

IMPROVEMENT IN THE EFFICIENCY OF OXIDATIVE PHOSPHORYLATION IN THE FRESHWATER EEL ACCLIMATED TO 10.1 MPa HYDROSTATIC PRESSURE

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Summary

Previous studies have suggested that the efficiency of oxidative phosphorylation in the freshwater eel (*Anguilla anguilla*) is increased after acclimation to high hydrostatic pressure. Analysis at atmospheric pressure of the respiratory chain complexes showed that, after 21 days at 10.1 MPa, the activity of complex II was decreased to approximately 50% ($P < 0.01$) of the control value and that cytochrome *c* oxidase (complex IV) activity was significantly increased to 149% of the control value ($P < 0.05$). ADP/O ratios calculated from mitochondrial

respiration measurements were significantly increased after acclimation to high hydrostatic pressure (2.87 versus 2.52, $P < 0.001$) when measured in the presence of pyruvate plus malate at atmospheric pressure. These results clearly show an increased oxidative phosphorylation efficiency in response to high-pressure acclimation.

Key words: fish, pressure, mitochondrial respiration, enzyme activity, energy metabolism, eel, *Anguilla anguilla*.

Introduction

High hydrostatic pressure has been shown to induce changes in energy metabolism in the freshwater eel (*Anguilla anguilla*). After a maximal value reached at the end of the compression period (compression to 10.1 MPa at a rate of 1 MPa min⁻¹), the rate of oxygen consumption decreases; concomitantly, tissue ATP content decreases and AMP content increases. These modifications in levels of energetic nucleotides are due to alterations to aerobic metabolism, only partially compensated by an increased use of anaerobic pathways (Sébert et al., 1993). It has therefore been suggested that high hydrostatic pressure induces a state resembling histotoxic hypoxia (i.e. poisoning of the respiratory chain). After 6–8 days at high pressure (10.1 MPa), oxygen consumption stabilises at a rate corresponding to approximately 65% of the control value (Simon et al., 1989) and muscle ATP and AMP levels are restored. The low observed ratio of pyruvate kinase to cytochrome *c* oxidase activity (PK/COX) shows that aerobic energy production predominates (Simon et al., 1992). These results have been correlated with the supposed recovery of membrane fluidity (which decreases during the early hours of pressure exposure: Sébert et al., 1995) and, hence, to the recovery of optimal conditions for membrane-bound enzymes.

The above results show that the metabolism of the eel, after acclimation to 10.1 MPa (absolute atmosphere) of hydrostatic pressure, is predominantly aerobic and, despite a reduced rate of oxygen consumption, is still able to maintain normal muscular energy production. This apparent contradiction suggests an improved oxidative phosphorylation efficiency

after exposure to high pressure (Simon et al., 1992). To confirm this, rates of mitochondrial oxygen consumption and the activities of the enzyme complexes of the respiratory chain were measured at atmospheric pressure and ADP/O ratios were calculated in red muscle of eels acclimated for 3 weeks to 10.1 MPa.

Materials and methods

Animals

Thirty-four yellow freshwater eels (*Anguilla anguilla* L., mass 133±6 g, mean ± S.E.M.) were obtained from a local fishery and kept without feeding in 40 l polyethylene tanks containing continuously renewed and aerated tap water.

To maintain water temperature and photoperiod as close as possible to natural conditions, the tanks were placed in a room open to the outside.

Experimental protocol

Two to four eels were placed in an experimental tank (14.7 l) connected to a high-pressure water circulation system, and the tank was placed in a hyperbaric chamber. The water circulation system allowed continuous renewal of the water, so that temperature and oxygen concentration could be controlled (Sébert et al., 1990).

The animals were brought to a hydrostatic pressure of 10.1 MPa at a rate of 0.2 MPa min⁻¹ and kept at this pressure for 21 days. During the same period, a control group of 2–4

eels was kept in a separate tank with water circulating under the same environmental conditions but at atmospheric pressure (0.1 MPa).

Water temperature T_w and oxygen partial pressure P_{wO_2} were monitored twice a day. Mean values (\pm S.E.M.) were: $T_w=18\pm 1^\circ\text{C}$, $P_{wO_2}\approx 20\text{ kPa}$; water flow was approximately 121 h^{-1} .

At the end of the period of pressure exposure, the animals were rapidly brought to atmospheric pressure (1 MPa min^{-1}) and killed by decapitation.

