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BRAIN BLOOD FLOW AND BLOOD PRESSURE DURING HYPOXIA IN THE EPAULETTE SHARK *HEMISCYLLIUM OCELLATUM*, A HYPOXIA-TOLERANT ELASMOBRANCH

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Summary

The key to surviving hypoxia is to protect the brain from energy depletion. The epaulette shark (*Hemiscyllium ocellatum*) is an elasmobranch able to resist energy depletion and to survive hypoxia. Using epi-illumination microscopy *in vivo* to observe cerebral blood flow velocity on the brain surface, we show that cerebral blood flow in the epaulette shark is unaffected by 2 h of severe hypoxia (0.35 mg O_2 l^{-1} in the respiratory water, 24 °C). Thus, the epaulette shark differs from other hypoxia- and anoxia-tolerant species studied: there is no adenosine-mediated increase in cerebral blood flow such as that occurring in freshwater turtles and cyprinid fish. However, blood pressure showed a 50 % decrease in the epaulette shark during hypoxia, indicating that a compensatory cerebral vasodilatation occurs to maintain cerebral blood flow. We observed an increase in cerebral blood flow velocity when superfusing the normoxic brain with adenosine (making sharks the oldest vertebrate group in which this mechanism has been found). The adenosine-induced increase in cerebral blood flow velocity was reduced by the adenosine receptor antagonist aminophylline. Aminophylline had no effect upon the maintenance of cerebral blood flow during hypoxia, however, indicating that adenosine is not involved in maintaining cerebral blood flow in the epaulette shark during hypoxic hypotension.

Key words: hypoxia, epaulette shark, *Hemiscyllium ocellatum*, adenosine, aminophylline, brain, blood flow.

Introduction

According to current understanding of hypoxia-tolerance, hypoxic survival demands that ATP use and ATP production are balanced. The necessity for balance has important implications for the survival of the brain; at least during the initial stages of hypoxia, the anaerobic production of ATP must increase to meet the high rate of ATP consumption of the brain. One way to overcome the energy deficit is to increase the rate of delivery of glucose to the brain, either by increasing blood glucose levels or by increasing cerebral blood flow. Anoxia-tolerant vertebrates such as crucian carp, goldfish and turtles show both increased blood glucose levels (Johnston and Bernard, 1983; Shoubridge and Hochachka, 1980; Walker and Johansen, 1977; Keiver et al., 1992; Penney, 1974) and increased cerebral blood flow (Nilsson et al., 1994; Hylland et al., 1994).

The anoxia-induced increase in cerebral blood flow seen in the crucian carp *Carassius carassius* (Nilsson et al., 1994) and the freshwater turtle *Trachemys scripta* (Hylland et al., 1994) can be blocked by the adenosine receptor antagonist aminophylline, indicating that adenosine is the vasodilator responsible for elevating cerebral blood flow during anoxia in these species. Mammalian systems utilise the same mechanism of elevated adenosine levels to increase cerebral blood flow during anoxia/hypoxia (Berne et al., 1974; Morii et al., 1987). Cerebral blood flow is determined by two factors: blood pressure and vasodilation/vasoconstriction of the arteries and arterioles responsible for regulating the vascular resistance in the brain. Adenosine acts to stimulate cerebral blood flow by being a potent vasodilator of cerebral resistance vessels (Morii et al., 1987). It is thought that a net breakdown of ATP and other phosphorylated adenosines during hypoxic energy deficiency results in rising levels of adenosine, which then act to stimulate glycolysis, partially by increasing cerebral blood flow, and to reduce metabolic rate (Lutz and Nilsson, 1997).

