

***In vitro* effect of hydrostatic pressure exposure on hydroxyl radical production in fish red muscle**

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The effects of hydrostatic pressure on reactive oxygen species (ROS) production have been studied *in vitro* on fish red muscle fibres. In the eel, *Anguilla anguilla*, previous studies have shown that hydrostatic pressure acclimatization improves oxidative phosphorylation efficiency together with a supposed concomitant decrease in electron leak and ROS production. In order to test the hypothesis of an electron leak decrease under pressure, hydroxyl radical (HO[•]) production and oxygen consumption were measured on fish red muscle fibres directly exposed to hydrostatic pressure. Experiments were performed under two conditions – atmospheric pressure and hydrostatic pressure (16.1 MPa) – on eel and trout (which exhibit low- and high-pressure sensitivity, respectively). This work has permitted, first, the validation of an indirect HO[•] measurement (*in vitro*) on fish red muscle and the documentation of reference values for fish. Second, at atmospheric pressure, results show higher oxygen consumption for trout (+40%) than for eel which is accompanied by increased HO[•] production (+90%); in addition, both species present a positive relationship between HO[•] production and oxygen consumption. Hydrostatic pressure exposure reverses this relationship for eel but not for trout. These preliminary results only partially verify the proposed hypothesis and further experiments are needed.

Keywords: Hydrostatic pressure, ROS production, fish red muscle, respiratory chain function

INTRODUCTION

Previous studies have shown several alterations to oxidative phosphorylation efficiency in fish red muscle by hydrostatic pressure, depending on the species studied.¹ In European eel (*Anguilla anguilla*), hydrostatic pressure acclimatization is accompanied by oxidative phosphorylation improvement (P/O ratio increase).² This improvement in metabolic efficiency has been recently linked to some changes in the activity of respiratory chain complexes (increased cytochrome oxidase activity, for example) by the effect of pressure on membrane

properties (such as temperature).² Pressure acclimatization would allow adjustment to the input of electrons into the respiratory chain to oxygen consumption possibilities which supposes a decreased electron leak, known to occur at the semi-ubiquinone level between complexes I and III.³ These observations have led us to suppose that, if electron leak decreases, there would be a concomitant decrease in mitochondrial reactive oxygen species (ROS) production. Indeed, electron leak is recognized as the starting point to ROS production in mitochondria, by reducing 1–3% of the oxygen.⁴ For this initial investigation, hydroxyl radical (HO[•]) was chosen among ROS and reactive nitrogen species (for example: superoxide anion, O₂^{•-}; hydrogen peroxide, H₂O₂; nitric oxide, NO[•]). In fact HO[•] is the end by-product of ROS and considered highly reactive.⁴⁻⁷ Effectively, HO[•] is produced by the Haber-Weiss reaction (O₂^{•-} + H₂O₂ → O₂ + OH⁻ + HO[•]) which is mainly iron catalysed.⁷ As the mitochondrial inner membrane is rich in metallic ions, it can help the reaction.⁸ So, HO[•] was estimated in fish red muscle directly exposed to hydrostatic pressure in order

Received 29 June 2004
Revised 4 December 2004
Accepted 23 December 2004

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to verify the hypothesis of an electron leak decrease. However, as the trout is more pressure sensitive than the eel and has a higher metabolic rate (inhibited by pressure¹), we have compared ROS production in both species under pressure and verified the relationship between ROS production and oxygen consumption.

MATERIALS AND METHODS

Permeabilised red muscle fibres of eel and trout were submitted to hydrostatic pressure (16.1 MPa) for 30 min. For *in vitro* measurements, the HO[•] determination technique used was adapted from Floyd *et al.*^{9,10} and Duine *et al.*¹¹ It involves trapping HO[•] by salicylate and then measuring 2-3- and 2-5-dihydroxybenzoic acid (DHBA), two stable metabolites which come from salicylate hydroxylation, by HPLC with electrochemical detection.

Eight European eels (*Anguilla anguilla*) from the Loire river (France), about 100 g in weight, and five trout (*Onchorynchus mykiss*) from a regional fishery (France), about 300 g in weight, were used. They were stored at 15°C without feeding in polyethylene tanks with continuously renewed and aerated tap water. After decapitation (trout were first stunned), trout (T) and eel (E) red muscle was immediately dissected along the lateral line and placed in an ice-cold extraction medium. The red muscle fibres were permeabilised using a method modified for fish by Sébert and Theron¹ according to Veksler *et al.*¹² and Letellier *et al.*¹³ Permeabilisation was obtained by a saponin treatment (150 µg/l), to perforate cell membranes without altering mitochondrial function.

Pressure exposure and O₂ consumption were performed at 15°C and HPLC measurements at ambient temperature.

Permeabilised red muscle fibres of trout (T) and eel (E) were exposed to 16.1 MPa (T161, E161) of hydrostatic pressure with salicylate for 30 min in a hyperbaric chamber (0.075 l). Compression and decompression were performed within 1 min. Control groups (T1, E1) were simultaneously tested in a similar hyperbaric chamber at atmospheric pressure (0.1 MPa). After 30 min under pressure, one part of the tissue was removed for O₂ consumption measurement and the other for ROS quantification.