Extraction of mitochondria

Red muscle (mass $10\pm 0.6\text{ g}$, mean \pm S.E.M.) was dissected from the animals immediately following decapitation and placed in ice-cold extraction medium 1 (EM1; Hepes, 4 mmol l^{-1} ; KCl, 140 mmol l^{-1} ; MgCl_2 , 5 mmol l^{-1} ; EDTA, 10 mmol l^{-1} ; ATP, 1 mmol l^{-1} ; pH 7.4 at 4°C). Samples were cut thinly with scissors, washed twice in EM1 and resuspended in EM1.

Trypsin was added to a final concentration of 15 units g^{-1} muscle; digestion proceeded for 15 min and was then stopped with 30 units g^{-1} of antitrypsin. The samples were washed twice with EM1 plus 0.5% (w/v) bovine serum albumin (BSA) and resuspended in EM1. The muscle was then homogenised using a hand-held Potter homogeniser (ten strokes at 4°C).

Mitochondrial enrichment was achieved using three identical series of two centrifugations. Each series consisted of a first centrifugation of 5 min at 4°C and 1200 g ; the resulting supernatant was then centrifuged at 7000 g for 10 min at 4°C . The resulting pellet was suspended in extraction medium 2 (EM2; Hepes, 20 mmol l^{-1} ; KCl, 170 mmol l^{-1} ; MgCl_2 , 5 mmol l^{-1} ; EDTA, 0.1 mmol l^{-1} ; pH 7.2 at 4°C). The three series were identical excepted that in the series 3 the final mitochondrial pellet was resuspended in respiratory medium ($100\ \mu\text{l g}^{-1}$ muscle; Tris, 20 mmol l^{-1} ; KCl, 150 mmol l^{-1} ; EDTA, 1 mmol l^{-1} ; Na_2HPO_4 , 10 mmol l^{-1} ; MgCl_2 , 7.5 mmol l^{-1} ; pH 7.2 at 18°C) and kept at 4°C .

Mitochondrial respiration

Mitochondrial respiration was measured in a 1 ml glass vessel with constant magnetic stirring (glass barrel) and at controlled temperature (18°C). The oxygen content in the vessel was measured using a Strathkelvin Instruments O_2 microelectrode (accuracy $\pm 0.2\%$ of saturation; Strathkelvin Instruments Ltd, 15 Lochend Road, Bearsden, Glasgow G61 1DX, Scotland). The solutions of ADP and NADH used were calibrated following the method of Lemasters (1984); the NADH solution was used to calibrate the respiratory medium (Robinson and Cooper, 1970); the molecular oxygen concentration at 18°C was $245\ \mu\text{mol l}^{-1}$.

Rates of oxygen consumption (\dot{M}_{O_2}) were measured, and calculation of ADP/O ratios was performed as described by Hinkle (1995) on freshly extracted mitochondria (0.3 mg of mitochondrial protein per millilitre of respiratory medium), using pyruvate plus malate (final concentrations 5 and 2.5 mmol l^{-1} , respectively), succinate plus rotenone, a complex

I inhibitor (final concentrations 10 mmol l^{-1} and $0.4\ \mu\text{mol l}^{-1}$, respectively; Lee et al., 1996), ascorbate (10 mmol l^{-1}), tetramethylphenylenediamine (TMPD, $65\ \mu\text{mol l}^{-1}$), myxothiazol ($0.1\ \mu\text{mol l}^{-1}$) and antimycin ($0.1\ \mu\text{mol l}^{-1}$; Stoner, 1987). Myxothiazol and antimycin are both complex III inhibitors.

Enzyme activities

The activities of the respiratory chain enzymes were determined at 18°C by spectrophotometry after rupture of the mitochondrial membrane by two freezing (in liquid nitrogen) and thawing (in ice-cold water) cycles. The mitochondrial extracts were kept at -80°C before measurements of enzyme activity. All enzyme activities are expressed in milliunits mg^{-1} mitochondrial protein; 1 unit of enzyme activity corresponds to the appearance of $1\ \mu\text{mole}$ of product, or the consumption of $1\ \mu\text{mole}$ of substrate, per minute.

Complex I activity was measured from the rate of rotenone-sensitive oxidation of NADH in respiratory medium supplemented with ubiquinone 2 ($85\ \mu\text{mol l}^{-1}$), EDTA (final concentration 1.9 mmol l^{-1}) and NADH ($80\ \mu\text{mol l}^{-1}$).

