Cell proliferation and gill morphology in anoxic crucian carp

Jørund Sollid,1 Aina Kjernsli,1 Paula M. De Angelis,2 Åsmund K. Røhr,1 and Gøran E. Nilsson1

1Department of Molecular Biosciences, University of Oslo and 2Institute of Pathology, Rikshospitalet, Oslo, Norway

Submitted 14 April 2005; accepted in final form 18 May 2005

MAMMALS, AND MOST other vertebrates, are intolerant to prolonged oxygen deprivation (8). However, there are a few vertebrates and invertebrates that are anoxia tolerant. Invertebrates like Caenorhabditis elegans, Drosophila melanogaster, and Artemia franciscana are able to survive anoxia through suspended animation and arrest of cell division (5, 7, 10, 27). Furthermore, this response to anoxia is also found in some vertebrates like zebra fish (Danio rerio) embryo and turtles of the genera Trachemys and Chrysemys (6, 28, 36). Crucian carp can survive anoxia for several months at 4°C (13). However, crucian carp is, unlike the other species, still active in anoxia (21, 22, 35). An active species will have a need for basic maintenance of organs, which is likely to demand maintained cell proliferation and therefore DNA synthesis.

Functional ribonucleotide reductase (RNR) enzymes are needed for DNA synthesis. They convert ribonucleotides to deoxyribonucleotides through a radical-based reaction. The RNR enzymes are divided into three main classes based on their utilization of metal cofactors (17). In vertebrates, RNR consists of four subunits (α2β2) where the oxygen-dependent radical formation occurs in the two β-subunits (R2). Previous findings on vertebrate RNR have shown radical half-life, which is essential for catalytic activity, of 30–60 min (4, 24). This suggests that DNA synthesis will stop in anoxia. Indeed, this has been found to be the case in cultured mammalian cells (11, 29, 30).

In the crucian carp’s habitat in Northern Europe, seasonal severe hypoxia and anoxia are common during winter. The anoxia is the result of a stop in photosynthesis combined with thick ice coverage that blocks oxygen diffusion. The crucian carp has evolved behavioral, physiological, and molecular adaptations to survive this environment. These include reduced brain activity and locomotion (20, 23), maintained cardiac activity (35), increased glycolysis (37), and avoidance of lactate self-pollution through generation and excretion of ethanol as a major glycolytic end product (15). The latter is especially critical because it enables the fish to survive on anaerobic metabolism without suffering from acidosis, as long as there is glycogen present. Nevertheless, survival is ultimately dependent on the glycogen stores. Hence, saving energy, whenever possible, is important.

Our group (34) previously found that crucian carp living in normoxic water have gills lacking protruding lamellae, the primary site of O2 uptake in fish. Such an unusual trait leads to a very small respiratory surface area. This reduces water and ion fluxes, thereby minimizing the cost of osmoregulation. However, if crucian carp experiences hypoxia, a large increase of the respiratory surface area occurs, enhancing oxygen uptake capabilities (34). The morphological alteration is due to hypoxia-induced apoptosis and cell cycle arrest in a tissue named interlamellar cell mass (ILCM), which fills the space between the lamellae in normoxia. The crucian carp is then able to cover its energy needs through aerobic metabolism at a lower oxygen tension, allowing it to postpone depletion of precious glycogen.

We have previously found that ILCM in normoxic gills has a relatively high rate of cell proliferation (34). Preliminary investigations have shown that anoxic crucian carp has gills resembling those seen in normoxia. Cell proliferation could thus still be present in anoxia, which, on the other hand, appears unlikely due to the oxygen dependence of RNR.

In this study, we exposed crucian carp to up to 7 days of anoxia and investigated gill morphology and cell proliferation.
in gills, intestine, and liver by using both 5′-bromo-2′-deoxyuridine (BrdU) and staining for proliferating cell nuclear antigen (PCNA). BrdU is a thymidine analog incorporated into DNA during DNA synthesis revealing cells in the S phase of cell cycle. PCNA is a DNA sliding clamp for replicative DNA polymerases. Subsequently, we partially cloned and characterized crucian carp RNRR2 to investigate possible differences of crucian carp RNRR2 compared with mammalian RNRR2 and measured its expression with real-time PCR.