Compared with freshwater habitats, hypoxia occurs rarely in the sea; this may explain why there appear to be few examples of hypoxia-tolerant elasmobranchs. The epaulette shark *Hemiscyllium ocellatum*, however, lives in a tropical marine environment where there are large fluctuations in the ambient oxygen concentration. On the Great Barrier Reef, this shark inhabits shallow reef platforms. During low tide at night, the water on the platform is cut off from the surrounding ocean water and since, no oxygen is formed at night by photosynthesis, the dissolved oxygen level may fall to hypoxic

levels of 2.1 mg l^{-1} (30 % of air saturation) (Kinsey and Kinsey, 1966) or less. In a recent study, this shark was found to be hypoxia-tolerant, surviving in water containing 0.35 mg O_2 l^{-1} (5 % of air saturation) for 3.5 h (Wise et al., 1998).

Since there is no increase in the blood glucose level during hypoxia in the epaulette shark (M. H. Routley, G. E. Nilsson and G. M. C. Renshaw, unpublished results), in contrast to *Carassius* spp. and turtles, one would expect the epaulette shark to respond to hypoxia using other compensatory mechanisms observed in hypoxia-tolerant vertebrates, for example with an increase in cerebral blood flow. However, the responses of cerebral blood flow to hypoxia remain to be studied in the epaulette shark. In fact, until now there have been no studies of cerebral blood flow regulation in elasmobranchs.

The aim of the present study was to determine whether hypoxia affects cerebral blood flow, blood pressure and heart rate in the epaulette shark and whether adenosine stimulates increases in cerebral blood flow in this species.

Materials and methods

The study was carried out at Heron Island Research Station (23°27′S, 151°55′E), located in the Capricorn Bunker group on the Great Barrier Reef, Australia. Epaulette sharks *Hemiscyllium ocellatum* (body mass 646.4±78.4 g, mean ± S.E.M., *N*=23) were caught by hand at low tide on the reef platform surrounding Heron Island. The animals were held in a 10 000 l flow-through seawater tank. The experiments were carried out between January and April.

Each shark was anaesthetised by adding benzocaine (60 mg l^{-1}) to the water. When the benzocaine anaesthesia had taken effect (after approximately 10 min), the shark was placed in a rectangular polyvinylchloride box and ventilated with aerated sea water (1 l min^{-1}, 24 °C, containing 40 mg l^{-1} benzocaine) *via* a tube inserted into the mouth. The shark was catheterised by inserting a PE 50 catheter through the caudal region into the dorsal aorta, as described by Axelsson and Fritsche (1994). The catheter was connected to a Gould Statham P23 Db pressure transducer to allow blood pressure and heart rate to be recorded. Pressure calibration was performed against a static water column. An 8 mm×8 mm hole was made in the skull above the brain, and a 2 mm×2 mm square of the meninx above the cerebellum was carefully removed. The surface of the brain was continuously superfused (200 µl min^{-1}) with a shark Ringer's solution (257 mmol l^{-1} NaCl, 7 mmol l^{-1} Na_2SO_4, 2.5 mmol l^{-1} $NaHCO_3$, 4 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} $MgSO_4$, 400 mmol l^{-1} urea, 70 mmol l^{-1} trimethylamine-*N*-oxide and 0.28 mmol l^{-1} Na_2HPO_4, pH 7.6, 24 °C) adopted from Simpson and Sargent (1985).

A Leitz Ortholux microscope with a Leitz Ultropak epi-illuminator and a water-immersion objective (6.5×) was used to observe and measure blood flow velocity (erythrocyte velocity) in vessels on the surface of one of the optic lobes, essentially as described by Nilsson et al. (1994). In brief, a video camera (Panasonic WV-CP410 with a 12 mm lens) was attached to the phototube (holding a 10× eyepiece) of the microscope. This gave 240× magnification on a 14 inch video screen, and single erythrocytes could be readily observed.

After 60–90 min of recovery from surgery, the sharks were exposed to hypoxia (0.35 mg O_2 l^{-1}) for 120 min. Hypoxia was achieved by replacing the air-equilibrated respiratory water with water bubbled with N_2. The equilibration of the respiratory water with N_2 was performed on-line in a 1.5 m high glass column that was bubbled from the bottom with N_2.

