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OXYGEN-DEPENDENT ENERGETICS OF ANOXIA-TOLERANT AND ANOXIA-INTOLERANT HEPATOCYTES

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Summary

The oxygen-dependence of cellular energetics was investigated in hepatocytes from goldfish *Carassius auratus* (anoxia-tolerant) and rainbow trout *Oncorhynchus mykiss* (anoxia-intolerant). In goldfish hepatocytes, an approximately 50 % reduction in the rate of oxygen consumption was observed in response to both acute and prolonged hypoxia, the latter treatment shifting the threshold for this reduction to a higher oxygen level. A concomitant increase in the rate of lactate production did not compensate for the decreased aerobic ATP supply, resulting in an overall metabolic depression of 26 % during acute hypoxia and of 42 % during prolonged hypoxia. Trout hepatocytes showed a similar suppression of cellular respiration after prolonged hypoxia but were unresponsive to acute hypoxia. Similarly, the rate of lactate production was unaltered during acute hypoxia but was increased during prolonged hypoxia, metabolic depression amounting to 7 % during acute hypoxia and 30 % during prolonged hypoxia. In both species, the affinity of hepatocytes for oxygen decreased during hypoxia, but this alteration was not sufficient in absolute terms to account for the observed decrease in aerobic ATP supply. Protein synthesis was suppressed in both cell types under hypoxia, whereas Na^+/K^+-ATPase activity decreased in trout but not in goldfish hepatocytes, emphasising the importance of membrane function in these cells during conditions of limited energy supply.

Key words: hypoxia, metabolic depression, protein synthesis, Na^+/K^+-ATPase, goldfish, *Carassius auratus*, rainbow trout, *Oncorhynchus mykiss.*

Introduction

The rate of oxygen consumption of most cell types has been found to be independent of O_2 concentration over a wide range (Wilson et al., 1979, 1988). Recently, however, Schumacker et al. (1993) and Chandel et al. (1997) reported a marked O_2-dependence of respiration in rat hepatocytes when hypoxia was maintained over several hours, an experimental approach not applied in previous studies. Furthermore, O_2-dependence even under acute hypoxia has been described in chick cardiac myocytes (Budinger et al., 1996). As pointed out by these authors, such a metabolic behaviour might represent an adaptive response to limited O_2 supply by slowing down the rate of O_2 depletion and thus delaying the onset of anoxia.

In a number of recent studies, the hepatocyte of the goldfish *Carassius auratus* has been characterized as highly tolerant to environmental anoxia, this tolerance being based on the capacity to shut down ATP-consuming functions in synchrony with ATP-producing pathways (Krumschnabel et al., 1994b, 1996, 1997). Considering the above report on anoxia-intolerant rat hepatocytes, the question arises as to whether a similar ability to suppress metabolic activity can also be found at low, as opposed to zero, levels of O_2 availability in goldfish hepatocytes. Moreover, the fact that O_2-conforming behaviour in chick cardiac myocytes could be elicited during a considerably shorter period of hypoxic exposure (approximately 2 min) than in rat hepatocytes (>2 h) may be related to the generally greater hypoxia-tolerance of cardiac tissue (Arai et al., 1991). Therefore, we hypothesize that, in goldfish hepatocytes, the O_2-dependence of respiration, if present, should also be more readily observable than in anoxia-intolerant cells.

We addressed this problem by comparing the O_2-dependence of the energetics of hepatocytes from goldfish and trout *Oncorhynchus mykiss*, the latter being well characterized as a model for anoxia-intolerant cells (Krumschnabel et al., 1996, 1997; Schwarzbaum et al., 1996). Respiratory rates were determined in cells that were exposed to normoxic or hypoxic conditions either acutely or for 1 h before measurements. Other variables evaluated were the cellular ATP pool, as a measure

of the energetic state of the hepatocytes, rates of lactate production, as an estimate of anaerobic ATP production, and the apparent O_2-affinity of cellular respiration, allowing insight into the intrinsic properties of mitochondria. In addition, the rate of protein synthesis and Na^+ pump activity were determined to provide information about the impact of hypoxia on ATP-consuming processes.

Materials and methods

Chemicals

Collagenase, bovine serum albumin (BSA), Leibovitz L-15 medium and luciferase–luciferin were obtained from Sigma. [^{3}H]leucine and $^{86}Rb^+$ were purchased from Du Pont NEN. All other chemicals were of analytical grade and were purchased from local suppliers.

Experimental animals and hepatocyte isolation

Goldfish *Carassius auratus* L. (50–100 g) were caught in a pond near Innsbruck, Austria, and were maintained in 200 l aquaria at 20 °C. Rainbow trout *Oncorhynchus mykiss* (Walbaum) (250–500 g) were obtained commercially and were kept in 200 l tanks at 15 °C. Fish were acclimated to the indicated temperature for at least 3 weeks before experimentation.

