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Oxygen consumption, ventilation frequency and cytochrome c oxidase activity in blue cod (*Parapercis colias*) exposed to hydrogen sulphide or isoeugenol

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ABSTRACT

The effects of hydrogen sulphide (H_2S) and isoeugenol exposure on activity, oxygen consumption (VO_2), ventilation frequency (Vf) and cytochrome c oxidase activity in a teleost fish are reported. In H_2S (200 μM Na_2S) exposed animals VO_2 and Vf decreased significantly (both to 40% of resting) after 30 min, concurrent with a loss of equilibrium and narcosis. Post-flushing, VO_2 increased to resting values, but Vf remained depressed ($P<0.05$) until 30 min of recovery. Subsequently, equilibrium and mobility were regained accompanied by increases in VO_2 (66%) and Vf (15%) between 60–70 min of recovery. Isoeugenol (0.011 g L^{-1}) exposed fish reached stage 4–5 of anaesthesia accompanied by decreases ($P<0.05$) in VO_2 (64%) and Vf (38%) by 35 min. Post-flushing, VO_2 and Vf recovered to resting values, followed by a rise ($P<0.05$) in VO_2 (45%) and Vf (25%). Overall, VO_2 in relation to the resting rate was reduced in isoeugenol treated animals. Conversely, VO_2 was increased ($P<0.05$) relative to the resting rate in H_2S exposed fish. 20 and 200 μM Na_2S reduced cytochrome c oxidase activity ($P<0.05$) in skeletal muscle and gill lamellae by between 69 and 97%, while isoeugenol had no effect in any tissue.

1. Introduction

Hydrogen sulphide (H_2S) is present in the atmosphere and aquatic environments. It has long been recognised as a toxic compound, produced through geological and biological processes (e.g. an end product of certain anaerobic bacteria) and by industrial activity (Beauchamp et al., 1984). Acute administration of H_2S, mostly in toxicity studies, has identified complex IV of the mitochondrial electron transport chain, cytochrome c oxidase, as the primary target in both mammals (Nicholls and Kim, 1982; Khan et al., 1990; Dorman et al., 2002) and fish (Smith et al., 1976; Torrans and Clemens, 1982). This inhibition appears to be similar to that produced by carbon monoxide and cyanide (Szabó, 2007).

H_2S has also been identified as an energy source for many photo- and chemoautotrophic bacteria as well as animals such as the lugworm (*Arenicola marina*) (Goubern et al., 2007). H_2S is also used as a substrate for ATP generation by colonocyte mitochondria in humans (Goubern et al., 2007). It is produced endogenously in vertebrate tissues from L-cysteine by two pyridoxyl 5′-phosphate-dependent enzymes: cystathionine β-synthase and cystathionine γ-lyase (Hosoki, et al., 1997; Szabó, 2007). This discovery along with intensive research over recent decades has led to the recognition of the biological importance of H_2S in vertebrate tissues; roles have been identified in brain, gastrointestinal, reproductive, pulmonary, vascular and heart tissues (see Dombkowski et al., 2005). For example, there is now strong evidence that the H_2S is involved in the oxygen sensing/signal-transduction cascade in hypoxic vasoconstriction and vasodilation in vertebrate smooth muscle (Dombkowski et al., 2004; Dombkowski et al., 2005; Dombkowski et al., 2006; Olson et al., 2006; Russell et al., 2007; Olson et al., 2008a; Olson, 2008).

It has also been suggested that H_2S exposure can induce hypometabolism. Inhaled H_2S has been reported to produce a completely reversible state of hypometabolism in mice (reducing VO_2, VCO_2, heart rate and core body temperature) (Blackstone et al., 2005; Blackstone and Roth, 2007; Haouzi et al., 2008; Volpato et al., 2008). The original study by Blackstone et al. (2005) reported a 90% decrease in VO_2 after 6 h of exposure to 80 ppm H_2S gas in mice. Subsequently, Blackstone and Roth (2007) reported that normally lethal levels of hypoxia (5% O_2) were survived for a 6.5 h period in mice pre-treated with 150 ppm H_2S, with 100% mortality in control animals after only 20 min. These findings were received with much excitement; since if human tissues respond in a similar manner, the reduction in metabolic rate could provide protection from ischemia-associated tissue damage to organs (e.g. infarction), which can occur after major trauma or cardiac arrest (Szabó, 2007; Volpato et al., 2008). Interestingly, recent evidence suggests that H_2S may also be directly involved in oxygen sensing associated with ischemic preconditioning in rainbow trout (*Oncorhynchus mykiss*) hearts (Whitfield et al., 2008).

