Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (*Carassius carassius*)

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Summary

Hypoxia-inducible transcription factor-1 (HIF-1) is a master regulator of hypoxia-induced gene responses. To find out whether HIF-1 function is involved in gene expression changes associated with temperature acclimation as well as in hypoxia adaptation in poikilotherms, we studied HIF-1 DNA binding activity and HIF-1α expression in normoxia and during hypoxia (0.7 mg l^{-1} O$_2$) in crucian carp at temperatures of 26, 18 and 8°C. Temperature had a marked influence on HIF-1 in normoxia. Although HIF-1α mRNA levels remained unaltered, cold acclimation (8°C) increased HIF-1α protein amounts in the liver, gills and heart and HIF-1 DNA binding activity in the heart, gills and kidney of crucian carp by two- to threefold compared to warm acclimated fish (26°C). In the heart and kidney HIF-1 activity was already significantly increased in the 18°C acclimated fish. Temperature also affected hypoxic regulation of HIF-1. Although hypoxia initially increased amounts of HIF-1α protein in all studied tissues at every temperature, except for liver at 18°C, HIF-1 activity increased only in the heart of 8°C acclimated and in the gills of 18°C acclimated fish. At 8°C HIF-1α mRNA levels increased transiently in the gills after 6 h of hypoxia and in the kidney after 48 h of hypoxia. In the gills at 26°C HIF-1α mRNA levels increased after 6 h of hypoxia and remained above normoxic levels for up to 48 h of hypoxia. These results show that HIF-1 is involved in controlling gene responses to both oxygen and temperature in crucian carp. No overall transcriptional control mechanism has been described for low temperature acclimation in poikilotherms, but the present results suggest that HIF-1 could have a role in such regulation. Moreover, this study highlights interaction of the two prime factors defining metabolism, temperature and oxygen, in the transcriptional control of metabolic homeostasis in animals.

Introduction

Hypoxia inducible factor-1 (HIF-1) is an evolutionarily conserved transcription factor that functions as a main regulator of gene expression in response to hypoxia (Wenger, 2002). The transcriptionally active HIF-1 consists of two constitutively expressed subunits, α and β. The β subunit is identical to ARNT (aryl hydrocarbon nuclear translocator), whereas the α subunit is unique for HIF-1, and confers the oxygen sensitivity to the molecule. HIF-1 mediated gene expression is oxygen-sensitive, in part because HIF-1α is rapidly degraded in normoxic conditions but stable in hypoxia. Degradation of HIF-1α in normoxia is mediated by the oxygen-dependent degradation domain (ODD), in which conserved proline residues are covalently modified by prolyl hydroxylase enzymes (Ivan et al., 2001). When hydroxylated, HIF-1α is recognized by the von-Hippel-Lindau protein, ubiquitinated, and degraded *via* the proteasomal pathway. In hypoxia the stabilized HIF-1α enters the nucleus and dimerizes with ARNT. The dimer binds to hypoxia-responsive elements (HRE) in the promoter/enhancer region of the target genes, and thereafter interacts with transcriptional co-activators. In part, the oxygen sensitivity of HIF function is caused by oxygen-dependent hydroxylation of a specific asparagine residue near the C terminus of HIF-1α (Lando et al., 2002). The asparagine residue is required for interaction with co-activator CBP/p300, which cannot occur if the amino acid is hydroxylated. This hydroxylation event is catalyzed by an asparaginyl hydroxylase, also known as factor inhibiting HIF-1 (FIH-1) (Mahon et al., 2001). In mammals, HIF-1 promotes transcription of genes encoding proteins involved in angiogenesis, erythropoiesis, glucose transport, glycolysis, iron transport, cell proliferation and cell survival (Semenza, 2002).

Although HIF-1 is the master transcriptional regulator of hypoxia-induced gene expression, its function is also modulated independently from oxygen. A variety of growth factors, hormones and cytokines regulate HIF-1 in mammalian cells by enhancing transcription and translation of HIF-1α and by affecting on DNA binding of HIF-1 dimer (Richard et al., 2000; Page et al., 2002; Treins et al., 2002; Ma et al., 2004). Moreover, HIF-1 appears to be needed for basal transcriptional levels of its target genes (Stroka et al., 2001; Mason et al., 2004; Huang et al., 2004). For example, in normoxic cardiac muscle, loss of HIF-1 transcriptional pathway impairs contractility, and reduces vascularization, ATP and phosphocreatine levels (Huang et al., 2004). Thus, HIF-1 activity appears also to be needed for maintenance of energetic homeostasis in tissues at normal physiological oxygen tensions. Given the fundamentality of thermal effects for metabolism, it could be expected that temperature also affects HIF-1. Indeed, exposure to heat, leading to conditions resembling fever, increases HIF-1α protein amounts in mice (Katschinski et al., 2002). However, heat-induction of HIF-1α appears to be insufficient for HIF-1 activation and alteration of gene expression, and thus the significance of the phenomenon has remained unclear (Katschinski et al., 2002).

The effects of temperature on HIF-1 function have not been studied in poikilothermic animals, although they constitute the vast majority of animals and even though they routinely face large fluctuations in their core body temperature. As an example, the body temperature of crucian carp, a temperate aquatic poikilotherm, varies from near freezing to above 20°C. Crucian carp are also commonly exposed to hypoxia or even anoxia during both winter and summer. During winter at freezing temperatures the hypoxia or anoxia is usually of long duration, up to many months, whereas in summer bouts of hypoxia in the absence of photosynthesis alternate with hyperoxic conditions when photosynthesis is active diurnally in eutrophic ponds. In the present study on crucian carp, we show that activity of hypoxia-inducible factor-1 is increased at low temperatures, and that body temperature and hypoxia interact markedly in the regulation of HIF-1 function in a poikilothermic animal.

Materials and methods
Animals

Crucian carp (*Carassius carassius* L., mean mass 46±17 g) were caught from a pond near Turku, Finland, in May–June and were held in tanks under circulation of aerated carbon filtered tapwater, the water temperature corresponding to that in the pond at the time of catching (8–13°C). The fish were acclimated to temperatures of 8, 18 or 26°C by changing the temperature 1°C per day and were kept at these temperatures for 2 weeks prior to experimentation. The fish were fed trout pellets (TESS, Raisio, Finland) regularly, but were fasted for 3 days prior to and during the experiments. The 3-day fasting may influence the physiological responses of fishes differently at different temperatures. At present the effects of food

deprivation on HIF function are not known, but one would expect to see more of a stress at high than at low temperatures, since the energy consumption of fish increases with temperature.