Hepatocytes were isolated as described previously (Krumschnabel et al., 1994b, 1996) and were suspended in Hepes-buffered saline containing (in mmol l^{-1}), for goldfish, 10 Hepes, 135 NaCl, 3.8 KCl, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 $NaHCO_3$ and 1 % BSA, pH 7.6 at 20 °C, and for trout, 10 Hepes, 136.9 NaCl, 5.4 KCl, 1 $MgSO_4$, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 5 $NaHCO_3$, 1.5 $CaCl_2$, 5 glucose and 1 % BSA, pH 7.6 at 20 °C. The omission of glucose from the medium used for goldfish was because the concentration of glycogen in normoxic goldfish hepatocytes is extremely high so that, even under severe metabolic inhibition, goldfish hepatocytes showed only a slight decrease in glycogen content (Dorigatti et al., 1996). In contrast, the glycogen content of trout hepatocytes is comparatively low and quite variable (Biasi, 1997). Thus, to ensure viability for the whole experimental period under the different conditions, the medium for trout was supplemented with glucose. However, it should be noted here that in preliminary experiments the absence or presence of glucose had no effect on the rate of lactate production under hypoxia (data not shown).

After isolation, the cells were left to recover for at least 1 h in a shaking water bath at the acclimation temperature, which was also the experimental temperature, before use. The viability of isolated hepatocytes (>95 %) was routinely assessed by Trypan Blue exclusion and remained unaltered by the experimental manipulations applied.

Incubation protocols for normoxic and hypoxic exposure

Cells were maintained in suspension in 40 ml incubation flasks shaken at 100 revs min^{-1} in a thermostatted water bath. Flasks were sealed with a rubber cap through which an 18 gauge syringe needle and a 3 mm plastic tube were inserted, the needle serving as an inlet for humidified gas mixtures and the tube serving both as an outlet for gas and for the removal of samples, as required, for each experiment.

Rates of O_2 consumption

Rates of O_2 consumption ($\dot{V}_{O_2}$) were determined using a Cyclobios oxygraph, a two-channel respirometer specifically designed for measurements of respiration at low O_2 partial pressures (Haller et al., 1994). Briefly, since the Oxygraph chamber was functionally airtight, the oxygen tension of the respiration medium decreased linearly with time as the cells consumed dissolved O_2. The rate of oxygen consumption was calculated from the time derivative of oxygen concentration measured in the closed respirometer. The O_2 signal was digitally stored on a computer using Datgraf software (Cyclobios, Austria), the sampling interval being 1 s. Rates of O_2 consumption were determined in cells maintained under normoxia or graded levels of hypoxia for 1 h. Oxygen levels corresponded to 100, 20, 10 or 5 % air saturation, equivalent to 21.1, 4.2, 2.1 or 1.1 kPa O_2 partial pressure. Hypoxia was created by mixing air and pure nitrogen using a Wösthoff gas-mixing pump (Wösthoff, Germany). After this pre-incubation period, hepatocytes were removed from the incubation flasks by means of a gas-tight Hamilton syringe (Hamilton-Bonaduz, Switzerland) and injected into an Oxygraph chamber containing either normoxic medium or medium that had previously been set to a defined O_2 content by removing the O_2 with nitrogen. In this way, respiration data were obtained for controls (cells incubated under normoxia and measured under normoxia), for cells acutely exposed to hypoxia (incubation under normoxia, measured under hypoxia) and for cells exposed to hypoxia after 1 h of adaptation to the same level of hypoxia. In the case of normoxic cells measured during hypoxia, the introduction of hepatocytes into the Oxygraph chamber tended to produce a slight increase in chamber O_2 content, so care was taken to minimize this O_2 overshoot by injecting these cells very slowly.

Determination of apparent P_{50} and data analysis

Determinations of apparent P_{50} values, i.e. O_2 partial pressures at half-maximal O_2 flux, were made on cells maintained under normoxia or at 5 % hypoxia for 1 h. In both groups of cells, the O_2 concentration in the experimental medium was adjusted to 5 % air saturation before injection of the hepatocytes, ensuring that a similar experimental time elapsed for normoxic and hypoxia-adapted cells before full anoxia was reached.

To calculate the rate of O_2 flux (J_{O_2}), the partial pressure of O_2 (P_{O_2}) was corrected to account for (i) the time response of the O_2 sensor, (ii) any O_2 leak and the blank rate of O_2 consumption and (iii) the time-dependent zero drift of the O_2 sensor (for details, see Gnaiger et al., 1995). The results were plotted as J_{O_2} *versus* P_{O_2}, with a single hyperbola fitted to the data according to the following equation:

$$J_{O_2} = J_{max} P_{O_2} / (P_{50} + P_{O_2}),$$

where J_{max} represents the respiration rate at saturating P_{O_2} and P_{50} represents the value of P_{O_2} that gives half-maximal J_{O_2} (i.e. 50 % of J_{max}). The hyperbolic nature of J_{O_2} as a function of P_{O_2} was similar to that observed in other cellular systems (Gnaiger et al., 1995). Hyperbolic fits were calculated by nonlinear regression.