**MATERIALS AND METHODS**

**Animals.** Crucian carp (*Carassius carassius* L.; weighing 12.5–31.5 g; all adults) were caught June 2003 in Tjernsund Pond (Oslo community, Norway). They were kept in tanks (~100 fish/500 liters), continuously supplied with aerated and dechlorinated Oslo tap water (10°C), on a 12:12-h diurnal regimen and fed daily with commercial carp food (Tetra Pond, Tetra, Melle, Germany).

**Exposures.** During anoxia exposures, fish were kept in dark, sealed tanks continuously supplied with N2-bubbled dechlorinated Oslo tap water. The oxygen level was monitored with an oxygen electrode (Model Oxi340i; WTW, Weilheim, Germany). The oxygen level was kept at <0.01 mg/l O2 (<0.1% of air saturation), which is the electrode’s detection limit. Control fish were kept in an identical tank but supplied with aerated water. The exposure groups, all consisting of seven fish, were kept for 7 days normoxia, 1 and 7 days of anoxia, and 7 days of anoxia followed by 7 days of reoxygenation.

**Scanning electron microscopy.** The scanning electron microscopy was done as previously described (34). Briefly, for scanning electron microscopy, gills were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer before being dried and Au/Pd coated. Gills were investigated with a JSM 6400 electron microscope (JEOL, Peabody, MA).

**Measurement of mitotically active cells with BrdU.** BrdU staining was done as previously described (34) with one modification: cross-sections were treated with boiling citric acid buffer in a microwave for 25 min at 750 W before they were stained. Briefly, BrdU was injected intraperitoneally (100 μg/g fish) 24 h before tissue sampling. Gills were fixed in 4% formaldehyde, paraffin embedded, and serially cross-sectioned before rehydration and citric acid treatment. Primary antibody, mouse IgG anti-BrdU (BD Biosciences, Franklin Lakes, NJ), was diluted 1:5 in 0.5% blocking solution (BS) (0.5% BSA and 0.5% Triton X-100 in PBS, pH 7.2). Secondary antibody was a biotinylated goat anti-mouse IgG, diluted 1:20 in 0.5% BS. The peroxidase conjugate was diluted 1:20 in 0.5% BS and applied before slides were incubated with diaminobenzidine solution. All chemicals were from Sigma (St. Louis, MO).

**Measurement of mitotically active cells by staining for PCNA.** PCNA has been used previously with success in other fish studies (19, 26). The PCNA staining was done as described for BrdU staining except primary antibody, anti-PCNA (Sigma), was diluted 1:10 in 0.5% BS.

**Counting of cells.** Stained (BrdU or PCNA) and unstained cells in gills were counted as previously described (34). In intestine, numbers of stained and unstained cells in the lower three-fourths of the crypts were counted. PCNA is present in most phases of the cell cycle (G1, S, G2, or M phase) but is not present in the G0 phase. We therefore used lack of PCNA staining to quantify number of G0 cells in gills and intestine. However, in liver, the low number of PCNA-positive cells resulted in us using data for this tissue as PCNA-positive cells.

**Partially cloning and characterization of the oxygen-dependent subunit of RNR (RNRR2).** RNA was extracted from normoxic crucian carp gill tissue (25 mg from each individual) was homogenized with TRIzol reagent (Invitrogen). Extracted RNA was quantified with NanoDrop (NanoDrop Technologies, Rockland, DE), and the RNA quality was confirmed by a BioAnalyzer (Agilent Technologies, Palo Alto, CA). The RNA was DNase I-treated (Sigma) before cDNA synthesis (Superscript III, Invitrogen) of 2 μg RNA.