For HO[•] measurement, permeabilised red muscle fibres (~150 mg) were placed in 30 volumes of respiratory buffer (Tris 20 mM, KCl 150 mM, EDTA 0.08 mM, NaH₂PO₄ 10 mM and MgCl₂ 7.5 mM, pH = 7.2) for 1 volume of tissue. Salicylate (0.5 mM) was added and the incubation was maintained for 30 min in darkness. Afterwards, perchloric acid (70%) was added (1.5 volumes for 1 volume of tissue). The tissue was homogenised in liquid nitrogen and finally returned to its incubation medium. After centrifugation (10 min at 12,000 g at 4°C), supernatants were neutralized (pH > 7)

with NaOH (6 M), filtered through 0.45 µm membranes and 50 µl injected into the HPLC system. The separation of 2-3-DHBA and 2-5-DHBA was performed on a Hypersil ODS 25 cm column. The mobile phase was 97% sodium acetate (48.2 mM), citric acid (10 mM), EDTA (0.13 mM) and sodium octyl sulphate (0.52 mM) buffer (filtered through a 0.45 µm membrane before use) and 3% of acetonitrile; the pH was adjusted to 3.8 with acetic acid. The flow rate was 0.8 ml/min and the mobile phase was continuously degassed by N₂ bubbling. Electrochemical detection was performed using an amperometric detector with a glass electrode and an Ag/AgCl reference electrode at a potential of 700 mV (sensitivity, 1 nA full scale). The tissue quantification was carried out with external calibration (standard of 2-3-DHBA and 2-5-DHBA containing, respectively, 2 ng/ml and 2.5 ng/ml).

Measurement of O₂ consumption has been previously described.^{1,2} Respiratory buffer containing pyruvate, malate and ADP in saturating concentrations (final concentrations were, respectively, 12 mM, 6 mM and 5 mM) was used. Maximal O₂ consumption was obtained using pyruvate and malate as substrates for the mitochondrial respiratory chain complex I and ADP addition stimulated the mitochondrial respiratory rate (oxidative phosphorylation). Before the addition of perchloric acid, one part of the permeabilised muscle fibres (~20–30 mg) was removed to measure O₂ consumption (in 20 volumes of respiratory buffer for 1 volume of tissue). O₂ consumption was calculated by measuring the oxygen content decrease over 2 min, using Strathkelvin Instrument O₂ microelectrodes.

The results are expressed as mean ± SEM. Statistical significance of the results was evaluated at the 5% level with Student's *t*-test.

RESULTS AND DISCUSSION

For this preliminary work on ROS production by fish red muscle, we have chosen to quantify the highly reactive HO[•] for the following reasons. It is the end by-product of ROS and the most toxic.^{4–7} Thus, it can be a good index of ROS production and, moreover, perhaps reactive nitrogen species production. Indeed, the reaction of O₂^{•-} with NO[•] results in peroxynitrite (ONOO⁻) formation¹⁴ which also leads to 2-3-DHBA formation by the salicylate hydroxylation technique used for HO[•] quantification.¹⁵

In order to test the hypothesis of a decrease in electron leak and thus ROS production during hydrostatic pressure exposure, it was necessary to carry out ROS analysis. HO[•] fugacity and ubiquity impede direct measurement and thus the Floyd *et al.* method was modified. Some precautions with salicylate have been used such as low concentration (0.5 mM rather than 2 mM in the work of Floyd *et al.*) in order to avoid oxidative

Table 1. O₂ consumption, HO[•] production and ratio of HO[•] production (30 min) to O₂ consumption.

	E1	E161	T1	T161
	<i>n</i> = 5		<i>n</i> = 4	
O ₂ consumption (nmol O ₂ /min/g)	335 ± 30	391 ± 29	480 ± 10	430 ± 10
HO [•] production (ng DHBA/g)	144 ± 13	152 ± 7	277 ± 48*	204 ± 21**
HO [•] production:O ₂ consumption (ng DHBA/μmol O ₂) ratio	13.4 ± 1	13.2 ± 1	20 ± 3	16.5 ± 2

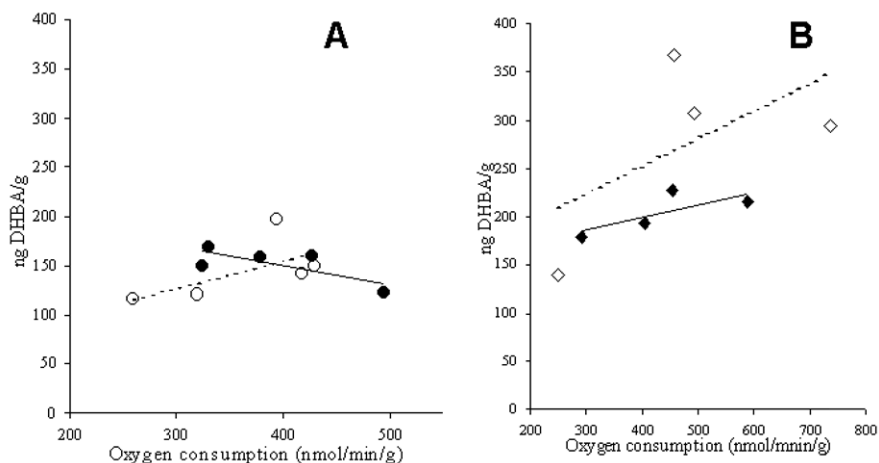
These measurements were performed on eel (E) and trout (T) fibres exposed (161) or not (1) to 16.1 MPa.