In a recent study, H_2S-induced metabolic rate depression was reproduced in mice, but not in sedated sheep (Haouzi et al., 2008). The difference was attributed to size-related metabolic rate differences

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associated with endothermy. However, adult pigs showed a reduction in VO_2, VCO_2 and cardiac output (a fall in heart rate, but not stroke volume) on intra-venous H_2S (Na_2S) infusion (Simon et al., 2008). Conversely, piglets showed an increase in metabolic rate during H_2S inhalation (Li et al., 2008). These apparently conflicting responses to H_2S exposure between species need to be resolved.

In the aquaculture industry there is significant scope for an agent capable of decreasing activity and metabolic rate in animals before they are handled and slaughtered. Such an agent could help reduce the metabolic exhaustion and stress which is associated with increased pre-harvest activity (Johnston, 1975; Milligan and Wood, 1987; Milligan and McDonald, 1988; Pagnotta and Milligan, 1991; Milligan, 1996; Schulte et al., 1992; Wang et al., 1994; Thomas et al., 1999). Furthermore, an agent capable of reducing metabolic rate post-mortem that can rapidly cross the gill epithelia pre-harvest and be delivered to muscle tissue is highly appealing, since maintaining tissue integrity by slowing autolytic degradation post-mortem has the potential to significantly improve product quality indicators and shelf-life. These kinds of improvements are evident in animals that have undergone "rested harvesting". Rested harvesting following isoeugenol anaesthesia, mediated via a competitive blockade of neuromuscular transmission (Ingvast-Larsson et al., 2003), has been shown to greatly reduce activity, stress and metabolic exhaustion, resulting in significant improvements in quality indicators over conventional harvesting techniques (Jerrett et al., 1996; Sigholt et al., 1997; Jerrett and Holland, 1998; Stehly and Gingerich, 1999; Fletcher et al., 2003; Iversen et al., 2003; Black et al., 2004; Kiessling et al., 2004; Small, 2004; Roth et al., 2006; Bagni et al., 2007; Bosworth et al., 2007; Ribas et al., 2007; Wilkinson et al., 2008).

The current study examines the metabolic response of a teleost species, the blue cod (*Parapercis colias*), to acute H_2S exposure. Activity, VO_2 and ventilation frequency (Vf) before, during and after exposure are reported. For comparative purposes, the responses to a commonly used fish anaesthetic, isoeugenol, were also assessed. The effects of the two compounds on cytochrome c oxidase activity in gill, red and white muscle in an *in vitro* study compliment these data. Furthermore, investigation of H_2S exposure in an ectothermic animal tests whether a reduction in thermogenesis (Haouzi et al., 2008) is the primary mechanism responsible for the H_2S-induced hypometabolism reported in mammals.

2. Materials and methods

2.1. Animals

Adult blue cod (*Parapercis colias* Forster 1801; Perciformes, Pinguipedidae) (379 ± 4 g; $n = 16$) of either sex were caught in baited cages set from the departmental research boat off Motunau beach, North Canterbury, New Zealand. Once captured, the fish were held in aerated seawater filled tanks while being transported back to the University's seawater aquaria (16 °C) and maintained on a 12:12 h (light:dark) cycle. The animals proved particularly placid and fed from the hand after only 2 weeks. They were fed a mixed diet of squid, mussels and commercial fish pellets (Hagen, Mansfield, MA, USA) twice weekly. Animals were not fed within the 48 h preceding an experiment. Animals were legally obtained, with a Ministry of Fisheries special permit. All manipulations described in this manuscript were approved by the University of Canterbury Animal Ethics Committee.