Succinate Q reductase (complex II) activity was measured at 600 nm in potassium phosphate buffer (50 mmol l^{-1} , pH 7.4 at 18°C), 0.1 mol l^{-1} EDTA, $45\ \mu\text{mol l}^{-1}$ ubiquinone 2, $12.5\ \mu\text{mol l}^{-1}$ rotenone, $2\ \mu\text{g l}^{-1}$ antimycin A, $75\ \mu\text{mol l}^{-1}$ dichloroindophenol (DCIP) and 20 mmol l^{-1} sodium succinate (Ziegler and Rieske, 1967; Ragan et al., 1987).

Complex III activity was measured by following cytochrome *c* reduction at 550 nm using the method of Ragan et al. (1987): potassium phosphate buffer, 45 mmol l^{-1} ; cytochrome *c*, $50\ \mu\text{mol l}^{-1}$; EDTA, 1 mmol l^{-1} ; ubiquinol, $50\ \mu\text{mol l}^{-1}$, modified by the addition of NaN_3 (5 mmol l^{-1}).

The cytochrome *c* oxidase activity of complex IV was measured at 550 nm in sodium phosphate buffer (0.33 mol l^{-1} , pH 7 at 18°C) with $50\ \mu\text{mol l}^{-1}$ reduced cytochrome *c* (Smith and Conrad, 1956) plus $2\ \mu\text{g l}^{-1}$ antimycin A.

The ATPase activity of complex V (activity inhibited by 0.4 mmol l^{-1} NaN_3) was measured by following NADH oxidation in an ATP-regenerating system (Ragan et al., 1987) after verification of the absence of NADH oxidase activity when the ATP concentration in the assay medium was zero. The ATP-regenerating medium was as follows: Hepes, 50 mmol l^{-1} (pH 8 at 18°C); sucrose, 0.25 mol l^{-1} ; MgCl_2 , 5 mmol l^{-1} ; NADH, 0.2 mmol l^{-1} ; phosphoenolpyruvate, 25 mmol l^{-1} ; pyruvate kinase, 40 units ml^{-1} ; lactate dehydrogenase, 20 units ml^{-1} ; ATP, 25 mmol l^{-1} .

Total protein concentration in the mitochondrial extract was measured using the method of Lowry as adapted by Peterson (1977).

Results are expressed as means \pm S.E.M. Statistical significance was evaluated at the 5% level using Mann-Whitney *U*-test or Student's *t*-test.

Results

The activities of the respiratory chain enzymes are given in

Table 1. Activities of the respiratory chain enzymes

Pressure (MPa)	Enzyme activity (milliunits mg ⁻¹)				
	Complex I	Complex II	Complex III	Complex IV	Complex V
0.1 (N=8)	10.5±2.0	44.4±5.2	367.5±73.0	166.6±27.4	55.2±3.0
10.1 (N=4)	6.1±0.9	20.2±4.0**	192.8±9.0	248.9±19.4*	53.1±4.1

Enzyme activities are expressed in milliunits mg⁻¹ mitochondrial protein, where 1 unit corresponds to the appearance of 1 µmole of product or the consumption of 1 µmole of substrate per minute.

Measurement temperature 18 °C

Values are means ± S.E.M.

Asterisks indicate a significant difference between pressures (**P*<0.05, ***P*<0.01).

Table 1. The activity of cytochrome *c* oxidase (complex IV) after pressure acclimation was significantly higher (49 % increase, *P*<0.05) than that of the control group (atmospheric pressure). Among the other enzymes, the activities of complexes I–III were reduced to approximately 50 % of the control values, but this was statistically significant only for succinate Q reductase activity (complex II, *P*<0.01). The ATPase activity of complex V was not modified after 21 days of exposure to 10.1 MPa.

ADP/O ratios using pyruvate plus malate determined by injection of a known amount of ADP (45±5 nmol, a saturating level since doubling it did not increase state 3 respiration rate) into the respiratory cell are given in Table 2 with states 3 and 4 respiratory rates and the respiratory control ratio (RCR: ratio of state 3 to state 4 respiration rates). After high-pressure acclimation, the ADP/O ratio was significantly

increased to 114 % of the control value (*P*<0.001) and the RCR was slightly, but not significantly, decreased. Concomitantly, rates of mitochondrial oxygen consumption (expressed in nmol O₂ min⁻¹ g⁻¹ fresh tissue) in both states 3 and 4 were slightly, but not significantly, decreased to approximately 90 % of control values. The results of mitochondrial respiration with succinate or ascorbate plus TMPD as substrates are also given in Table 2. With succinate, ADP/O ratios were increased (not significantly) to 109 % of the control value; with ascorbate, ADP/O ratios of control and pressure-acclimated animals were similar (0.86 and 0.83, respectively). Mitochondrial respiration rates in states 3 and 4 were decreased (not significantly) to approximately 90 % of the control value with succinate but were increased with ascorbate (to 116 % and 132 % of control values, respectively; differences not significant). RCRs were