Results are expressed as mean ± SEM. **P* < 0.05 comparison between E1 and T1; ***P* < 0.005 comparison between E161 and T161.

phosphorylation uncoupling at concentrations higher than 1 mM.¹⁶ Likewise, as salicylate hydroxylation is a photosensitive reaction, incubation was performed in darkness. The linearity, reproducibility and sensitivity of the HPLC method were also tested. The detection limit obtained was 10⁻¹⁴ moles of injected DHBA and calibration curves for both metabolites were established between 0.1–25 ng injected DHBA (*r* = 0.999 for 2-3- and 2-5-DHBA). In practice, this range of DHBA concentrations tallies with the assumed concentration ranges in permeabilised red muscle fibre samples. This tissue preparation has the advantage of maintaining good physiological conditions and of being sensitive to hydrostatic pressure. The HO[•] determination has been previously tested on rat muscle fibres by Hasegawa *et al.*¹⁷ who obtained similar values (about 200 ng DHBA/g). This application in mammals allowed us to validate the HO[•] determination technique because there are no available data for fish, oxidative stress in fish being mainly studied by antioxidant enzyme activity determination.^{18,19}

Hydrostatic pressure is both an environmental and thermodynamic factor. It is known to modify membrane properties (changes in fluidity and unsaturation index) and to induce proton leak variations.^{2,20} Moreover, some

authors have suggested that a proton leak tends to decrease mitochondrial ROS formation by acting as an antioxidant.^{21,22} Therefore, hydrostatic pressure is supposed to induce ROS production by altering respiratory chain functioning. According to their different pressure resistance, two fish species (eel and trout) were chosen. The eel is known to acclimatize to pressure effects by improving oxidative phosphorylation²³ but to produce ROS when exposed for short periods (6 h) to high pressure.²⁴ In contrast, the trout has a higher metabolic rate (+40%) and is very sensitive to hydrostatic pressure 1 (Table 1). At atmospheric pressure, trout have higher DHBA production (+90%; *P* < 0.05) than eel which supports the existence of a relationship between HO[•] production and oxygen consumption (Fig. 1), a relationship previously demonstrated in vertebrates and recently in invertebrates by Abele *et al.*²¹ and Heise *et al.*²⁵ However, the DHBA production/oxygen consumption ratio is higher in trout than in eel (not significant, due to trout value dispersion, see Fig. 1) showing for a given oxygen consumption a significant trout HO[•] production. This could be due to a lower antioxidant capacity (Amérand, unpublished data) and/or to a difference in ROS production regulation by proton leak and the


Fig. 1. Relationship between HO[•] production (ng DHBA/g of tissue) and O₂ consumption (nmol/min/g) in eel (A) and trout muscle fibres (B) which are exposed (closed symbols: E161 and T161) or not (open symbols: E1 and T1) to hydrostatic pressure. The same scale is used in order to compare eels and trout.

uncoupling of oxidative phosphorylation.²⁵ Pressure experiments can provide more information. Trout mitochondrial O₂ consumption decreases with pressure exposure (not significant) according to previous results.¹ As their muscle cells are known to have an apparent respiratory over capacity with substantial uncoupling,²⁶ we cannot exclude the hypothesis that, in this species, high pressure decreases the respiration rate but also modifies the proton leak and/or the electrochemical proton gradient and consequently ROS production. In contrast, pressure has no effect on the eel respiration rate or DHBA production, which confirms the pressure resistance of the eel. Unfortunately, the hypothesis for a HO[•] production decrease under pressure is not totally confirmed (Table 1). Muscle fibres are briefly exposed to hydrostatic pressure (30 min) whereas the improvement in oxidative phosphorylation (the basis of our hypothesis) requires 15–21 days.² However, for eel the reversal of the DHBA/O₂ consumption relationship under pressure suggests that, in these conditions and for a given O₂ consumption rate, ROS production would be decreased. Further experiments are needed to confirm this hypothesis.

CONCLUSIONS

We suggest that electron leak and consequently ROS production should be considered, at least in ectotherms as target systems, in the regulation of mitochondrial functioning under pressure. The correlation between proton leak, ROS production and antioxidant enzymatic defences is in progress in our laboratory.

ACKNOWLEDGEMENTS

The authors acknowledge the advice and technical assistance of E Girin, L Nicolas and M Belhomme. G Sinquin helped with translation of the manuscript. This work was supported partially by the European contract EELREP N°Q5RS-2001-01836.

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