Hypoxia exposure

For hypoxia exposure the oxygen concentration of the water was decreased to 0.7 mg l^{-1} (6–8% of air saturation) over a 1–2 h period by regulated nitrogen bubbling. The oxygen concentration was monitored with an oxygen probe throughout the experimentation. The fish were removed from the tank for sampling after 6, 24 or 48 h of hypoxia. They were immediately killed by a blow to head and the heart, liver, gills and kidney were dissected, frozen in liquid nitrogen and stored at –80°C. Normoxic fish were kept in an aerated tank and sampled at random times during the hypoxia exposure.

Preparation of protein extracts

Protein extracts for immunoblot analyses and electrophoretic mobility shift assay were prepared from the tissue samples as earlier described (Semenza and Wang, 1992) with small modifications. Tissue pieces were homogenized on ice in buffer containing 10 mmol l^{-1} KCl, 1.5 mmol l^{-1} MgCl$_2$, 10 mmol l^{-1} Tris/HCl, pH 7.8, 0.5 mmol l^{-1} phenylmethyl-sulphonyl fluoride, 0.5 mmol l^{-1} dithiothreitol, 1 mmol l^{-1} Na$_3$Vo$_4$, 2 μg ml^{-1} approtein, 2 μg ml^{-1} leupeptin, 2 μg ml^{-1} antipain and 2 μg ml^{-1} pepstatin. The homogenates were centrifuged at 1500 *g* for 20 min at +4°C. The supernatants containing cytosol were frozen in liquid nitrogen and stored at –80°C. The nuclear pellets were resuspended in two volumes of buffer containing 420 mmol l^{-1} KCl, 1.5 mmol l^{-1} MgCl$_2$, 20 mmol l^{-1} Tris/HCl, pH 7.8, 0.5 mmol l^{-1}, 25% glycerol, 0.5 mmol l^{-1} phenylmethylsulphonyl fluoride, 0.5 mmol l^{-1} dithiothreitol, 1 mmol l^{-1} Na$_3$Vo$_4$, 2 μg ml^{-1} approtein, 2 μg ml^{-1} leupeptin, 2 μg ml^{-1} antipain and 2 μg ml^{-1} pepstatin, incubated on ice with frequent vortexing for 30 min and centrifuged at 10 000 *g* for 30 min at +4°C. The supernatants were dialyzed against a buffer containing 100 mmol l^{-1} KCl, 0.2 mmol l^{-1} EDTA, 20 mmol l^{-1} Tris/HCl, pH 7.8 and 20% glycerol overnight at 4°C. The dialysates were centrifuged at 10 000 *g* for 30 min at +4°C and supernatants containing nuclear proteins were frozen in liquid nitrogen and stored at –80°C. To assess the cellular localization of heat shock proteins (Hsps), immunoblot analyses of Hsps was carried out in both nuclear and cytoplasmic protein extracts. All other analyses were carried out in nuclear extracts.

Immunoblot analysis and co-immunoprecipitation

Nuclear or cytoplasmic protein (30 μg) was separated on an 8% SDS-polyacrylamide gel and transferred into nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked in 3% non-fat dry milk in PBS with 0.3% Tween20 for 1.5 h and incubated in primary antibodies. The primary antibodies and the dilutions used were the following: polyclonal N-terminal rainbow trout HIF-1α (Soitamo et al., 2001) 1:200, monoclonal rat anti-Hsp90 (SPA-

835, Stressgen Biotechnologies, Victoria, Canada) 1:500, and monoclonal mouse anti-Hsp70 (clone 3a3, Affinity Bioreagents, Golden, CO, USA) 1:10000. Membranes were incubated in horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotechnologies, Buckinghamshire, UK) and the proteins were detected using an enhanced chemiluminescense assay (ECL, Amersham Pharmacia Biotechologies). The signals were captured on X-ray film, and analysed with Chemi-imager (Alpha Innotech Corp., San Leandro, CA, USA). Equal loading was confirmed by staining gels with Coomassie Brilliant Blue.

For co-immunoprecipitation, 1 mg of liver nuclear protein was incubated with 3 μg of Hsp90 antibody at 4°C for overnight. Protein A immobilized on Sepharose CL-4B in PBS (60 μl, 50 μg μl^{-1}, Sigma) was added and the sample was incubated for a further 1 h at 4°C. The immunoprecipitate was collected by centrifugation and washed two times with 200 μl of ice-cold buffer containing 100 mmol l^{-1} KCl, 0.2 mmol l^{-1} EDTA, 20 mmol l^{-1} and Tris/HCl, pH 7.8. The interacting proteins were detached from the beads by suspending the pellet into the sample loading buffer containing lithium dodecyl sulphate and incubating the sample for 10 min at 75°C. The precipitated proteins were subjected to immunoblot analyses as described above.

Electrophoretic mobility shift assay (EMSA)

The binding activity of HIF-1-dimer on conserved HIF-binding sites in DNA (HRE, hypoxia response element) was analysed in nuclear extracts using electrophoretic mobility shift assay carried out as earlier described (Kvietikova et al., 1995). Since no fish sequences were available, the sense and antisense strands for the HIF-1 binding sites in the promoter region of human erythropoietin gene (Kvietikova et al., 1995) were used for generation of γ-^{32}P-labelled oligonucleotide probe. The specificity of crucian carp HIF-1 binding to HRE of human erythropoietin was ensured by supershift experiments and by experiments using γ-^{32}P-labelled mutated oligonucleotide probe of the same gene (5′GCCCTA-AAAGCTGTCTCA3′, mutated bases underlined). Reaction mixtures containing 10 μg of nuclear protein, 0.1 μg of poly(dI-dC) and γ-^{32}P-labelled oligonucleotide in 10 mmol l^{-1} Tris, pH 7.5, 50 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl, 1 mmol l^{-1} MgCl$_2$, 1 mmol l^{-1} EDTA, 5% (v/v) glycerol and 5 mmol l^{-1} DTT were incubated for 30 min on ice. For supeshift gels, N-terminal rainbow trout HIF-1α antibody (1 μl), actin antibody (clone AC-40, Sigma, 100 ng) or in some reactions bovine serum albumin (100 ng), was added to reaction mixture. The samples were run in a native 4% polyacrylamide gel for 1.5 h at 150 V at room temperature. The gel was dried under vacuum and the protein–DNA complexes were visualized by autoradiography (Fuji Super RX), and analysed with Chemi-imager.