ATP and lactate contents

Cellular ATP contents were determined using the luciferase–luciferin method described by Brown (1982). Lactate levels were measured in duplicate samples taken from cell suspensions, i.e. as the sum of intracellular and extracellular lactate accumulated, using a standard enzymatic technique as described in Bergmeyer (1984). The experimental protocol for ATP and lactate sampling was as follows. Controls were incubated under normoxia for 1 h, with samples being removed 0, 30 and 60 min into the experiment. At this point, the cells were briefly centrifuged and the supernatant aspirated, and the hepatocytes were resuspended in medium previously equilibrated with 5 % air-saturated gas (acute normoxic–hypoxic transition). Sampling was then continued at 0, 30 and 60 min after this transition. In another group of cells, the addition of hypoxic gas at 5 % air saturation to incubation flasks was initiated at time zero, allowing hepatocytes to experience a transition period of several minutes (5–10 min) before full equilibration of the gas phase with the incubation medium (slow normoxic–hypoxic transition). These cells were sampled every 30 min over a period of 2 h.

Rate of protein synthesis and Na^+ pump activity

The rate of protein synthesis and Na^+ pump activity were measured by following the incorporation of [^{3}H]leucine into cellular protein and by determination of cellular uptake of $^{86}Rb^+$, respectively. We have repeatedly shown that more than 95 % of Rb^+ uptake is sensitive to inhibition by ouabain (Krumschnabel et al., 1994b, 1996, 1997), making Rb^+ uptake a fairly good estimate of Na^+/K^+-ATPase activity. For these measurements, the cells were suspended in modified Leibovitz L-15 medium (L-15 medium containing 10 mmol l^{-1} Hepes, 5 mmol l^{-1} $NaHCO_3$, pH adjusted to 7.6 at 20 °C) and incubated under normoxia for 1 h before experimentation. Following this period, cell suspensions were divided and incubated under normoxia or hypoxia for another hour as described above. After this hour, $^{86}Rb^+$ (555 Bq ml^{-1}) and [^{3}H]leucine (18.5×10^3 Bq ml^{-1}) were added to the suspensions, and duplicate samples were then removed every 10 min. After three samplings, cells maintained under normoxia were switched to hypoxic conditions and *vice versa*, and sampling continued for another 30 min.

For $^{86}Rb^+$ uptake measurements, 50 µl samples of hepatocyte suspensions were removed into small reaction tubes, briefly centrifuged, and the supernatant was carefully aspirated. The remaining cell pellet was washed three times with iso-osmotic saline and finally broken up by vigorous vortexing in 1 ml of distilled water. Finally, the reaction tubes were placed into scintillation vials, and $^{86}Rb^+$ was determined using a Rackbeta Wallac counter by Cherenkov counting.

Incorporation of [^{3}H]leucine into trichloroacetic acid (TCA)-precipitable protein was determined by pipetting 30 µl samples of cell suspensions onto Whatman GF/A filters, which were then briefly air-dried. The filters were placed into ice-cold 10 % TCA solution containing 5 mmol l^{-1} unlabelled leucine, where they were left for at least 10 min. This was followed by two 15 min washes in 5 % TCA solutions at room temperature (20 °C) and three consecutive 10 min washes in pure ethanol. Finally, filters were air-dried, placed into 5 ml scintillation vials containing scintillation cocktail and counted for radioactivity.

In preliminary experiments, we had established (i) that both $^{86}Rb^+$ uptake and [^{3}H]leucine incorporation were linear for at least 1 h under normoxic conditions and (ii) that the simultaneous presence of both isotopes in a sample did not cause any interference during measurements of radioactivity.

Calculation of the rate of ATP production

The rate of anaerobic ATP production was estimated from the amount of lactate accumulated over the first 60 min by cells exposed to the different treatments. To compare aerobic and anaerobic ATP production, rates of respiration and lactate production were expressed as ATP equivalents. The following stoichiometries were assumed: 6 ATP/O_2 and 1.5 ATP/lactate derived from endogenous glycogen and 1 ATP/lactate derived from glucose.

Statistical analyses

Values are presented throughout as means ± S.E.M. of N independent cell preparations. Differences between experimental groups were evaluated using Student's t-tests with $P<0.05$ considered significant. Hyperbolic fittings for the calculation of P_{50} values were performed using the Sigmaplot software package (Jandel Scientific).

Results

O_2-dependence of respiration

Normoxic rates of respiration of goldfish and trout hepatocytes amounted to 0.512±0.039 nmol O_2 10^6 $cells^{-1}$ min^{-1} (N=46) and 0.799±0.065 nmol O_2 10^6 $cells^{-1}$ min^{-1} (N=31), respectively. In goldfish hepatocytes, acute exposure of previously normoxic cells to graded levels of hypoxia did not result in any alteration in the rate of O_2 consumption at 20 % and 10 % air saturation, but caused a significant decrease to approximately 60 % of normoxic controls at 5 % air saturation (Fig. 1A). In contrast, normoxia-adapted trout hepatocytes maintained control rates of O_2 consumption at both 10 % and 5 % air saturation (Fig. 1B). Incubation of cells under hypoxic conditions for 1 h before measurements evoked a different response pattern of cellular respiration. The rate of O_2 consumption of hepatocytes after prolonged hypoxic exposure showed a decrease to 52 % and 45 % (goldfish) and to 55 % and