**Setup and quantification of real-time PCR.** A Lightcycler and a LC FastStart DNA MasterPlus SYBR Green I kit was used (Roche Diagnostics, Indianapolis, IN). We followed the protocol recommended by the supplier. RNRR2 primers (5′-TGAAAGGAACCCCCAC-3′ and 5′-GTAATCGCTCCACCAA-3′) were picked from the consensus sequence using Lightcycler probe design software version 1.0 (Roche Diagnostics) and had a final concentration of 0.5 μM. The following PCR amplification program was applied: 10 min of denaturation at 95°C before 40 cycles of a three-segment program of (1) 10 s at 95°C for denaturation, (2) 10 s at 60°C for annealing, and (3) elongation time set to 25 bp/s at 72°C. Efficiency of each single reaction was analyzed with the LinRegPCR program (31). The crossing point of each reaction was determined by using the second derivative method in the Roche Lightcycler software version 3.5. The latter program was also used to analyze melting points of products to assure specificity and purity of the PCR reactions.

**Statistics.** Statistical significance was tested with one-way ANOVA with Tukey’s post hoc test, where *P < 0.05* was considered significant. Data were analyzed by GraphPad InStat (GraphPad, San Diego, CA).

**RESULTS**

**Gill morphology.** Scanning electron microscopy revealed that, after 7 days in anoxia, crucian carp gills were indistinguishable from normoxic gills (Fig. 1). Thus ILCM remained intact during anoxia.

**Cell proliferation in the gills.** During anoxia exposure, the fraction of S-phase cells stained with BrdU decreased significantly (*P < 0.0001*) in ILCM, from 12.2 ± 1.3% in normoxia to 5.0 ± 0.9% after 7 days in anoxia (Fig. 2A). After 7 days of reoxygenation, there was a nonsignificant increase to 6.9 ± 0.5%. In both normoxia and anoxia, BrdU-positive cells were only found in ILCM, confirming previous findings showing a low mitotic activity in other gill structures (34).

The PCNA staining also indicated a reduced, but continued, mitotic activity. The number of G0 cells (PCNA negative) increased significantly (*P < 0.01*) from 6.8 ± 1.2% in normoxia to 11.1 ± 0.5% after 7 days of anoxia (Fig. 2A). There was no significant change in the number of G0 cells after 7 days of reoxygenation (11.5 ± 0.3%). Like that observed for BrdU-
positive cells, PCNA-positive cells were only found in ILCM (Fig. 3, c and d).

Cell proliferation in the intestine. In intestine, the number of S-phase cells decreased significantly \((P < 0.01)\) from 8.1 ± 0.7% in normoxia to 1.8 ± 0.3% after 7 days in anoxia. After reoxygenation, the amount of S-phase cells had increased significantly to 10.2 ± 2.3% (Fig. 2B). BrdU-positive cells were only found in intestinal crypts (Fig. 3, e and f). PCNA staining indicated no significant change in number of G0 cells during anoxia (Fig. 2B).

Cell proliferation in the liver. The amount of BrdU- and PCNA-positive cells remained constant at ~1% in liver throughout anoxia (Fig. 2C).

RNRR2 characterization and regulation. A sequence alignment between RNRR2 from several species demonstrated a very high sequence identity (Fig. 4). From the three-dimensional structure of mouse RNRR2 and crucian carp sequence, we could determine that all amino acids involved in coordinating the diiron-oxygen cluster, transporting electrons, and surrounding the tyrosyl radical were conserved (Fig. 5). Furthermore, amino acids suggested to influence accessibility of the diiron-oxygen cluster and the tyrosyl radical (16) were also found to be conserved between mouse and crucian carp (Fig. 4).

The real-time PCR revealed no significant change in the amount of RNRR2 mRNA between normoxia and 7 days of anoxia (Fig. 6).

DISCUSSION

The results show that anoxia induces a reduction of cell proliferation in ILCM and in intestinal crypts, two tissues with a relatively high proliferative activity. In liver, with a more moderate mitotic activity, no changes in the number of S-phase or G0-phase cells were detected. Most importantly, the results indicate that DNA synthesis continues after 7 days in anoxia. Surprisingly, amino acid residues in RNRR2, especially those surrounding the iron center, were identical in crucian carp and mouse. There was no change in the amount of RNRR2 mRNA after anoxia exposure. Finally, the gill morphology did not change in response to anoxia, in contrast to previous findings in hypoxia.
The reduction in number of S-phase cells mirrored the increase of G0-cells in ILCM (Fig. 2A). In normoxia, the mitotic activity in ILCM was very high, whereas the number of cells in G0-phase was only 7%. During anoxia exposure, the number of S-phase cells fell from 12 to ~5%. The drop in S-phase cells is approximately equal, as previously found in hypoxia-exposed crucian carp (34), indicating similar proliferative response situations. Still, ILCM remained intact after 7 days of anoxia, although a previous study showed 50% reduction after the same time period in hypoxia (34). This indicates that anoxia, unlike hypoxia, does not induce apoptosis. The maintenance of a large ILCM in anoxia should help keep osmoregulatory costs low. Because no oxygen is present, there would not be any advantage of increasing the respiratory surface area.