At specific times, adenosine and aminophylline (both from Sigma) were administered by dissolving them in the Ringer's solution superfusing the brain. Adenosine was superfused over the brain for 20 min, while aminophylline was superfused for 50 min starting 30 min before a second adenosine superfusion.

The time for single erythrocytes to travel a specific distance in the centre of a blood vessel was recorded using a stopwatch. Before each experiment, 1–3 venules long enough to allow observation of the same erythrocytes for at least 1.5 s at basal flow conditions were selected (if more than three vessels fulfilled this criterion, then three of them were chosen randomly). Every 5, 10 or 20 min, the velocity in each vessel was recorded five times to obtain a mean value, thus minimising the effects of short-term variation in blood flow and recording error. The basal flow velocity in the vessels studied was 224.1±26.3 µm s^{-1} and the diameter (lumen) of the blood vessel was between 25 and 66 µm (*N*=23).

In the first experiments (see Fig. 1), 14 animals were used; cerebral blood flow velocity was measured in one set of seven animals, while blood pressure and heart rate were measured in another set of seven animals. In the second experiment (see Fig. 2), we used five animals, and four animals were used in the third experiment (see Fig. 3).

Blood flow velocities (µm s^{-1}) were normalised to percentage values for each vessel, and the mean velocity measured for each individual during the control period was set to 100 %. Changes during hypoxia or drug exposure were first evaluated using Friedman non-parametric analysis of variance (ANOVA) for repeated measures followed by Dunn's *post-hoc* test (for cerebral blood flow velocity) or repeated-measures ANOVA followed by a Bonferroni *post-hoc* test (for blood pressure and heart rate). One animal was considered as one observation. Values are given as means ± S.E.M.

Results

Hypoxia (0.35 mg O_2 l^{-1}) for 120 min had no significant effect on cerebral blood flow velocity in the epaulette shark (Fig. 1A). However, both dorsal aortic blood pressure and heart rate decreased significantly during hypoxia (ANOVA, $P<0.0001$) (Fig. 1B,C). Blood pressure stabilised at approximately 50 % of the normoxic value after approximately 15 min of exposure to hypoxia. Upon re-oxygenation, normal blood pressure was regained within 55 min. The heart rate returned to basal values within 45 min of re-oxygenation.