Northern blot analysis

Total RNA was isolated from the tissue samples using Sigma Tri Reagent according to the manufacturer's instructions. Equal amounts (20 μg) of glyoxylated total RNA were separated on a 1.25% agarose gel, and transferred to nylon membrane (Hypond-N, Amersham Corp., UK). Membranes were hybridised with [α-^{32}P]dCTP-labelled DNA-probes specific for crucian carp HIF-1α sequence (Sollid et al., 2005), GenBank accession number DQ306727) and for rainbow trout β-actin sequence (accession number AJ438158). After stringent washing (5× SSC 0.1% SDS, 2× SSC 0.1% SDS, 1× SSC 0.1% SDS and 0.2× SSC 0.1% SDS for 10 min each, all at room temperature), the membranes were visualized by autoradiography (Kodak X-OMAT AR) and analysed with Chemi-imager. Ribosomal 18S RNA was stained from the membrane using Methylene Blue after transfer. Since changes in β-actin mRNA in response to temperature were often opposite to HIF-1α and 18S, mRNA data were normalized to ribosomal 18S RNA.

Statistical analyses

Statistical significance of the Gaussian distributed data was tested by analysis of variance (ANOVA) followed by Holm–Sidak or Dunnet's *post* test, as appropriate, using SigmaStat software. The significance of the data not following Gaussian distribution (HIF-1α protein data) was analysed by the generalized linear model (GENMOD, log link function, Poisson distribution) with fish mass as covariate, using SAS Enterprise software.

Results

Crucian carp HIF-1α

HIF-1α protein and DNA binding activity of HIF-1 were detected at high levels in normoxic crucian carp tissues. The antibody recognized three HIF-1α bands with molecular masses of approximately 85, 91 and 94 kDa (Fig. 1). The latter two probably represent post-translational modifications of the protein, while the 85 kDa band corresponds to the predicted size of native HIF-1α protein (Sollid et al., 2005). The 91 kDa band is possibly a modified but phosphatase-resistant HIF-1α form and the 94 kDa band could be the phosphorylated HIF-1α form (Katschinski et al., 2002). Supershift experiments and experiments with mutated probe ensured that crucian carp HIF-1 binds in the HRE of human erythropoietin gene (supplementary material, Fig. S2).

The 91 kDa HIF-1α form was the most dominant in amount, and only this band was quantified. There was a very high
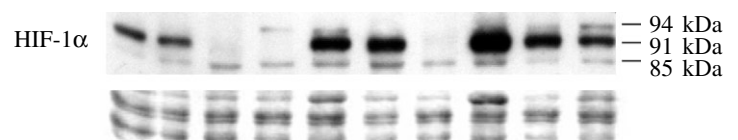

Fig. 1. An immunoblot showing three HIF-1α forms and individual variation of HIF-1α protein amounts in crucian carp. In the top panel, each lane represents HIF-1α detected in nuclear extracts of liver from normoxic fish at 8°C. The bottom panel shows the gel stained with Coomassie Brilliant Blue.
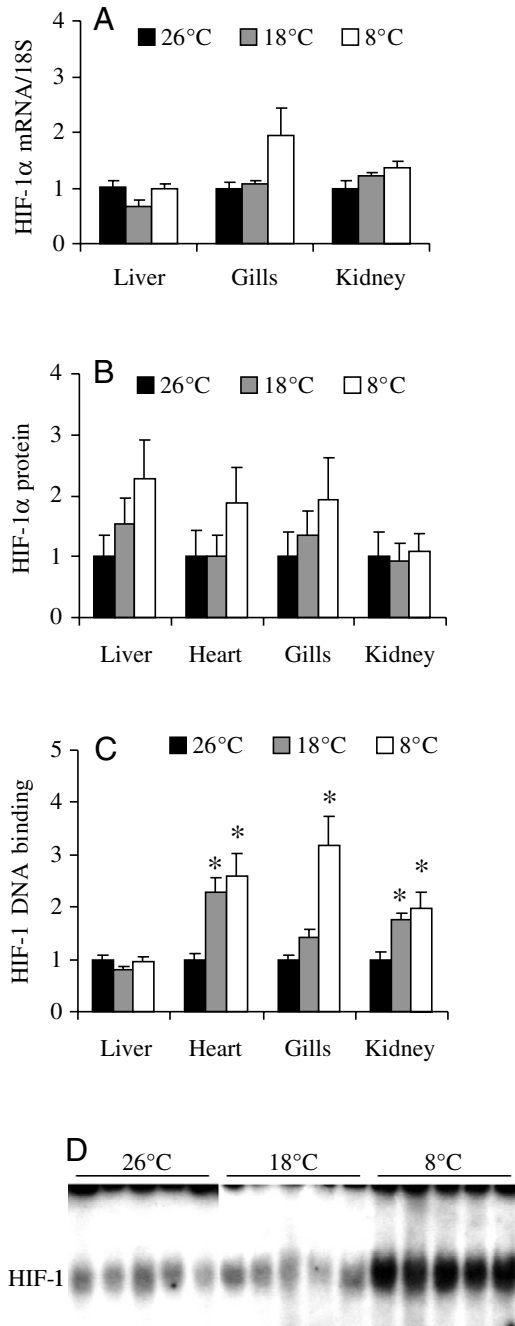
Fig. 2. Cold body temperature activates HIF-1. (A) HIF-1α mRNA amounts, (B) HIF-1α protein amounts, (C) HIF-1 DNA-binding activities in normoxic fish acclimated to 26°C, 18°C or 8°C. Results are shown as fold increase compared to fish acclimated to 26°C and are means ± s.e.m. of 10 fish. HIF-1α protein data includes variation caused by differences in fish mass. Asterisks denote significant ($P<0.05$, $P<0.001$, $P<0.001$, $P<0.05$ and $P<0.01$ for heart 18°C, heart 8°C, gills 8°C, kidney 18°C and kidney 8°C, respectively) differences to fish acclimated to 26°C (two-way ANOVA followed by Holm–Sidak *post* test). (D) Representative electrophoretic mobility shift assays showing binding of HIF-1 on HRE of human erythropoietin gene in nuclear extracts from gills of fish acclimated to 26°C, 18°C or 8°C (5 individuals for each temperature).