Table 2. Mitochondrial respiration in media containing pyruvate and malate or succinate or ascorbate

Substrate	Pressure (MPa)	ADP/O ratio	RCR	\dot{M}_{O_2} state 3 (nmol min ⁻¹ g ⁻¹)	\dot{M}_{O_2} state 4 (nmol min ⁻¹ g ⁻¹)
Pyruvate plus malate	0.1 (N=20)	2.52±0.04	3.06±0.20	5.28±0.76	1.85±0.24
	10.1 (N=10)	2.87±0.05*	2.57±0.25	4.80±1.34	1.75±0.47
Succinate	0.1 (N=6)	1.31±0.08	2.30±0.16	6.18±2.00	2.85±0.84
	10.1 (N=5)	1.43±0.07	2.53±0.33	5.45±1.33	2.61±0.90
Ascorbate	0.1 (N=6)	0.86±0.03	1.52±0.17	4.60±1.17	3.32±0.88
	10.1 (N=5)	0.83±0.03	1.23±0.04	5.43±0.78	4.38±0.64

Final concentrations of substrates were 5 mmol l⁻¹ pyruvate and 2.5 mmol l⁻¹ malate, 10 mmol l⁻¹ succinate, 0.4 µmol l⁻¹ rotenone, 10 mmol l⁻¹ ascorbate, 65 µmol l⁻¹ tetramethylphenylenediamine, 0.1 µmol l⁻¹ myxothiazol and 0.1 µmol l⁻¹ antimycin.

RCR (respiratory control ratio): ratio of state 3 to state 4 respiration rate.

Rates of oxygen consumption (\dot{M}_{O_2}) are expressed in nmol O₂ min⁻¹ g⁻¹ fresh tissue.

Measurement temperature 18 °C.

Values are means ± S.E.M.

Asterisks indicate a significant difference from the control (atmospheric pressure) value (**P*<0.001).

increased with succinate and decreased with ascorbate (neither difference was significant).

Discussion

The ADP/O values obtained in the controls of the present study are similar to those reported previously (Moon and Ouellet, 1979; Moyes et al., 1989). Together with the RCR results, they confirm the validity of the mitochondrial extraction and respiration techniques used here. Moreover, preliminary results using electron microscopy (not shown) confirm the normal shape of mitochondria in the control muscle samples. Measurements of citrate synthase activity in the mitochondrial extract (results not shown) show that this matrix enzyme was not released (extract citrate synthase activity was less than 14 % of the total activity present), ruling out any substantial damage to the mitochondrial inner membrane (Kashfi and Cook, 1992; Letellier et al., 1992). Finally, the significant increase in COX (complex IV) activity of 49 % in response to high pressure is consistent with previous results on red muscle from the same species, in which an increase of 46 % in COX activity was found (Simon et al., 1992).

The main objective of the present study was to investigate how high-pressure acclimation is able to maintain, with a predominantly aerobic metabolism, a normal muscular energy charge despite a reduced rate of oxygen consumption. Our results confirm the suggestion of Simon et al. (1992) that pressure acclimation improves oxidative phosphorylation efficiency: ADP/O ratios measured at atmospheric pressure in mitochondria from high-pressure-acclimated animals are significantly higher than those of controls with pyruvate plus malate as the substrate (2.87 versus 2.52, respectively, $P < 0.001$). A trend towards an increase also seems to exist with an FADH₂-producing substrate, succinate (non-significant 9 % increase), but no change in the ADP/O ratio was found with ascorbate as the substrate. Hence, the mitochondria of pressure-acclimated eels appear to be able to respond to the same energy demand while consuming less oxygen when oxidising NADH-producing substrates.