When the brain was superfused with 50 µmol l^{-1} adenosine

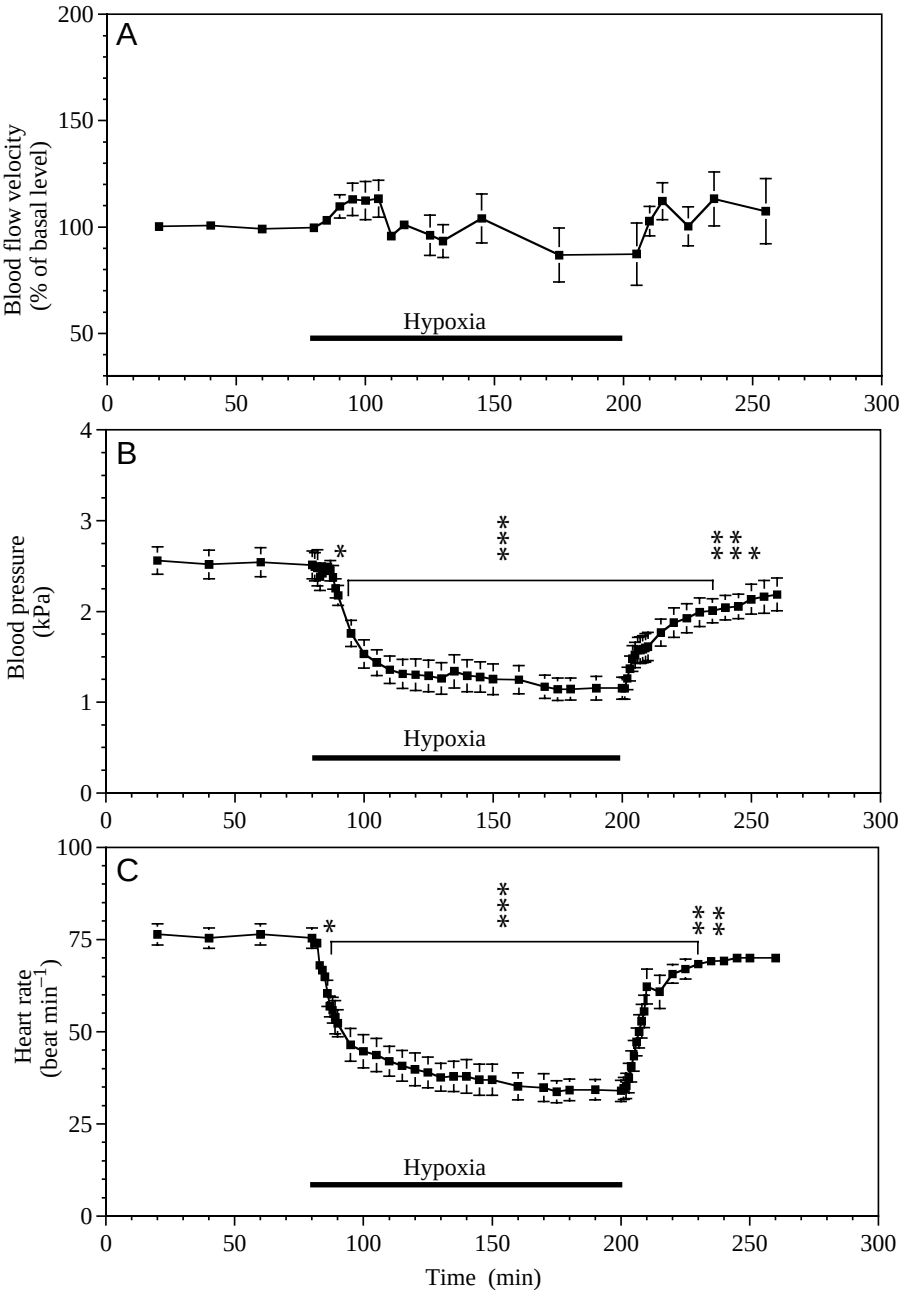

Fig. 1. (A–C) Effects of hypoxia ($0.35\,mg\,O_2\,l^{-1}$ for 120 min) on (A) cerebral blood flow velocity measured in the venules on the surface of the cerebellum, (B) systemic blood pressure and (C) heart rate. Values are means ± S.E.M. Cerebral blood flow velocity was measured in one set of seven animals, while blood pressure and heart rate were measured in another set of seven animals. No significant changes were seen in cerebral blood flow velocity. The decrease in systemic blood pressure became significant ($P<0.05$) after 10 min of hypoxia, and blood pressure was no longer significantly different from the initial values after 50 min of re-oxygenation. Heart rate was significantly ($P<0.05$) decreased after 6 min of hypoxia and was no longer significantly different from the initial values after 45 min of re-oxygenation. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the final pre-treatment value (Bonferroni *post-hoc* test).

during normoxia, cerebral blood flow velocity increased significantly (ANOVA, $P<0.0001$), reaching 178.5±8.1 % of the control velocity (Fig. 2). When adenosine was removed from the Ringer's solution superfusing the brain, the cerebral blood flow velocity returned to basal values within 10 min. Superfusing the brain with $1\,mmol\,l^{-1}$ aminophylline significantly (ANOVA, $P<0.001$) reduced the effect of $50\,\mu mol\,l^{-1}$ adenosine, whereas aminophylline alone had no significant effect on the normoxic cerebral blood flow velocity (Fig. 2). Aortic blood pressure and heart rate did not change when the brain was superfused with adenosine (results not shown).

Cerebral blood flow velocity remained unaffected by hypoxia when the brain was superfused with $1\,mmol\,l^{-1}$ aminophylline (Fig. 3). Aminophylline was also shown to be without effect in the normoxic control situation.

No changes in the diameter of the venules were seen during hypoxia or adenosine superfusion.