Table S1), showing that the amount of the HIF-1α protein was characteristic for each fish.

Cold body temperature activates HIF-1

Although temperature did not have any significant effect on HIF-1α mRNA levels (Fig. 2A) in normoxia, both HIF-1α protein amounts and especially the DNA binding activity of HIF-1 increased with decreasing temperature (Fig. 2B,C). Temperature had a significant effect on HIF-1α protein amounts in the liver and gills of crucian carp ($\chi^2=25.74$, $P<0.0001$ and $\chi^2=21.68$, $P<0.0001$, Figs 2B, 4). In the heart, acclimation to cold (8°C) increased HIF-1α protein amounts slightly, but not significantly ($\chi^2=4.94$, $P<0.0846$, Figs 2B and 4B), whereas in the kidney temperature had no effect on HIF-1α amounts (Figs 2B and 4D). Cold acclimation (8°C) increased HIF-1 DNA-binding activity significantly in the heart, gills and kidney of crucian carp (Fig. 2C). In the heart and kidney HIF-1 DNA binding was already significantly increased in the 18°C acclimated compared to 26°C acclimated fish. Notably, temperature did not affect HIF-1 DNA binding activity in the liver (Fig. 2C).

Cold induces heat shock proteins

In mammalian cells, Hsp90–Hsp70 complex binds HIF-1α protein and in that way protects HIF-1α against oxygen-independent proteosomal degradation in both normoxia and hypoxia (Isaacs et al., 2002; Katschinski et al., 2004; Zhou et al., 2004). Thus increased amounts of HIF-1α in response to elevated body temperature are presumably a direct consequence of induction of heat shock proteins of the 90 and 70 family (Katschinski et al., 2002). In view of this interaction, we studied whether decreasing body temperature influenced Hsp70 and 90 levels in crucian carp.

Two Hsp90 forms, likely representing the constitutively expressed β form and weakly expressed, but stress-inducible α form, were detected in crucian carp tissues. Yet it was not possible to distinguish these two forms quantitatively. Amounts of Hsp90 proteins in nuclear extracts from all studied tissues were significantly higher at 8°C than at 26°C, with the most pronounced induction in the gills (Fig. 3A). In the liver, Hsp90 proteins were already significantly increased in 18°C acclimated compared to 26°C acclimated fish.

individual variation in the amount of HIF-1α protein in both normoxic and hypoxic fish, which also caused zero density values in immunoblots (Fig. 1). Part of the variation was related to the mass of the fish; small fish tended to have more HIF-1α in all studied tissues (supplementary material Fig. S1 and Table S1). Fish mass also affected the time course of HIF-1α response to hypoxia; the response was faster in small fish (GENMOD, $\chi^2=10.86$, $P<0.05$; $\chi^2=18.50$, $P<0.001$; $\chi^2=22.73$, $P<0.0001$ and $\chi^2=10.51$, $P<0.05$, in the liver, heart, gills and kidney, respectively; data from heart in supplementary material Fig. S1). HIF-1α amounts in all studied tissues of normoxic fish correlated significantly (supplementary material
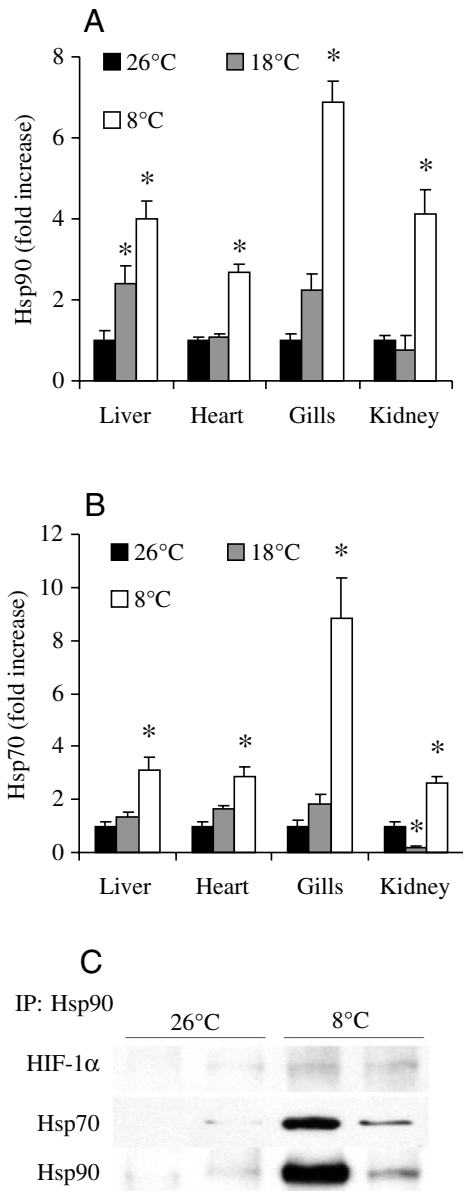
Fig. 3. Cold temperature induces heat shock proteins. (A) Hsp90 amounts in normoxic fish at 26°C, 18°C or 8°C. Results are shown as fold increase compared to levels in normoxic fish at 26°C and are means ± s.e.m. of 5–10 fish. Asterisks denote significant ($P<0.001$) differences to fish at 26°C (ANOVA and Dunnet's). (B) Hsp70 amounts in normoxic fish at 26°C, 18°C or 8°C. Results are shown as fold increase compared to levels in normoxic fish at 26°C and are means ± s.e.m. of 5–10 fish. Asterisks denote significant differences to fish at 26°C (ANOVA and Dunnet's, $P<0.001$). (C) Interaction of Hsp90 and Hsp70 with HIF-1α in crucian carp. Nuclear extracts from the liver of normoxic crucian carp were immunoprecipitated (IP) with Hsp90 antibody, thereafter HIF-1α, Hsp70 and Hsp90 were detected from the precipitates by immunoblot analysis.

