slide represents a comparison of normoxia vs. hypoxia samples. Hypoxia samples were labeled with Cye 3 and normoxia samples with Cye 5 (1, 3, 4, 5) except for *number 2*, which was a dye swap experiment (corresponding to *number 5*) (the color indication was later changed so that it is consistent between all 6 vertical columns). The complete annotated data set is available at NCBI Geo Gene Expression Omnibus (see MATERIALS AND METHODS for details) and a complete list of the 367 differential expressed genes and an expression profile (supplemental data for this article may be found at http://ajpregu.physiology.org/cgi/content/full/00089.2005/DCI).

2*L*). If oxygen supplies do not meet demands, enhanced anaerobic metabolism leads to accumulation of lactate, a phenomenon best known in muscle tissue. The monocarboxylate transporter can help to remove accumulated lactate and transport it to the bloodstream, particularly to the heart and other aerobic tissues. Further studies are necessary to show that increased transport of monocarboxylates is indeed an important adaptive mechanism to chronic hypoxia.

Another important physiological adaptation to hypoxia is indicated by our findings of enhanced expression of the oxygen transporter myoglobin (Fig. 2*L*). Myoglobin increases the oxygen diffusion rate through tissues (38). Thus by increasing myoglobin levels, the organism could compensate for the gradient decline and stabilize the oxygen flux to its tissues.

A novel mechanism of adapting to low oxygen levels is suggested by our findings that genes causative for two genetic metabolic disorders affecting cholesterol trafficking and degradation showed enhanced expression under hypoxia (Fig. 2*C*). One is the Niemann-Pick disease gene C (NPC) and the other is lysosomal acid lipase (LAL; cholesterol esterase), which is linked to Wolman disease. The Niemann-Pick disease is an inherited metabolic disorder. NPC is a neurovisceral disorder characterized by accumulation of cholesterol and glycolipids in the lysosomal/late endosomal system, which is due to mutations on either the NPC1 or the NPC2 genes (31). Although NPC1 and NPC2 proteins appear essential for proper cellular cholesterol trafficking, their precise functions and relationship have remained elusive (31).

LAL is essential for the hydrolysis of cholesteryl esters and triglycerides in the lysosome (49). Defective LAL activity in Wolman disease results in large amounts of lipids, particularly cholesteryl esters and glycerides, to accumulate in the brain, liver, spleen, lymph nodes, and other tissues (36). Strikingly, both Niemann-Pick and Wolman disease lead to accumulation

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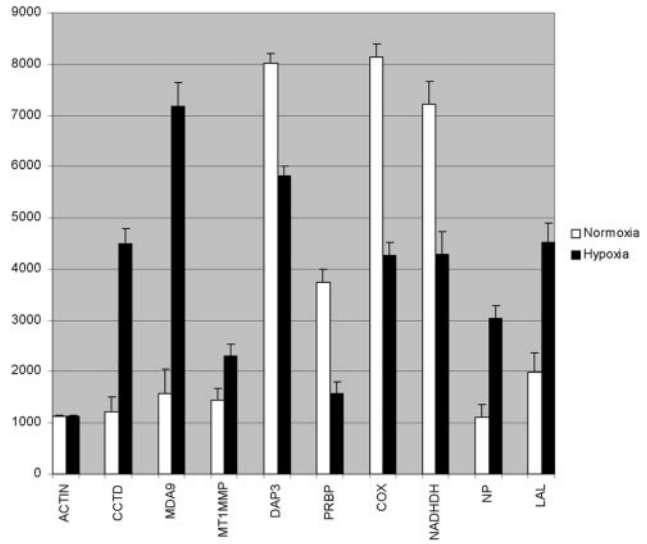

Fig. 3. Selected genes found to be differentially expressed on the microarrays, were analyzed by quantitative real-time RT-PCR. Relative expression is given based on normalization to β-actin. A standard curve for β-actin was included in each experiment, and data represent 3 independent experiments, each done in triplicates. Gene abbreviations are defined in Table 1.

of cholesteryl esters and glycerides in several tissues, and both are connected to lysosomes. Lysosomes represent the machinery for cellular turnover. We speculate that both NPC and LAL are involved in biomembrane turnover, transport, or generation of nonmetabolizable lipids, such as cholesterol and sphingosine. Thus upregulation may be expected in active tissues and tissues exposed to wear and tear, especially in their membranes. Hypoxia would lead to a higher need for biomembranes possibly due to higher wear and tear because of the increase in ventilation rate. This hypothesis could explain our observation of ruffled cell membranes on the surface of the secondary lamellae (Fig. 1). Both genes have so far not been implicated in a response to hypoxia. We have confirmed our microarray results for both genes by quantitative PCR (Fig. 3). The upregulation of these genes corroborates clearly with the above-mentioned downregulation of genes for lipid oxidation. Additional studies are necessary to further clarify the role of the Niemann-Pick disease gene and of LAL in adaptation to hypoxia.