Discussion

The results of these experiments show that there is no change in the cerebral blood flow when the epaulette shark is exposed to hypoxia. This result is surprising in view of the response to hypoxia of mammals and of anoxia-tolerant vertebrates (such as crucian carp and freshwater turtles), which increase their brain blood flow during hypoxia or anoxia (Table 1). The increase in cerebral blood flow in the anoxia-tolerant

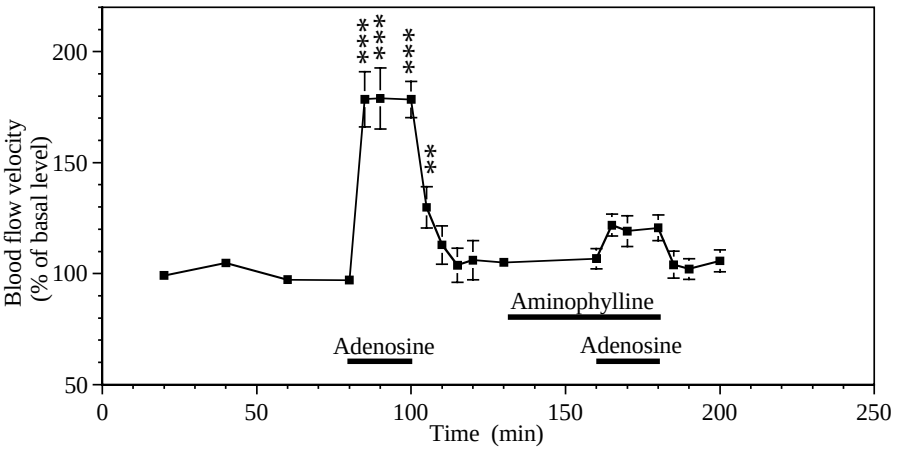

Fig. 2. Effects of superfusing the brain with adenosine (50 μmol l^{-1} for 20 min) and aminophylline (1 mmol l^{-1} for 50 min) on cerebral blood flow velocity measured in the venules on the surface of the cerebellum. Values are means ± S.E.M. from five animals. ***P*<0.01, ****P*<0.001 compared with the final pre-treatment value (Dunn's *post-hoc* test).

species has been interpreted as increasing the supply of glucose to the brain, thereby facilitating an increased glycolytic ATP production. In the hypoxic situation, elevated cerebral blood flow will also increase the oxygen delivery to the brain. The common carp (*Cyprinus carpio*), a species that is moderately tolerant to hypoxia, doubles its cerebral blood flow during hypoxia (0.8 mg O_2 l^{-1} at 23 °C) (Yoshikawa et al., 1995). In fact, even the rainbow trout *Oncorhynchus mykiss*, a teleost with a very limited tolerance to hypoxia, elevates its cerebral blood flow in response to hypoxia (V. Söderström and G. E. Nilsson, unpublished results).

Another means of increasing the rate of glucose delivery to the brain during hypoxia would be to increase the concentration of glucose in the blood, as has been shown to occur in both anoxic crucian carp and anoxic freshwater turtles. However, blood glucose levels have been found to remain constant during hypoxia in the epaulette shark (M. H. Routley, G. E. Nilsson and G. M. C. Renshaw, unpublished results); this is also the case in the dogfish *Scyliorhinus canicula* (Butler et al., 1979). It is possible that elasmobranchs lack the ability to increase blood glucose levels in response to hypoxia. A release of red blood cells from the spleen to increase the haematocrit is a mechanism used by teleosts (and other vertebrates) to increase the uptake and delivery of oxygen during hypoxia, but it is unclear whether elasmobranchs utilise this mechanism (Nilsson, 1993). Indeed, the epaulette shark displays a constant, and relatively low, haematocrit during hypoxia (M. H. Routley, G. E. Nilsson and G. M. C. Renshaw, unpublished results). The tolerance of the epaulette shark to hypoxia, without elevating cerebral blood flow, blood glucose concentration or haematocrit, appears to be quite remarkable.