Likewise cold acclimation (8°C) significantly increased amounts of Hsp70 family proteins in nuclear extracts of all crucian carp tissues studied (Fig. 3B). Additionally, the Hsp70 protein amount was significantly lower in the kidney of 18°C

acclimated than in the 26°C acclimated fish (Fig. 3B). The antibody recognizing both the constitutive and the stress-inducible proteins of the Hsp70 family detected one protein band in the liver and kidney and two bands in the gills and heart of crucian carp (data not shown). In the gills and heart the amounts of both fractions appeared to increase upon cold acclimation. Yet, due to the close proximity of molecular masses of Hsp70-family proteins it remains unknown which proportions of the expression are due to the different Hsp70 isoforms.

To assess the cellular localization of Hsps we measured Hsp70 and Hsp90 in cytoplasmic extracts of crucian carp tissues. Hsp expression in cytoplasm was in accordance with that in the nucleus. Yet in the gills the cold induction of Hsps was weaker in the cytoplasm (only three- to fourfold induction compared to seven- to ninefold induction in the nucleus, data not shown), suggesting that in gills at cold temperatures more Hsps are localized in the nucleus.

We thereafter used a co-immunoprecipitation assay to determine the interaction of Hsps and HIF-1α in crucian carp tissues. After immunoprecipitation with Hsp90 antibody, both HIF-1α and Hsp70 were detected in crucian carp liver nuclear extracts by immunoblot analysis (Fig. 3C). This suggests that the Hsp90–Hsp70 complex also binds HIF-1α in crucian carp tissues. Note that the reliability of co-immunoprecipitation in demonstrating interactions between proteins is limited by the possibility that protein complexes are formed during the assay rather than *in vivo* in the tissues (Sambrook, 2001). To control this the nuclear extracts were prepared in a way causing minimum disruption to protein–protein interactions occurring *in vivo*, i.e. buffers contained low salt concentrations and no detergents were used.

Temperature affects hypoxic regulation of HIF-1

Crucian carp were exposed to a water oxygen concentration of 0.7 mg l^{-1}. This oxygen level is below their critical O$_2$ concentration (~1.0 mg l^{-1} at 8°C for crucian carp) (Nilsson, 1992), which represents the P_{O_2} at which oxygen delivery to the tissues becomes seriously compromised and fish can no longer fulfil their energy requirements by aerobic metabolism alone. Consequently adaptive responses, including activation of anaerobic energy production and remodelling of gill epithelia to enhance oxygen uptake, are turned on (Vanwaversveld et al., 1989; Sollid et al., 2003).

Exposure to hypoxia initially increased HIF-1α protein amounts, with the exception of liver at 18°C, in all studied tissues at every temperature (Fig. 4). However, the DNA-binding activity of HIF-1 increased in hypoxia only at 8°C and 18°C (Fig. 5). In the heart of 8°C acclimated fish, HIF-1 DNA binding activity was significantly increased after 6 and 24 h of hypoxia, and in the gills of 18°C acclimated fish HIF-1 activity increased after 48 h of hypoxia (Fig. 5). In addition, temperature had a significant effect on the hypoxic response of HIF-1 in the kidney of crucian carp ($P<0.001$, two-way ANOVA). Similar to the temperature response, hypoxia did not affect HIF-1 DNA-binding activity in the liver. HIF-1α protein
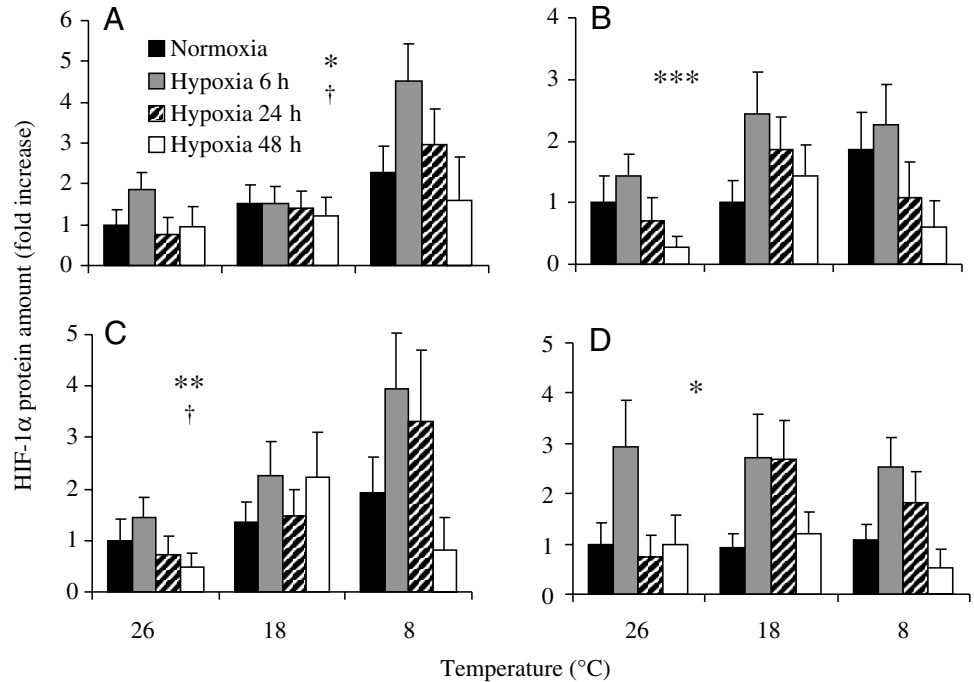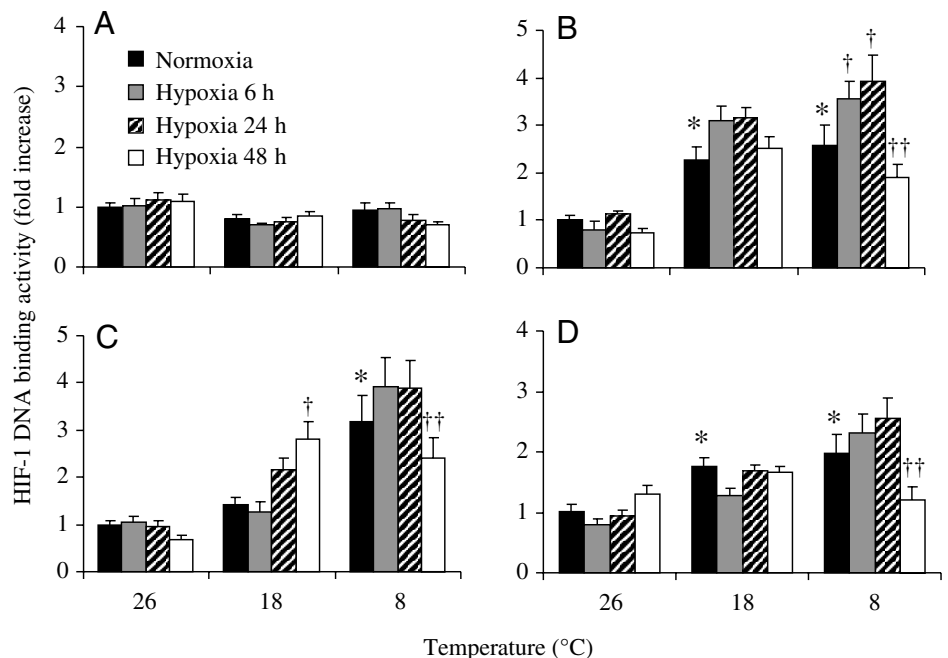
Fig. 4. HIF-1α protein amounts in the liver (A), heart (B), gills (C), and kidney (D), of fish exposed to hypoxia at 26, 18 or 8°C. Results are shown as fold increase compared to levels in normoxic fish at 26°C and are means ± s.e.m. of 10 fish (includes variation caused by differences in fish mass). Asterisks denote significant effect of hypoxia on HIF-1α amounts (GENMOD, *P<0.01, **P<0.001, ***P<0.0001). †Significant effect of temperature on HIF-1α amounts (GENMOD, P<0.0001). GENMOD analysis does not allow *post* tests for testing differences between study groups.