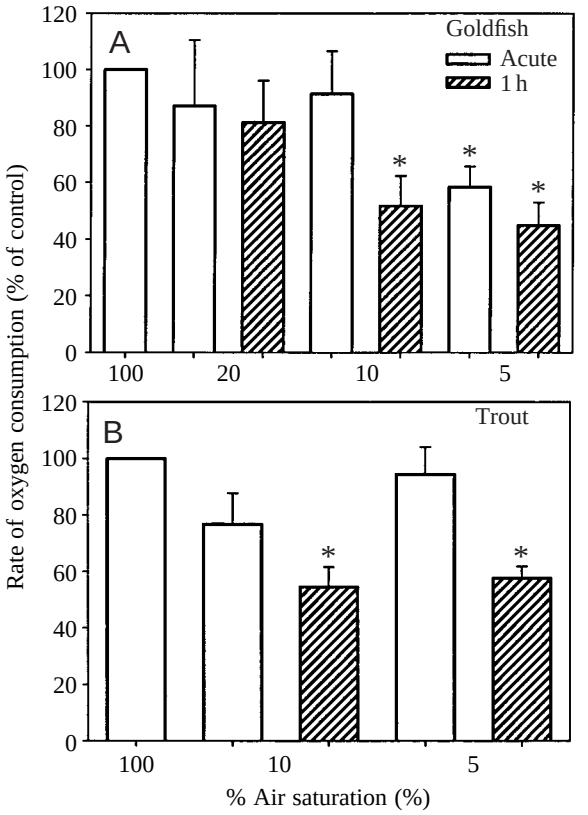

Fig. 1. Rates of oxygen consumption ($\dot{V}_{O_2}$) of hepatocytes from goldfish (A) and trout (B) at defined levels of O_2 availability. Cells were exposed to hypoxia after 1 h of incubation under 100 % (acute, open columns) or 20 %, 10 % or 5 % air saturation of O_2 (1 h, hatched columns). Values are expressed as a percentage of normoxic controls and are presented as means + S.E.M. of 4–14 independent preparations. An asterisk indicates a significant difference (P<0.05) compared with normoxic controls at 100 % air saturation.

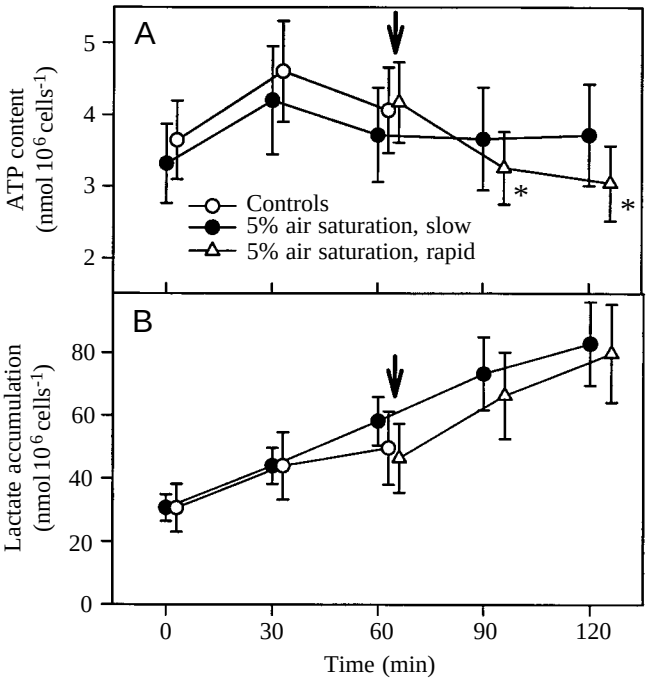

Fig. 2. ATP content (A) and lactate accumulation (B) of goldfish hepatocytes during normoxia (open circles), after rapid transfer to 5 % air-saturated medium (triangles) and after slow deoxygenation with a 5 % air-saturated gas mixture (filled circles). The arrow indicates the time of transfer from normoxic to hypoxic conditions of the rapid transition group. For the slow transition group, the addition of hypoxic gas was initiated at time zero. Values are means ± S.E.M. of eight independent preparations (N=7 at 120 min). An asterisk indicates a significant difference (P<0.05) compared with the value at time zero.

57 % (trout) at 10 % and 5 % air saturation, respectively (Fig. 1). Incubation at 20 % air saturation for 1 h did not affect cell respiration (determined for goldfish only). Importantly, even after 1 h of incubation at 5 % air saturation, cell viability was 97.6±1.7 % (N=5) in hepatocytes from goldfish and 98.2±0.4 % (N=10) in hepatocytes from trout.