Hypoxic conditions represent high stress levels that can lead to the induction of genes involved in the cellular stress response, like heat shock proteins (HSPs). Ischemia-induced expression of HSP70 has been demonstrated in tissues of intact animals (8) and in mammalian cell culture cells (2). HSP70 functions as a chaperone and is also known to protect cells against apoptosis (18). We observed increased expression of genes for HSP70 and HSP40 (Fig. 2*K*). We also observed decreased expression for other chaperones like chaperonin 10 (Fig. 2*K*), which is involved in the folding of newly synthesized proteins (12). Because protein biosynthesis appears to be lowered, it fits that chaperonin 10 expression is also downregulated. In contrast to HSP70 and HSP40, other genes involved in the cellular stress response showed decreased expression (Fig. 2*D*). This could be due to the time point we have chosen; different stress proteins are switched on and off at different times during exposure to stress. We investigated the long-term response to hypoxia. A future study including earlier time points could shed light on the temporal induction of stress response and other genes.

As pointed out in the introduction, anoxia induces apoptosis in the gills of the Crucian carp leading to an increase in gill surface area. We have not found any indication of hypoxia-induced cell death neither by apoptosis nor necrosis in zebrafish gills (data not shown). We have discovered expression changes for several genes involved in apoptosis (Fig. 2*F*). However, the majority of the observed gene regulations tend to be antiapoptotic.

We discovered that hypoxia induced expression levels of four different phosphatases, whereas no phosphatase was found with repressed expression (Fig. 2*H*). This was similar for the clustering of disease-related genes, which exclusively showed enhanced gene expression (Fig. 2*C* and Table 2).

Clustering for genes of the immune response revealed that this group solely showed a decrease in gene expression under hypoxic conditions (Fig. 2*I*). However, these observations should not lead to general conclusions, because the numbers of phosphatases, disease-related genes, and immune response genes found are not large enough.

Several of the genes we found differentially expressed in the zebrafish had been described as hypoxia-responsive genes in other systems. To further verify our results, we used quantitative real-time PCR for nine transcripts. We confirmed the gene expression changes of these nine transcripts found in the

Table 2. *Different disease-related genes with enhanced expression upon long-term hypoxia treatment in the zebrafish gills*

Hypoxia-Induced Gene	Related Disease	Potential Gene Function	Reference No.
NPC gene	Nieman-Pick disease	Lipid transport	31
LAL	Wolman disease	Lipase	49, 9
Lissencephaly-1 (LIS-1)	Lissencephaly	Cytoskeletal dynamics, cell division and motility	22
B-cell CLL/lymphoma 7A (Bcl7a)	B-cell non-Hodgkin lymphoma (B-NHL)	Unknown (translocation found in B-NHL)	47
Peroxisomal membrane protein 1 (Pxmp1; ABC3)	Zellweger syndrome	Peroxisomal ABC transporter	24
Myosin heavy chain 7 (Myh7)	Hypertrophic cardiomyopathy	Molecular motor protein	20
Dystroglycan	Congenital muscular dystrophy	Cell adhesion molecule	4
Tartrate acid phosphatase 5 (TR AP type 5)	Metabolic bone diseases	Acid phosphatase	21
Nebulin	Congenital myopathy	Determines length of thin filament in muscle	45

Potential gene functions are given and future experiments are necessary to delineate their role in hypoxia. Abbreviations for gene names or disease are in parentheses.

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microarray studies by this independent method (Fig. 3). The fold induction values were not directly comparable, but in all cases, induction or reduction was confirmed. Quantitative differences between array data and qPCR results have been reported previously (26, 43).

Our study has shown that cDNA microarray analysis is well suited to studying the responses to physiological stresses, such as low oxygen levels. In this study, we have identified specific changes in gene expression in the gills of adult zebrafish and identified possible novel mechanisms for the long-term adaptive response to hypoxia. We identified several genes that currently have not been linked to hypoxia. It remains to be determined which of these changes are tissue specific. We are currently investigating adaptive gene expression changes to long-term exposure to hypoxia in zebrafish heart and muscle tissues. Future studies will be very useful to obtain a global view of tissue- and species-specific gene expression changes and to delineate the role of the individual genes found. This will lead to a better understanding of the adaptive responses to hypoxia and findings in zebrafish might have clinical implications in humans.

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