amounts diminished after 24 or 48 h of hypoxia (Fig. 4). Following the same time course, HIF-1 DNA binding activities in the heart, gills and kidney of 8°C acclimated fish diminished significantly after 48 h of hypoxia (Fig. 5).

Hypoxia also had an influence on HIF-1α mRNA amounts in crucian carp tissues. In 8°C acclimated fish, HIF-1α mRNA amounts increased transiently in the gills after 6 h of hypoxia and in the kidney after 48 h of hypoxia (Fig. 6B,C). In the gills of 26°C acclimated fish HIF-1α mRNA amounts increased significantly after 6 hours of hypoxia, and

were still above normoxic levels after 48 h of hypoxia (Fig. 6B). Note that the transient changes in HIF-1α mRNA amounts in the gills and in the kidney were accompanied by concomitant significant increases in β-actin mRNA (data not shown) while the induction in the gills of 26°C acclimated fish was specific to HIF-1α mRNA. Additionally HIF-1α mRNA amounts decreased significantly after 48 h of hypoxia in the liver of 8°C acclimated fish (Fig. 6A). Since there were no significant differences in HIF-1α or β-actin mRNAs between normoxic fish at different

Fig. 5. HIF-1 DNA-binding activities in the liver (A), heart (B), gills (C), and kidney (D), of fish exposed to hypoxia at 26°C, 18°C or 8°C. Results are shown as fold increase compared to levels in normoxic fish at 26°C and are means ±s.e.m. of 10 fish. Temperature has a significant effect on hypoxic activation of HIF-1 in the heart (P<0.05), gills (P<0.01) and kidney (P<0.001) of crucian carp (two-way ANOVA). *Significant difference to normoxic fish at 26°C (for P-values, see Fig. 2), †significant difference (P<0.05, P<0.01 and P<0.05 for heart 6 h hypoxia, heart 24 h hypoxia and gills 48 h hypoxia, respectively) to normoxic fish at the same temperature, ††significant difference (P<0.05, P<0.05 and P<0.001 for heart, gills and kidney, respectively) to fish exposed to hypoxia for 24 h at the same temperature (Holm–Sidak *post* test).
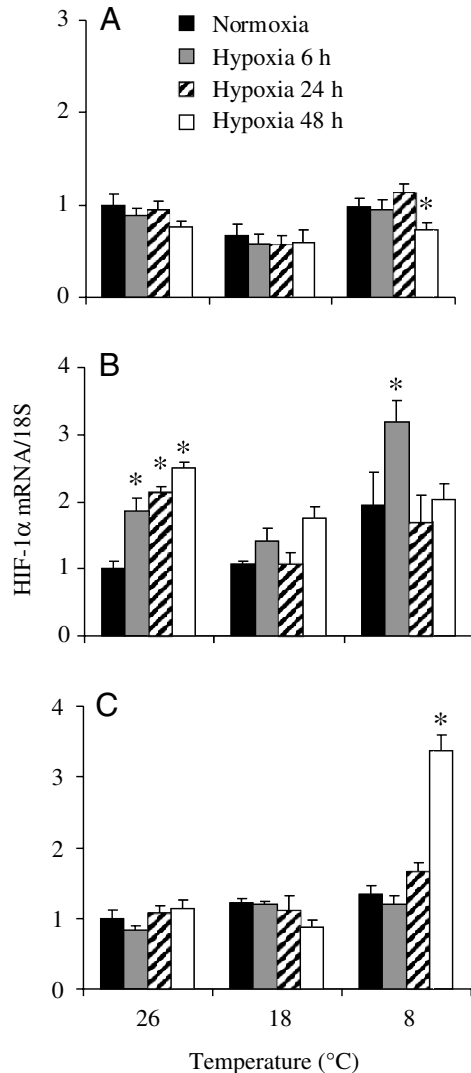
Fig. 6. HIF-1α mRNA amounts in the liver (A), gills (B) and kidney (C) of fish exposed to hypoxia at 26°C, 18°C or 8°C. Results are shown as fold increase compared to levels in normoxic fish at 26°C and are means ± s.e.m. of 5–10 fish. Temperature has a significant effect on the hypoxia response of HIF-1α mRNA in the liver ($P<0.05$), gills ($P<0.01$) and kidney ($P<0.01$) of crucian carp (two-way ANOVA). Asterisks denote significant difference ($P<0.05$, $P<0.01$, $P<0.01$, $P<0.001$, $P<0.01$ and $P<0.001$ for liver 8°C 48 h, gills 26°C 6 h, gills 26°C 24 h, gills 26°C 48 h, gills 8°C 6 h and kidney 8°C 48 h, respectively) to normoxic fish at the same temperature (Holm–Sidak *post* test).