ATP contents and lactate accumulation in response to rapid and slow normoxic–hypoxic transitions

Normoxic goldfish hepatocytes maintained cellular ATP contents of approximately 4 nmol 10^6 cells^{-1} for 1 h. When these cells were subjected to a rapid (<1 min) transfer from normoxic to 5 % hypoxic medium, the ATP contents showed a significant decline of approximately 25 % within 30 min and remained significantly reduced throughout the experimental period (Fig. 2A). By comparison, no change in cellular ATP level was observed for 2 h when the O_2 content of the medium was reduced more slowly, i.e. over a period of 5–10 min (Fig. 2A).

The rate of anaerobic ATP generation, as estimated from the rate of lactate accumulation, was 0.47 nmol ATP 10^6 cells^{-1} min^{-1} in control cells and 0.83 and 0.69 nmol ATP 10^6 cells^{-1} min^{-1} in acutely and chronically exposed hepatocytes, respectively (Fig. 2B). The lactate contents of cell suspensions immediately before and after transfer from normoxic to hypoxic medium were the same, indicating that any lactate produced up to this point had been retained within the cells.

The ATP and lactate dynamics of trout hepatocytes are shown in Fig. 3. As in the goldfish cells, the ATP content remained constant at approximately 2.7 nmol 10^6 cells^{-1} in normoxic controls and showed a significant decline of 30 %, without subsequent recovery, upon rapid transfer to 5 % hypoxia (Fig. 3A). Slow reduction of the O_2 supply did not affect ATP levels for approximately 30 min, but prolonged exposure to low O_2 levels caused a reduction in ATP content by approximately 25 %, this decrease being significant after 60 and 120 min of hypoxia. Rates of lactate production were similar in trout hepatocytes during normoxia and after a rapid switch to hypoxia, yielding an anaerobic rate of ATP output of approximately 0.40 nmol ATP 10^6 cells^{-1} min^{-1} (Fig. 3B). In contrast to goldfish hepatocytes, lactate seems to have been exported to the extracellular environment during incubation, since changing the incubation medium restored the initial lactate level of the trout cell suspensions. In cells subjected to

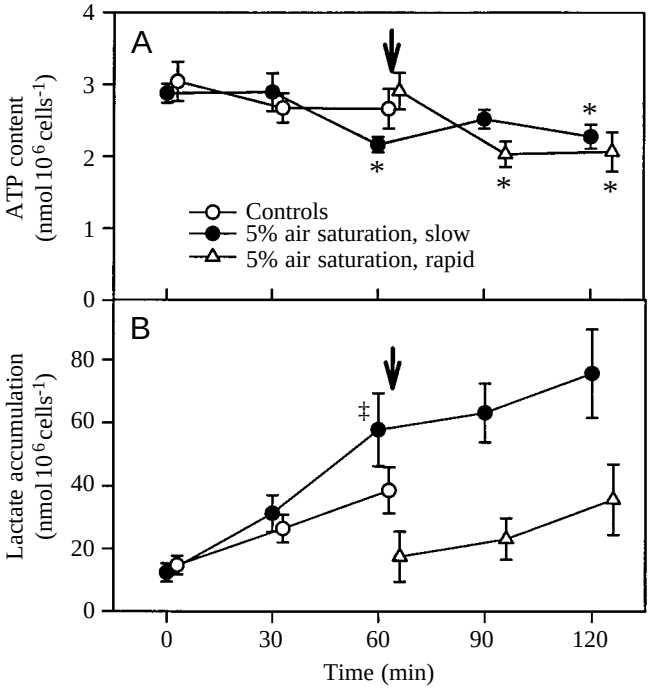

Fig. 3. ATP content (A) and lactate accumulation (B) of trout hepatocytes under the conditions described for Fig. 2. Values are means ± S.E.M. of seven independent preparations. An asterisk indicates a significant difference (P<0.05) compared with the value at time zero. A double dagger indicates a significant difference (P<0.05) compared with time-matched controls.

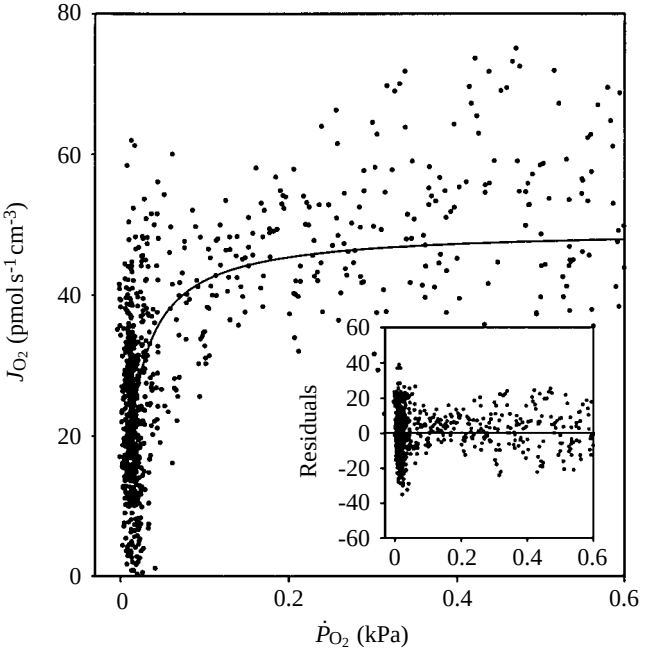

Fig. 4. Results of a representative experiment for the determination of the apparent P_{50} for O_2 of goldfish hepatocytes. O_2 flux (J_{O_2}) is plotted against P_{O_2}, with a single hyperbola being fitted to the data according to the equation given in Materials and methods. Values plotted are data points sampled at 1 s intervals. The inset illustrates the residuals obtained from the regression as a function of P_{O_2}, the random distribution of residuals validating the hyperbolic function.