Hypoxia induced a marked hypotension and bradycardia in the epaulette shark, as has been demonstrated in other elasmobranchs and teleost fishes (Butler and Taylor, 1971; Satchell, 1961, 1991; Piiper et al., 1970). Cerebral blood flow is determined by two factors: blood pressure and the diameter of the cerebral resistance vessels. Because blood pressure fell by approximately 50 % during hypoxia in the epaulette shark, the maintenance of cerebral blood flow indicates that a compensatory cerebral vasodilatation must have occurred. Because we could only observe venules on the dorsal brain surface, and not the deeper resistance vessels, we could not verify this vasodilatation by direct observation. The resistance vessels involved in such a vasodilatation are probably arteries and arterioles on the ventral side of the brain and within the brain. It is possible that the maintained cerebral blood flow is the result of cerebral blood flow autoregulation, which is the

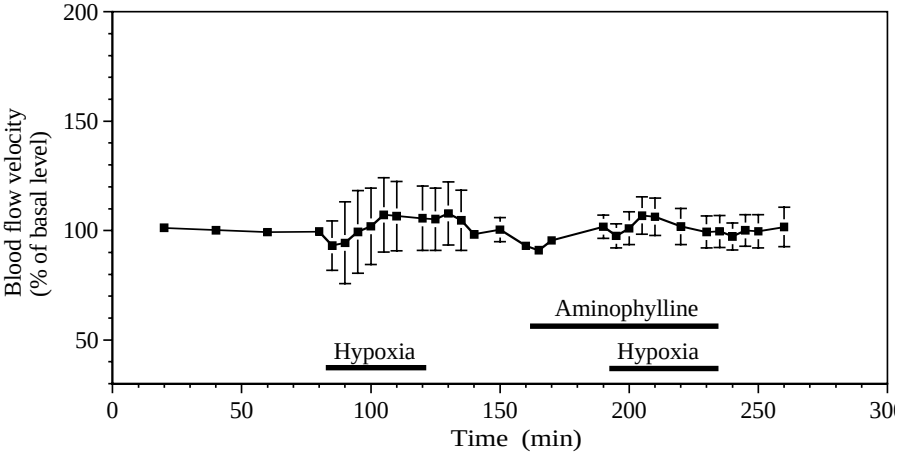

Fig. 3. Effects of hypoxia (0.35 mg O_2 l^{-1} for 40 min) and of superfusing the brain with aminophylline (1 mmol l^{-1} for 70 min) on cerebral blood flow velocity measured in the venules on the surface of the cerebellum. Values are means ± S.E.M. from four animals. No significant changes were found.

Table 1. *Effect of hypoxia/anoxia on cerebral blood flow and blood pressure in various vertebrates*

Species	Hypoxic/anoxic regime	% Increase in cerebral blood flow	% of the hypoxic/anoxic increase in cerebral blood flow that can be blocked by an adenosine receptor antagonist	Effect on blood pressure	Reference
Crucian carp (*Carassius carassius*)	360 min of anoxia (10 °C)	116	100	ND	Nilsson et al. (1994)
Common carp (*Cyprinus carpio*)	60 min of hypoxia (0.8 mg O_2 l^{-1}, 23 °C)	160	ND	ND	Yoshikawa et al. (1995)
Freshwater turtle					
(*Chrysemys scripta*)	30 min of anoxia (25 °C)	260	ND	No change	Davies (1989)
(*Trachemys scripta*)	75 min of anoxia (20 °C)	170	100	No change	Hylland et al. (1994)
Dog	10 min of hypoxia (Pa_{O_2} = 26 mmHg)	130–150	ND	50 % increase	McPherson et al. (1994)
	15–20 min of hypoxia (Pa_{O_2} = 18 mmHg)	176	ND	16 % increase	Traystman et al. (1978)
	2–3 min of hypoxia				Emerson and Raymond (1981)
	(Pa_{O_2} = 47 mmHg)	17	100	No change	
	(Pa_{O_2} = 20 mmHg)	98	50	No change	
Rat	5 min of hypoxia (Pa_{O_2} = 33 mmHg)	135	ND	19 % decrease	Kozniewska et al. (1992)
	2–5 min of hypoxia				Morii et al. (1987)
	(Pa_{O_2} = 45 mmHg)	39	100	No change	
	(Pa_{O_2} = 34 mmHg)	195	73	No change	
Piglet	10 min of hypoxia				Laudington et al. (1990)
	(Pa_{O_2} = 47 mmHg)	112	100	No change	
	(Pa_{O_2} = 25 mmHg)	188	74	No change	
Cat	3 min of hypoxia (Pa_{O_2} = 39 mmHg)	10	100	6 % increase	Isozumi et al. (1994)
Man	15 min of hypoxia (Pa_{O_2} = 35 mmHg)	70	ND	5 % decrease	Cohen et al. (1967)