temperatures, the effect of temperature on both mRNAs is apparently directed to the hypoxia response (Fig. 6; β-actin data not shown). A representative northern blot is shown in supplementary material Fig. S3)

Discussion

Role of HIF-1 in cold acclimation

While the prominent role of HIF-1 in hypoxia adaptation and in pathological conditions is often emphasized, less attention has

been given to the significance of this transcription factor in normoxic tissues. Yet, growing evidence indicates that HIF-1 also has important roles in metabolic control of tissue function at high oxygen tensions. Although HIF-1α amounts in mammalian cells upon normoxia are generally less than the detection limits of immunoblot analysis, immunohistochemistry reveals the presence of nuclear HIF-1α protein in normoxic mammalian tissues (Stroka et al., 2001). Most notably, recent studies (Mason et al., 2004; Huang et al., 2004) persuasively show the consequences of loss of the HIF-1 transcriptional pathway on gene expression, energetics and function of normal cardiac and skeletal muscle in mice. Cardiac myocyte-specific deletion of HIF-1α impairs metabolism-related gene expression and reduces vascularization as well as ATP, phosphocreatine and lactate levels in normoxic heart (Huang et al., 2004). Subsequently, contractile function and Ca^{2+} handling in the heart are impaired. In skeletal muscle, loss of HIF-1α abolishes the induction of glycolytic enzymes during exercise and enhances aerobic energy metabolism (Mason et al., 2004). Moreover, disruption of HIF-1α has been shown to decrease the basal expression of HIF-1 target genes in normoxic embryonic stem cells (Iyer et al., 1998) and to reduce basal ATP levels in other cell types (Seagroves et al., 2001). Thus, normoxic HIF-1 activity is significant for maintenance of energetic homeostasis in tissues.

Change in body temperature indisputably challenges tissue homeostasis. Since a reduction of temperature decreases the rates of biochemical reactions two- to threefold for each 10°C drop, the transition to cold is accompanied by depression of metabolism and physical activity. Usually poikilotherms do not completely submit to these Q_{10} effects, but use a variety of compensatory modifications to maintain higher level of activity and energy production. In eurythermic fish such compensatory responses include changes in the enzyme isoform pattern and a general increase in both specific activities and expression of enzymes involved in energy metabolism (Somero, 2004; Gracey et al., 2004). Thus, cold acclimation requires reorganization of metabolism with an increased capacity for cellular energy production, and, therefore, profound changes in gene expression are required. No overall transcriptional control mechanism has been described for this process, but the present results suggest that HIF-1 could have such a role.

Although cold temperature increased HIF-1 DNA-binding markedly in several crucian carp tissues, it remains obscure which genes are induced by HIF-1 upon cold acclimation. First, it is presently unknown whether target genes of normoxic HIF-1 activation differ from those induced by hypoxia. Second, HIF-1 regulated gene expression is poorly known in fish in general. Only the HRE in the lactate dehydrogenase-B (LDH-B) gene promoter region of killifish *Fundulus heteroclitus* has been determined (Rees et al., 2001). However, a cDNA microarray study on hypoxic goby fish *Gillichthys mirabilis* showed tissue-specific induction of genes involved in anaerobic energy metabolism and glucose homeostasis, suggesting that HIF-1 in fish may induce the same genes in hypoxia as mammalian HIF-

1 (Gracey et al., 2001). Moreover, a recent cDNA microarray study on the responses of common carp to reduced temperature indicates similarities to the observed HIF-1 responses in crucian carp tissues (Gracey et al., 2004). Although induction of genes coding for proteins of the electron transport chain was common to all tissues in the cold acclimated phenotype, transcriptional changes of other energetic pathways showed tissue specificity. While expression of most glycolytic genes was increased in the gills, kidney and, to a lesser extent, in the heart of cold acclimated fish, transcript profiles of liver showed cold-induced transition to lipid metabolism and activation of the pentose phosphate pathway (Gracey et al., 2004). Thus, it is possible that the lack of HIF-1 response in the liver of crucian carp indicates different metabolic responses both to hypoxia and to cold between liver and other tissues.

Interestingly, increased amounts of HIF-1α protein at cold body temperatures have recently also been reported in a hibernating mammal, the thirteen-lined ground squirrel *Spermophilus tridecemlineatus*, and in freeze-tolerant insect larvae, goldenrod gall fly *Eurosta solidaginis* (Morin and Storey, 2005; Morin et al., 2005). These findings suggest that HIF-1 may have a conserved function in transcriptional response to cold among organisms.

Mechanisms for cold activation of HIF-1

Activation of HIF-1 includes transcription, translation and stabilization of HIF-1α as well as its phosphorylation, nuclear translocation, dimerization with ARNT, DNA-binding, recruitment of transcriptional cofactors and target gene *trans*-activation. In addition to oxygen supply and cellular redox state, a variety of oxygen-independent factors including nutrients, cytokines, growth factors and hormones have been shown to control these different activating steps (Wenger, 2002).

Here we describe a novel oxygen-independent activator of HIF-1, cold body temperature. Higher HIF-1 activities in cold acclimated compared to warm acclimated fish cannot be attributed to tissue hypoxia. Moreover, the additive responses and divergent time courses in HIF-1 regulation by cold and hypoxia suggest that distinct pathways are utilized.

Earlier studies of temperature effects on HIF-1 were restricted to homeothermic mammals. It is not known how the activation of HIF-1 at cold body temperatures in the hibernating thirteen-lined ground squirrel occurs (Morin and Storey, 2005). In mouse, an elevated body temperature is associated with increased amounts of HIF-1α protein in several tissues (Katschinski et al., 2002). Hsp90 levels were shown to correlate with HIF-1α protein levels. Hsp90 bound HIF-1α, and was required for HIF-1α induction by heat. Later it was found that association of HIF-1α with the Hsp90–Hsp70 complex reduces the proteosomal degradation of HIF-1α in both normoxia and hypoxia (Isaacs et al., 2002; Katschinski et al., 2004; Zhou et al., 2004). Thus, heat induction of HIF-1α is presumably a direct consequence of increased Hsp70 and Hsp90 amounts. Our results suggest that increased Hsp90 and Hsp70 amounts could similarly stabilize HIF-1α protein at cold temperatures in crucian carp tissues. The effects of

reduced temperatures on these chaperones have been little studied. Yet, in addition to cold-shock treatment, where the temperature is rapidly reduced for a few hours (Ali et al., 2003), increased expression of Hsps of the 90 and 70 families have also been observed when fish have been acclimated to lowered temperatures at a range that is normally experienced by the animals (Gracey et al., 2004; Deane and Woo, 2005). This response likely serves to protect cells from increased misfolding and denaturation of proteins at cold temperatures.