a slow normoxic–hypoxic transition, the rate of glycolytic ATP production amounted to 0.77 nmol ATP 10^6 cells^{-1} min^{-1}, lactate contents of cell suspensions being significantly higher after 60 min of hypoxic incubation than in the normoxic controls. However, from 60 to 120 min of hypoxia, the rate of lactate production decreased again (to 0.30 nmol ATP 10^6 cells^{-1} min^{-1}).

Effects of hypoxia on P_{50} of cellular respiration

Fig. 4 illustrates that, in the P_{O_2} range below 5 % air saturation, J_{O_2} followed a hyperbolic function with an increase in P_{O_2}. The inset of the figure shows the residuals obtained from the regression as a function of P_{O_2}. The residuals are distributed randomly around zero, thus validating the hyperbolic function. The values of P_{50} were derived from the hyperbolic function (see Materials and methods).

To characterize further the O_2-dependence of cellular respiration, the effect of a 1 h exposure to 5 % air saturation on the P_{50} of the cells was studied. Table 1 summarizes the results of four such experiments on hepatocytes from the two species. It can be seen that P_{50} values in normoxic controls were quite dissimilar in the cells from goldfish and trout, the mean P_{50} value in the latter species exceeding that in the former by more than fourfold. However, the response to 1 h of exposure to 5 % air saturation was almost the same for both species in that the P_{50} values increased by approximately twofold compared with the normoxic controls. Differences between the normoxic and hypoxic groups were not quite significant in goldfish (P=0.06), but were statistically significant in trout hepatocytes.

Effects of hypoxia on ATP-consuming processes

An attempt was made to assess the impact of hypoxia on protein synthesis and the Na^+ pump, which represent the most important ATP-requiring processes in the cells. We studied the response of these processes to a brief (⩽30 min) and to a more extended (⩽90 min) hypoxic episode. As shown in Table 2, neither short nor prolonged exposure to 5 % air saturation caused a significant decrease in Na^+ pump activity or protein synthesis in goldfish hepatocytes, although the latter showed a tendency to be reduced during hypoxia. In trout hepatocytes, Rb^+ uptake decreased in response to both short and prolonged hypoxia, the

Table 1. *P_{O_2} at half-maximal respiration rate (P_{50}) in hepatocytes from goldfish and trout after a 1 h exposure to normoxia or hypoxia*

	Normoxia (kPa)	Hypoxia (kPa)	Hypoxia/normoxia
Goldfish	0.0110±0.0028	0.0190±0.0038	2.03±0.61
Trout	0.0526±0.0178	0.0918±0.0254*	1.89±0.38

*An asterisk denotes a value significantly different from the normoxic control.

Values are means ± S.E.M. of four independent preparations.

Table 2. *Na^+ pump activity (measured as $^{86}Rb^+$ uptake) and the rate of protein synthesis (measured as [^{3}H]leucine incorporation) in hepatocytes from goldfish and trout before and after normoxic/hypoxic or hypoxic/normoxic transitions*

	Goldfish				Trout			
Function	NOR → HYP		HYP → NOR		NOR → HYP		HYP → NOR	
$^{86}Rb^+$ uptake (nmol 10^{-6} cells min^{-1})	0.499±0.120	0.651±0.176	0.410±0.062	0.342±0.062	0.382±0.073	0.181±0.054*	0.184±0.061	0.320±0.050*
[^{3}H]leucine incorporation (pmol 10^6 cells min^{-1})	25±6	14±3	16±3	17±4	13±2	11±2	7±1‡	12±2

NOR → HYP and HYP → NOR indicate the metabolic activities of the cells before and after normoxic/hypoxic (90 min normoxic/30 min hypoxic) or hypoxic/normoxic (90 min hypoxic/30 min normoxic) transitions. Cells were maintained under normoxic or hypoxic conditions for 1 h before starting measurements by the addition of radioactive tracers.

*Significantly different from the value before the transition; ‡significantly different from the normoxic controls.

Values are given as means ± S.E.M. of 3–6 independent preparations.

decrease being significant in the former case but not quite in the latter case (P=0.05). However, reoxygenation of hypoxic cells caused a significant increase in rates of Rb^+ uptake to a mean value similar to initial normoxic rates, confirming the effect of prolonged hypoxia. Protein synthesis was not affected by brief hypoxic exposure but was depressed by 45 % after prolonged hypoxia. Again, reoxygenation reversed this effect, restoring normoxic rates of protein synthesis.