Over the recent years, it has become clear that PCNA not only functions as a DNA sliding clamp for replicative DNA polymerases but also has a marked ability to interact with multiple partners involved in metabolic pathways, DNA repair, and cell cycle regulation (18). The results in the present paper show that ~90% of the cells in ILCM still had PCNA present after 7 days in anoxia (Figs. 2A and 3d). This indicates that cell cycle slows down, but the cells remain prepared to proceed through mitosis when conditions improve. This conclusion is supported by the concomitant sustained expression of RNRR2 (Fig. 6). However, the number of S-phase and G0-phase cells had not returned to preanoxic levels after 7 days of reoxygenation. This could indicate that there was no immediate need to replace ILCM cells after 7 days in anoxia. Indeed, no reduction in the ILCM was apparent at the end of the anoxic episode (Fig. 1b).

There was a significant fall in the number of S-phase cells in intestine, which returned to normoxic levels during reoxygenation. This is in contrast to what was observed in gills. This may indicate a need for new intestinal cells for getting the organ back into a fully functional state. In the crucian carp's natural habitat, the increase of water oxygen tension during spring, after a period of anoxia, is likely to trigger feeding behavior, since they then have very low-energy reserves (14).

As previously mentioned, the oxygen-dependent oxidoreductase activity of RNR is located on the R2 subunit. Previously, mammalian RNRR2 was only found to be expressed in the S phase of the cell cycle (1, 9). However, as shown in Fig. 2A and Fig. 6, the significant reduction of S-phase ILCM cells in anoxia was not correlated with a reduction of RNRR2 mRNA. Thus not only does RNRR2 appear not to follow the number of S-phase cells but its expression is maintained for 7 days in anoxia. This suggests either that a reduction of cell proliferation in anoxia does not necessarily affect expression of RNRR2 gene or that there is, in
conjunction with a reduction of cell cycle-related RNRR2, an increased expression of p53-induced R2 (12, 39), believed to be important in DNA damage repair. However, RNRR2 protein degradation is dependent on transition into mitosis, and the level of RNRR2 protein is not affected by DNA damage or replication block (3).

The partial sequence of crucian carp RNRR2 indicated no important differences in key amino acids between crucian carp and mouse RNRR2. This seems puzzling because mammalian RNRR2 is inactivated in anoxia (2, 4). We still found new S-phase cells after 7 days of anoxia exposure revealing RNR activity in anoxic crucian carp. The iron center of RNRR2 is essential for production and stabilization of the tyrosyl radical. If iron is lost, the radical is lost and must be regenerated through an oxygen-demanding reaction. The amino acid configuration has been used to explain the low stability of the diiron-oxygen cluster and tyrosyl radical in mouse RNRR2 (16). The sequence homology between crucian carp and mouse RNRR2 could suggest that the enzymes have similar properties regarding radical stability. The mouse RNRR2 radical is readily inactivated by radical scavengers and has a low stability (25). However, because no structural or spectroscopic data are presently available for crucian carp RNR, no direct measurements of the radical stability in this species exists. In mammals, 60% of the iron is lost after 30 min at 37°C and 30% is lost after 30 min at 0°C (24). When extrapolated, the data suggest that virtually no iron is left in the R2 subunit after 2 h at 0°C.

Thus, even at the relatively low temperature (10°C) in the present study, the iron center is lost and no radical should be present, that is, if crucian carp RNR functions as mammalian RNR. Because we found no differences in key amino acids outside areas with known importance affect RNR function in anoxia.