ND, not determined.
1 mmHg = 0.133 kPa.

ability to maintain cerebral blood flow relatively constant during arterial blood pressure changes (Busija, 1993).

Adenosine appears to be responsible for increasing cerebral blood flow during hypoxia in several vertebrates (Table 1). However, in the epaulette shark, the maintenance of cerebral blood flow velocity during hypoxia apparently did not rely on local adenosine release since aminophylline did not cause a reduction in cerebral blood flow during hypoxia-induced hypotension. However, cerebral blood flow increased when adenosine was superfused over the brain, and this response could be inhibited by aminophylline treatment. This response is similar to those of the crucian carp and the freshwater turtle, in which adenosine causes an aminophylline-blockable increase in cerebral blood flow (Hylland et al., 1994; Nilsson et al., 1994). Superfusing the brain with adenosine caused no change in blood pressure or heart rate in the epaulette shark, showing that the increase in cerebral blood flow during adenosine superfusion was due to the vasodilatation of resistance vessels in the brain and was not a result of increased blood pressure. It should be pointed out that teleosts are the oldest group of vertebrates in which an adenosine-mediated increase in cerebral blood flow has been demonstrated. The finding that adenosine is able to stimulate cerebral blood flow in a shark pushes the origin of this mechanism further back in vertebrate evolution. Because sharks and bony fish have had separate evolutionary trajectories from the Ordovician (500 million years before the present), adenosine-mediated cerebral vasodilatation is probably at least some 500 million years old (Long, 1995).

The present study adds the regulation of cerebral blood flow

to the list of differences found in responses to hypoxia between teleost fishes and elasmobranchs. During hypoxia, teleost fishes raise the levels of glucose in their blood (McDonald and Milligan, 1992), while the dogfish (Butler et al., 1979) and epaulette shark (Wise et al., 1998) appear to be unable to do this. Elasmobranchs may also lack the ability of teleosts to increase their haematocrit during hypoxia. In addition, under normoxic conditions, teleosts seem to have a higher haematocrit than elasmobranchs (Butler et al., 1979; Short et al., 1979; Perry and Gilmour, 1996). Finally, teleost fishes in general seem to be able to regulate their rate of oxygen uptake over a wider range of water oxygen levels than elasmobranchs (Randall, 1970).

To conclude, in contrast to mammals, turtles and teleosts, the present data suggest that hypoxia does not increase cerebral blood flow in the epaulette shark. However, blood pressure and heart rate are significantly reduced when the shark is exposed to hypoxia. Nevertheless, despite the decrease in blood pressure, the cerebral blood flow velocity remained constant; this result suggests that there is a cerebral vasodilatation during hypoxia. Although we found that adenosine can trigger an increase in cerebral blood flow velocity in the epaulette shark, this vasodilator is apparently not responsible for maintaining cerebral blood flow during hypoxic hypotension. Compared with teleosts, elasmobranchs appear either to be more limited in their repertoire of physiological adaptations for hypoxic survival or to have a different set of adaptations. This makes the existence of hypoxia-tolerant elasmobranchs such as the epaulette shark particularly interesting.

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