Discussion

Oxygen-dependence of ATP turnover

The rate of O_2 consumption of hepatocytes from both goldfish and trout was clearly responsive to O_2 availability when cells were incubated under hypoxic conditions for prolonged periods (Fig. 1). In goldfish hepatocytes, a critical threshold for the initiation of metabolic depression was found to lie between 20 % and 10 % air saturation, but even at 5 % hypoxia the decrease in the rate of respiration was not associated with a decrease in ATP content (Fig. 2). Furthermore, although the rate of ATP production by glycolysis increased in relative contribution to total cellular ATP production from 13 to 33 % during chronic hypoxia, glycolysis could not make up for the decrease in aerobic ATP output. Taken together, metabolic depression amounted to approximately 42 % (Table 3).

In trout hepatocytes, the decrease in the rate of respiration under prolonged hypoxia was similar to that in goldfish hepatocytes, but it was accompanied by a significant decline in cellular ATP content (Fig. 3). Again, the concomitant increase in the rate of lactate accumulation did not make up for the deficit generated by the decrease in the rate of oxygen consumption, causing an overall metabolic depression of approximately 30 % (Table 3).

Under conditions of acutely imposed hypoxia, a somewhat different response pattern was observed. In goldfish hepatocytes, cellular respiration responded immediately to 5 % air saturation (Fig. 1A), but ATP levels also fell within

Table 3. *Total and glycolytic rates of ATP production and metabolic depression in hepatocytes from goldfish and trout during acute and prolonged hypoxia*

	Total ATP production (nmol 10^6 $cells^{-1}$ min^{-1})	Glycolytic ATP production (%)	Metabolic depression (%)
Goldfish			
Normoxia	3.5	13.4	–
Acute hypoxia	2.6	31.8	25.9
Chronic hypoxia	2.1	33.3	41.8
Trout			
Normoxia	5.2–5.4	7.6–11.0	–
Acute hypoxia	4.8–5.0	6.3–9.1	6.8–7.5
Chronic hypoxia	3.5–3.9	21.7–29.1	27.6–32.0

Total ATP production was determined from rates of oxygen consumption and lactate accumulation measurements. These rates were converted to rates of ATP production assuming stoichiometries of 6 ATP/O_2 and 1.5 ATP/lactate derived from endogenous glycogen and 1 ATP/lactate derived from glucose.

For trout cells, a range of values is given, accounting for the possibility that either glucose or glycogen could have been used as a glycolytic fuel.

30 min of hypoxia, suggesting that mitochondrial ATP production was more rapidly modulated than ATP-requiring processes. This compares well with our previous findings that, after the addition of cyanide (which causes an immediate inhibition of oxidative phosphorylation), ATP levels showed an initial decrease but remained constant during a slow transition to anoxia induced using a N_2/CO_2 gas mixture (Krumschnabel et al., 1994b, 1997). The trout cells were found to differ from the goldfish cells in that their rate of oxygen consumption remained unaffected by acute hypoxia down to 5 % air saturation (Fig. 1B). The differences in the responses of the cells to short- and long-term exposure to low P_{O_2} may reflect the well-known fact that thresholds for physiological responses are defined by both the intensity and the duration of the stimuli.

Comparison with other cells

These observations are in good agreement with numerous studies reporting that cellular rates of O_2 consumption remain constant down to very low O_2 tensions (Wilson et al., 1979, 1988). The studies of Schumacker et al. (1993) and Budinger et al. (1996), together with the findings reported here, provide evidence that respiration is observed to be O_2-independent only when the duration of hypoxic exposure is brief (a few minutes). Prolonged exposure to hypoxia (>2 h) caused a significant decrease in the rate of O_2 consumption at an O_2 tension as high as 9.3 kPa (approximately 45 % air saturation) in rat hepatocytes, which were unresponsive to acute hypoxia down to at least 2.0 kPa (approximately 10 % air saturation) (Schumacker et al., 1993). When compared with the present results, the responses of rat hepatocytes to hypoxia were similar to those of trout hepatocytes in that the reduction in the rate of aerobic metabolism occurred after prolonged but not after acute hypoxia and was accompanied by a decline in ATP content. In contrast, in goldfish hepatocytes and in chicken cardiac myocytes, a significant reduction in the rate of cellular O_2 uptake took place during both brief (1 min) and prolonged (2 h) hypoxia (Budinger et al., 1996). However, whereas in the myocytes the ATP content remained constant, this was not the case in goldfish hepatocytes, in which ATP levels fell upon exposure to acute hypoxia (Fig. 2). Thus, although the capacity for metabolic depression seems to be a common feature of hypoxia-tolerant cells, it appears that no uniform response pattern can be defined as characteristic of these cells.