2.2. Respirometry

Approximately 12 h before respirometry measurements, a fish was netted and placed into a custom perspex closed-box respirometer connected to an aerated reservoir of filtered seawater (see below for detail) and held in a temperature controlled room at acclimation temperature (16 °C). The time between set-up and experimentation allowed the fish to recover from the disturbance associated with capture and settle in to its new surroundings. The set-up was covered with a black cotton sheet, so as to screen the fish. The respirometer was lit with a Leica CLS 150X fibre-optic light (Wetchar, Germany), that was switched on at least an hour prior to the initial resting rate measurements. This did not cause outward signs of discomfort. The light/dark cycle of the respirometer conformed to that in the holding aquarium. The contents of the respirometer were mixed with two large magnetic stirrer bars, separated from the animal by a perspex grate and rotated by IKA-Werke lab disc (Staufen, Germany) magnetic stirrers. The change in the partial pressure of oxygen (PO_2) in the respirometer was measured with a Strathkelvin Instruments (Strathkelvin, Glasgow, Scotland) oxygen electrode (model IL1302) and meter (model 781), with the signal fed via a 4-channel bioamplifier into an ADInstruments Powerlab 4SP (Waverley, Australia) archiving on a PC laptop running Chart 5 software (ADInstruments). The change in PO_2 was used to calculate VO_2, according to the equation:

$$VO_2 = \frac{\Delta PO_2 \times \alpha O_2 \times V}{t \times M} \times 22.39$$

Where VO_2 is oxygen consumption (mL O_2 kg^{-1} h^{-1}), ΔPO_2 is the change in the partial pressure of oxygen (mmHg, 1 mmHg = 0.133 kPa), αO_2 is the capacitance (seawater 1.66 μmol L^{-1} $mmHg^{-1}$), V is the volume of the respirometer (5.5 L), t is the time (hours), M is the mass of the fish (kg) and 22.39 is a constant used to convert moles to litres.

Preliminary experiments showed that VO_2 and Vf were independent of PO_2 when >90 mmHg. As an increased ventilation rate and amplitude and a reflex bradycardia, due to stimulation of neuroepithelial cells, below a PO_2 of about 100 mmHg might have changed cardiorespiratory dynamics (Reid and Perry, 2003), the PO_2 in the respirometer chamber was never allowed to drop below 100 mmHg.

2.3. Introduction of chemicals

The respirometer was connected to three independently controlled reservoirs (approximately 25 L each) filled with filtered seawater using Maxi-Jet MJ1000 water pumps (Aquarium Systems, Loreggia, Italy). With this arrangement, it was possible to switch between reservoirs and thus replace the contents of the respirometer by diverting the outflow to a drain pipe. The set-up allowed sequential control, exposure and recovery VO_2 measurements to be made without moving the fish, with the seawater in the second and third reservoirs used to flush out either H_2S or isoeugenol from the respirometer by sequential dilution. This was achieved by flowing 25 L of fresh seawater through the respirometer over a 15 min period. Any residual concentration of chemical in the respirometer was then further diluted when connected to the third reservoir. VO_2 in response to the chemicals was measured only after the resting rate had remained stable for at least 1 h.

A 10 ppt stock solution of AQUI-S™ anaesthetic (AQUI-S NZ, Lower Hutt, New Zealand), containing isoeugenol, was introduced into the reservoir (total of 4 aliquots) every 5 min, creating a final concentration of 20 ppm AQUI-S™ (0.011 g L^{-1} isoeugenol). The concentration used was based on the manufacturer's recommendations. The respirometer chamber was then closed and the VO_2 measured for 40 min. Subsequently, the contents of the respirometer was gradually replaced with fresh seawater over a 15 min period and recovery VO_2 and Vf recorded until resting values were again restored. ($n = 5$).

H_2S in solution was generated using hydrated sodium sulphide (Na_2S). A 1 M stock solution of Na_2S was introduced into the reservoir (total of 3 aliquots) every 5 min, creating a final concentration of 200 μM Na_2S. The aeration of the reservoir was ceased during mixing in order to avoid "gassing-off" the H_2S in solution. The chamber was then closed and VO_2 measured for 30 min. Following this, the contents