There are three ways of increasing the ADP/O ratio and thus the oxidative phosphorylation efficiency. First, the proton leak can be reduced; this has been shown to correlate positively with the mitochondrial membrane unsaturation index (Brookes et al., 1998). As pressure adaptation is known (measurements at atmospheric pressure) to increase this index and also to increase membrane fluidity and to decrease the saturation index (Cossins and MacDonald, 1984; DeLong and Yayanos, 1985; Sébert, 1997; Sébert and Macdonald, 1993), the proton leak is unlikely to be reduced in pressure-acclimated fish (see Brown and Brand, 1991). Moreover, as there was no significant change in state 4 respiration rate between 0.1 and 10.1 MPa, with any substrate used, the proton leak does not appear to change with high-pressure acclimation. In addition, increased complex V efficiency and/or a reduced proton leak will result in an increased ADP/O

ratio irrespective of the respiratory chain substrate used; however, in the present study, ADP/O ratios with ascorbate as substrate did not increase after pressure acclimation (control 0.86, pressure-acclimated 0.83). This also rules out the second route by which the ADP/O ratio can be increased: an increase in F₁F₀-ATPase efficiency. This modification of complex V will result in an increased oxidative phosphorylation yield regardless of the substrate used.

The final possibility is to increase the efficiency of the coupled proton translocation/electron transfer (global respiratory chain H⁺/2e⁻ ratio). The present results seem to support this pathway. Enzyme activities are measured following the oxidation of substrates or reduction of products but in conditions of substrate saturation, i.e. the activities measured give an indication of maximal possible electron transfer rates. Table 1 shows that, in high-pressure-acclimated fish, there is a reduction of approximately 50 % in the activity of complexes I–III (result significant only for complex II), whereas complex IV (COX) activity is significantly increased (by 50 %). Thus, the potential for electron transfer is decreased in the first part of the respiratory chain (in particular in complex II), while it is increased for complex IV. From this, it appears that in control fish the first steps of the respiratory chain transfer more electrons than COX can accept, leading to an electron leak and a relatively high production of superoxide radicals (Liu, 1997), as has been demonstrated in fish exposed to high pressure for a short period (i.e. 3 h; Sébert et al., 1995). In contrast, in the high-pressure group, the first steps of the respiratory chain convey fewer electrons, all or most of which are transferred to COX. This leads to a decreased electron leak and a possible improvement of the H⁺/2e⁻ ratio (Liu, 1997). In other words, high-pressure acclimation allows optimisation of the respiratory chain, thus balancing electron transfer and electron utilisation.

The ADP/O ratios determined here allow us to localise in the respiratory chain the element responsible for the increased efficiency. There are three sites of proton translocation in the respiratory chain: complex I, the ubiquinone cycle and cytochrome *c* oxidase. The ADP/O ratio with pyruvate plus malate as a substrate is increased and, because the ADP/O ratio also shows a tendency to increase when the substrate is succinate (which feeds complex II), the site involved is unlikely to be complex I. Because the ADP/O ratio with ascorbate as substrate is not altered after pressure acclimation, the stoichiometry of H⁺ transfer by COX (complex IV) is not modified. The only other possible candidate is the ubiquinone cycle (known to be a strong superoxide radical producer; Liu, 1997). In mitochondria of pressure-acclimated animals, the H⁺/2e⁻ ratio of this step could be increased, reducing its electron leak as a consequence of both the reduced maximal electron inflow and increased COX activity.

To summarise, we propose that the greater efficiency of the respiratory chain and oxidative phosphorylation in eels acclimated to high pressure is due both to a reduced electron transfer potential at the entry point of the respiratory chain and to enhanced COX activity; this situation permits a reduction in

the electron leak and, hence, an optimisation of the respiratory chain. Thus, as suggested by Brand (1993), pressure acclimation is able to modify the relative control of respiratory rates by ATP turnover and/or the respiratory chain. The fact that measurements were performed at atmospheric pressure means that we could not confirm that the ADP/O ratio is increased under pressure. However, pressure strongly affects aerobic metabolism during the first few hours of exposure (see Sébert et al., 1993) and triggers processes that, during the acclimation, restore normal ATP production despite the observed decrease in \dot{M}_{O_2} (giving a predicted increase in ADP/O ratio). Thus, the decompression that preceded measurements at atmospheric pressure reveals, on an amplified scale, the phenomenon that exists at pressure. In a recent review, Sébert (1997) has shown that acclimation of eels to high pressure causes structural, biochemical and physiological changes that mimic those believed to occur during the migration towards the breeding area. The modifications to the mitochondrial respiration reported in the present work are in agreement with these observations.

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