HIF-1α protein stabilization alone, however, is not sufficient for HIF-1 activation and consecutive gene expression in normoxic conditions (Richard et al., 1999; Katschinski et al., 2002). Thus, interaction with Hsps cannot be the only mechanism responsible for cold activation of HIF-1. As the endocrine system is involved in compensatory responses of thermal acclimation in aquatic poikilotherms (Larsen et al., 2001; Gabillard et al., 2003a; Gabillard et al., 2003b), increased normoxic HIF-1 activity could result from hormonal or growth factor stimulation, as in mammalian cells. The main mechanism implicated in normoxic activation of HIF-1 is PI$_3$-kinase pathway-mediated increase in translation of HIF-1α protein (Dery et al., 2005). Some normoxic inducers also increase HIF-1α mRNA transcription, possibly through activation of diacyl-glycerol sensitive protein kinase C (Page et al., 2002). Similar to the hibernating ground squirrel (Morin and Storey, 2005), and to tissues other than skeletal muscle in cold-acclimated common carp (Gracey et al., 2004), temperature did not affect HIF-1α mRNA amounts in normoxic crucian carp. This renders increased translation of HIF-1α as the likely mechanism contributing to cold activation of HIF-1. Still, it is important to note that temperature has major impact on transcription and translation in general and a temperature change may affect protein levels *per se* by changing the relative rates of synthesis and degradation of proteins (Somero, 1995; Hochachka and Somero, 2002).

Interactions of temperature and hypoxia

Hypoxia increased HIF-1α protein amounts in crucian carp tissues equally at all temperatures, indicating that HIF-1α protein responds to reduced tissue oxygen levels. However, HIF-1 DNA-binding activity increased during hypoxia in the heart, gills and kidney of fish acclimated to 8 and 18°C, whereas in warm-acclimated (26°C) fish it remained unaltered in all tissues studied, showing a temperature-dependent difference in the induction of hypoxia-sensitive genes. Yet, owing to higher oxygen demand due to the Q_{10} effect, tissue hypoxia is more severe in warm than in cold acclimated fish. This shows that crucian carp does not utilize the hypoxic HIF-1 response at the oxygen level used in the experiment at temperatures close to the optimum temperature (27°C) for the species, but the response is connected to the cold acclimated phenotype. The reason for this may be related to the life history of crucian carp. This species has evolved its exceptional hypoxia tolerance in response to overwintering at freezing temperatures. While winter acclimatized crucian carp survive for about 5 months in anoxia at 2°C, anoxic survival of summer acclimatized individuals at 18°C is limited to a few days (Piironen and Holopainen, 1986).

Moreover, even in cold acclimated fish, severe hypoxia increased HIF-1 activity only weakly. Thus HIF-1 responds more to acclimation to the lowered temperature than to hypoxia at the lowered temperature. This is in accordance with the well-known benefits of cold body temperature for animals in limiting oxygen (Wood, 1991). Evidently, the gene expression pattern of cold adapted phenotype, including high HIF-1 activity, also promotes hypoxia-tolerance in cold.

The transient hypoxic HIF-1 response is in accordance with the results of mammalian studies (Stroka et al., 2001), and is thought to result from HIF-1-induced increase in the expression of prolyl hydroxylases degrading HIF-1α protein, which forms a direct negative feedback mechanism to control HIF-1 activity under prolonged hypoxia (Marxsen et al., 2004). In addition to protein stability, hypoxia also seems to regulate the HIF-1 response of crucian carp at the mRNA level. Although this is apparently not the case in cultured mammalian cells, *in vivo* reports on the regulation of HIF-1α expression in hypoxia are contradictory (Wiener et al., 1996; Wenger et al., 1997). Interestingly, in a study comparing the most hypoxia-tolerant mammal known, the subterranean mole rat (*Spalax* sp.), and rat (*Rattus norvegigus*), HIF-1α mRNA amounts increased under severe hypoxia in the kidney of mole rat, whereas in rat kidney HIF-1α mRNA amounts remained unaltered (Shams et al., 2004). Since the normoxic HIF-1α expression was also two times higher in mole rat than in rat, it is tempting to speculate that high basal expression of HIF-1α, and hypoxic regulation of its mRNA levels, would be common adaptive features which enhance hypoxia tolerance of animals. In crucian carp hypoxic regulation of HIF-1α mRNA was temperature-dependent. This further emphasizes interaction of the two prime factors defining metabolism, temperature and oxygen, in the transcriptional control of metabolic homeostasis in poikilohermic animals.

Conclusion

This is the first study where the effect of temperature on HIF-1 function has been studied in a poikilothermic vertebrate. We show that HIF-1 activity increases with falling temperature, and is apparently more sensitive to lowered temperature than to hypoxia *per se*. No overall transcriptional control mechanism has been described for low temperature acclimation in poikilotherms, but the present results suggest that HIF-1 could have a role in such regulation. Moreover, we show a pronounced interaction between body temperature and hypoxia in the regulation of HIF-1 function in a poikilothermic animal, crucian carp.

List of abbreviations

ARNT	aryl hydrocarbon nuclear translocator
FIH-1	factor inhibiting HIF-1
HIF-1	hypoxia inducible factor-1
HIF-1α	hypoxia-inducible-factor-1α
HRE	hypoxia-response elements
Hsp	heat shock protein
LDH-B	lactate dehydrogenase-B
ODD	oxygen-dependent degradation domain

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Table S1. *Correlation of HIF-1α protein amounts between different tissues in crucian carp*

	Liver	Heart	Gills	Kidney
Liver	1			
Heart	0.804635	1		
Gills	0.836895	0.803971	1	
Kidney	0.783969	0.751661	0.845377	1

Correlation matrix of HIF-1α protein amounts in different tissues of normoxic crucian carp. Values are correlation coefficients.