There are two methodological considerations that must be discussed in the present paper. First, could there be traces of oxygen in the water allowing crucian carp to regenerate the RNR radical? The oxygen electrode sensitivity is 0.1% O₂, and it is possible that water oxygen level was just below this detection limit. However, it has been shown that below 0.1% O₂ the radical is abolished in mammalian cell cultures exposed for 5 h (2). Second, when the fish is injected with BrdU, it is exposed to air for a few seconds. Although crucian carp is not an air breather, the short contact with air could supply tissues with some oxygen that could reconstitute the RNR radical and

The partial sequence of crucian carp RNRR2 indicated no important differences in key amino acids between crucian carp and mouse RNRR2. This seems puzzling because mammalian RNRR2 is inactivated in anoxia (2, 4). We still found new S-phase cells after 7 days of anoxia exposure revealing RNR activity in anoxic crucian carp. The iron center of RNRR2 is essential for production and stabilization of the tyrosyl radical. If iron is lost, the radical is lost and must be regenerated through an oxygen-demanding reaction. The amino acid configuration has been used to explain the low stability of the diiron-oxygen cluster and tyrosyl radical in mouse RNRR2 (16). The sequence homology between crucian carp and mouse RNRR2 could suggest that the enzymes have similar properties regarding radical stability. The mouse RNRR2 radical is readily inactivated by radical scavengers and has a low stability (25). However, because no structural or spectroscopic data are presently available for crucian carp RNR, no direct measurements of the radical stability in this species exists. In mammals, 60% of the iron is lost after 30 min at 37°C and 30% is lost after 30 min at 0°C (24). When extrapolated, the data suggest that virtually no iron is left in the R2 subunit after ~2 h at 0°C. Thus, even at the relatively low temperature (10°C) in the present study, the iron center is lost and no radical should be present, that is, if crucian carp RNR functions as mammalian RNR. Because we found no differences in key amino acids between mouse and crucian carp R2, it is possible that crucian carp has other means of ensuring radical stability and RNR activity. This could involve other parts of the enzyme or supporting cellular systems. However, we cannot exclude that some of the differences seen in amino acids outside areas with known importance affect RNR function in anoxia.

There are two methodological considerations that must be discussed in the present paper. First, could there be traces of oxygen in the water allowing crucian carp to regenerate the RNR radical? The oxygen electrode sensitivity is 0.1% O₂, and it is possible that water oxygen level was just below this detection limit. However, it has been shown that below 0.1% O₂ the radical is abolished in mammalian cell cultures exposed for 5 h (2). Second, when the fish is injected with BrdU, it is exposed to air for a few seconds. Although crucian carp is not an air breather, the short contact with air could supply tissues with some oxygen that could reconstitute the RNR radical and

Fig. 4. Amino acid sequences of the ribonucleotide reductase (RNR)R2 subunit for several species: CC, Carassius carassius; DR, Danio rerio (accession no. AAH75746); XL, Xenopus laevis (accession no. AAH41209); MM, Mus musculus (accession no. AAH85136). Amino acids holding the iron center are highlighted in red. The radical is generated on Tyr177 (shown in yellow). Amino acids involved in transport of the radical to the radical active site on the R1 subunit are highlighted in blue, whereas amino acids involved in generating the entrance to and surrounding the radical site are highlighted in gray.

Fig. 5. Diiron-oxygen cluster of mouse RNRR2, the metal coordinating amino acids, and amino acids involved in the electron transport pathway. The radical is created on Tyr177 (mouse RNRR2 numbering) and transported to the R1 subunit through a pathway consisting mostly of hydrogen-bonded side chains (33). The first part of the pathway in RNRR2 is indicated with yellow dots.
promote some DNA synthesis. We find this unlikely because such a short and minute supply of oxygen would probably not produce radicals in more than a fraction of the RNRs. We did not find any change of cell proliferation in liver throughout the exposures or any incidences of weak staining indicating partial DNA synthesis. In summary, it is unlikely that new S-phase cells are present in anoxia due to a small, undetectable, transient supply of oxygen.

ACKNOWLEDGMENTS

We thank Guro Sandvik, Aasa R. Schjølberg, and Liza Kravik for technical assistance and Lars Thelander and Kristoffer K. Andersson for valuable input.

GRANTS

This work was funded by The Research Council of Norway.

REFERENCES