Adaptation at the mitochondrial level

In principle, the observed decrease in cellular respiration rate could have been caused by O_2 supply being limiting or by an alteration in mitochondrial function. From the present data, it is clear that the former option is unlikely because (i) during acute exposure, $\dot{V}_{O_2}$ remained unresponsive at O_2 levels that produced a decrease in $\dot{V}_{O_2}$ after prolonged hypoxia (Fig. 1), and (ii) the P_{50} for oxygen of cellular respiration was 2–3 orders of magnitude below the lowest level of hypoxia applied in this study (Table 1). Thus, during prolonged hypoxia, O_2 uptake must have been actively down-regulated, allowing mitochondrial function to proceed at slower rates even though enough O_2 was available. Previous studies (Chandel et al., 1995, 1996) suggest that, in both rat hepatocytes and chick myocytes, down-regulation of O_2 consumption is based on a mechanism involving a reversible inhibition of cytochrome *c* oxidase. Our present observation that the apparent P_{50} for O_2 increased approximately twofold after prolonged hypoxia (Table 1) would be in line with such an interpretation. However, since even the increased P_{50} in hepatocytes from both goldfish and trout is extremely low in absolute terms, it seems that the apparent V_{max}, rather than P_{50}, must have been modulated to account for the decrease in the rate of O_2 consumption by approximately 50 %. In fact, the P_{50} values found in the present study are among the lowest reported in the literature (Gnaiger et al., 1995). Unfortunately, no comparable data on fish cells have been published, but the low P_{50} values may well reflect a fundamental difference of cellular metabolism between higher and lower vertebrates or between cells from homeothermic and poikilothermic animals.

Since both Steinlechner-Maran et al. (1996) and Costa et al. (1997), working with mammalian cells and mitochondria, using a nearly identical respirometer and applying the same mathematical corrections as used in the present study, obtained consistent P_{50} values that agree with other data in the literature, we are confident that our present data do not reflect an experimental artefact.

Is there a hierarchy for ATP-consuming functions?

To be an effective means of prolonging survival during hypoxia, depression of cellular ATP production must be accompanied by a corresponding decrease in the rate of ATP consumption. But whilst this demand follows from energetic necessity, there is no general rule as to which functions must be down-regulated and in which order ATP consumers have to be shut down. Analyzing the data of Table 2, it can be seen that, in trout hepatocytes during acute hypoxia, Na^+ pump activity was immediately reduced, but protein synthesis was initially unaffected. In contrast, when cells were kept hypoxic for a prolonged period, Na^+ pumping was not reduced any further but protein synthetic activity was decreased. Interestingly, the extent of reduction of both processes, amounting to approximately 50 %, was roughly equivalent to the reduction in the rate of O_2 consumption, emphasizing the important role of these ATP consumers for total cellular energy turnover.

Inspection of the goldfish data provides a different picture. Whereas the rate of protein synthesis shows a clear tendency to be reduced during hypoxia, Na^+ pump activity was maintained at the control level during both brief and prolonged hypoxia (Table 2). This finding once more emphasises the unique role of Na^+/K^+-ATPase in these cells, in which even under fully anoxic conditions Na^+ pumping is reduced by no more than 50 % and consumes up to 90 % of the anaerobically generated ATP (chemical anoxia, Krumschnabel et al., 1994b, 1996; true anoxia, W. Weiser and G. Krumschnabel, unpublished observation). A similar exceptional position of

Na^+/K^+-ATPase activity within the metabolic network has been reported in turtle hepatocytes, another well-described model for anoxia-tolerant cells (Buck and Hochachka, 1994). Thus, it appears that there exists, in the sense of Buttgereit and Brand (1995; see also Guppy and Withers, 1999), a hierarchy of ATP-consuming functions in cellular metabolism, the nature of which differs in cells from hypoxia-tolerant and hypoxia-intolerant animals (Wieser and Krumschnabel, 1999). We do not yet know why Na^+/K^+-ATPase activity is maintained in preference over other ATP consumers in some species. There is, however, some indication as to how this is achieved, namely by preferential coupling of Na^+ pumping to glycolytic ATP production (Krumschnabel et al., 1994a,b, 1997). In general, the energy metabolism of goldfish hepatocytes appears to rely more on glycolysis than that of trout hepatocytes, since the relative contribution of glycolytic ATP production was generally somewhat higher in the former than in the latter (Table 3). Considering that glycolysis will become increasingly important as hypoxia proceeds towards anoxia, such a coupling could at least partly explain why, in some cells, Na^+/K^+-ATPase activity can be sustained at the expense of other functions. In addition, other ATP consumers, particularly protein synthesis, may be generally more sensitive to changes in energy supply (Buttgereit and Brand, 1995) or be subjected to other control mechanisms, possibly involving O_2-sensing proteins (Lefebvre et al., 1993; Land and Hochachka, 1995; Tinton et al., 1997).

In summary, the present study shows (i) that the rate of oxygen consumption is dependent on oxygen availability in isolated cells within a range relevant under physiological conditions, (ii) that this decrease in the rate of oxygen consumption is more readily observable in anoxia-tolerant than in anoxia-intolerant cells, (iii) that it is not based on a drastic alteration in the apparent oxygen affinity of mitochondrial respiration, but on some as yet unknown respiratory rearrangement, and (iv) that the down-regulation of ATP production is accompanied by a partial reduction in the rate of ATP-consuming functions which, however, do not include Na^+/K^+-ATPase activity in goldfish hepatocytes, emphasising the unique role of this enzyme in these cells.

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