Nitric oxide (NO) in vascular regulation of the spiny dogfish, *Squalus acanthias*

Kai E. Swenson[1], Randy L. Eveland[1], Chris D. Reiter[2],
Mark T. Gladwin[2], and Erik R. Swenson[1]

[1]University of Washington, VA Puget Sound Health Care System, Seattle, WA. 98195
[2]Critical Care Medicine Department, National Institutes of Health, Bethesda, MD. 20892

There has been an explosion in research on the involvement of nitric oxide (NO) in mammalian vascular biology. NO is produced by the vascular endothelium and acts as a local vasodilator[4]. It is produced by 3 isoforms of NO synthase (NOS) in mammals of which endothelial NOS (eNOS) is the most important in normal vasoregulation, but in some circumstances neuronal NOS (nNOS) and inflammatory NOS (iNOS) may also be involved. Among the many stimuli known to upregulate NOS activity and gene expression, hypoxia is a potent inducer of NO generation. The importance of NO in other terrestrial vertebrates has been established for birds, reptiles and amphibians [1,9,16].

In contrast, vascular regulation by NO in fish has neither been as well defined, nor extensively studied. The literature in teleosts, indeed, is contradictory, depending upon the class, species, specific organ vascular bed, and size of vessel studied; and whether in vivo or in vitro studies were performed. Olson and Villa[14] argue against NO-mediated vasodilation, because acetylcholine (which stimulates endothelial NO production) vasoconstricts isolated large arteries and a perfused-trunk preparation of the trout. In contrast, McGeer and Eddy[10] found an NO-donor (nitroprusside) lowered blood pressure in salmon. Evidence in heart[14] and brain[8] suggests vascular NO production, since acetylcholine vasodilates and NOS inhibitors block the effect. This does not appear to the case in the gills[15], however, where dose-dependent directional differences in responses to acetylcholine, NO donors and NOS inhibition suggest a more complex level of vascular regulation and a lesser role of NO[14].

Data in elasmobranchs are even more limited. Evans and Gunderson[2] and Evans[3] showed in isolated large conduit arteries (ventral aorta and mesenteric arteries) of *S. acanthias* that acetylcholine is vasoconstricting and l-arginine, NO and nitroprusside are mildly vaso-constricting. It appears that prostaglandins are the critical endothelial-derived vasodilators in large conduit vessels, as is the case in the trout aorta[11]. At the whole organ level, where small arteriolar resistance vessels are included, evidence points to a more typical mammalian-like response of endogenous NO-mediated vasodilation. Renshaw and Dyson[17] found low levels of eNOS expression by immuno-cytochemistry in brain vascular endothelium of epaulette sharks (*H. oscellatum*), which could be markedly up-regulated when sharks were made hypoxic. The authors proposed that this response and likely increased NO synthesis and cerebral vasodilation is neuroprotective in hypoxia. They did not study responses in other vascular beds. eNOS expression has been shown in teleost retina, that is also hypoxia-sensitive[7] and in developing zebrafish[5]. Whole animal studies of the role of NO in elasmobranchs have never been undertaken.

Spiny dogfish (Squalus acanthias) of both sexes weighing between 1.5 and 3.0 kg were transferred from holding tanks to small plexiglass boxes with special securing rods that prevented the fish from moving. Fresh seawater was pumped continuously into the tank. Via a ventral approach we percutaneously cannulated the dorsal artery in the tail. The catheter was then passed cephalad 20 cm into the central circulation. The proximal end was attached to a 3-way stopcock connected to pressure transducer and recorder (Space Labs 511). The catheter was continuously perfused with heparinized dogfish Ringer's solution (1000 units per liter). The tank was then closed and covered by black

polylvinyl sheeting to minimize external stimuli. Animals were allowed at least one hour to recover down before any experiments began. For experiments involving hypoxia exposure, a 3-foot high vertical plexiglass cylinder was placed in line with the seawater source. The inspired seawater was made hypoxic by continuously bubbling 100% nitrogen through the cylinder, and then pumped to the fish tank. This lowered the oxygen concentration from 21 to 3%. To return to normoxia, the recirculating system was closed off and the fish was again provided with normoxic seawater.

Drugs or agents with known differing effects on NO metabolism and vascular tone in mammals were administered slowly into the dorsal artery catheter over 5 minutes. These drugs were dissolved in 10 ml dogfish Ringer's solution, then frozen until needed. All solutions were brought to a pH of 7.4, if necessary, with NaOH. These included: acetylcholine bromide (stimulates endothelial NO production); L-arginine (the substrate for NO generation by NOS); L-NAME (l-nitro L-arginine methylester, an analogue of arginine which inhibits NOS); sodium nitroprusside (SNP, a drug that releases NO); and cell-free hemoglobin (a potent scavenger of NO, via reduction to nitrate (NO_3) in reaction with heme iron). Cell-free hemoglobin was prepared by taking human red cells and washing them 3 to 4 times with 0.9% saline. After washing in this manner, one ml of distilled water per ml of red cells was used to lyze the cells. Lysates were then spun in a high-speed ultracentrifuge for 90 minutes at 9000 rpm. The supernatant, free of cell membranes, was used without further purification. The partial pressure of oxygen (PO_2) of ambient water and arterial blood was measured by a blood gas analyzer (Radiometer ABL 77) at 37 ^{o} C and values were temperature-corrected. Plasma nitrite (NO_2), which is generated by reaction of NO with O_2 and whose concentration serves as an index of NO production, was measured by reduction to NO with acidified KI [6], with detection by chemiluminesence (Sievers 280 NO Analyzer).

The first series of experiments were performed in normoxic fish. Various drugs were studied (but not always every drug) in the following sequence in order to use drugs with rapid onset and clearance first, followed by those with longer or irreversible action. A baseline period of 30 minutes was then followed by sequential administration every 23 to 30 minutes of acetylcholine bromide at 75 and 750 ug/kg, L-arginine at 20 mg/kg, L-NAME at 25 and 100mg/kg and SNP at 12 mg/kg. Blood pressure and heart rate were recorded every five minutes, with blood sampling before a new drug was introduced. A second set of normoxic fish were studied in a baseline state for one hour and then after 1.5 umol/kg of cell-free hemoglobin. Blood pressure and heart rate were recorded every 5 minutes. At the end of each one-hour period, blood was drawn for plasma nitrite and arterial blood gases.

A third series of fish were studied in hypoxia, with blood pressure and heart rate measured every 5 minutes. After an hour of normoxia and collection of water and arterial blood for nitrite, PO_2, PCO_2 and pH measurements, seawater was made hypoxic as described above. It took 40 minutes to reach a steady state 3% O_2. After 1 hour of hypoxia, repeat samples were taken and the fish was returned to normoxic water for 30 minutes. Then the fish was given either L-NAME (25 mg/kg) or cell-free hemoglobin (1.5 umol/kg), and made hypoxic for one hour, after which a final set of samples was taken.

The findings in normoxia are presented in Table 1. Baseline normoxic systolic and diastolic blood pressures were 35 $\pm$ 4/ 27 $\pm$ 3 mmHg and heart rate is 27 $\pm$ 4 per min. The finding of marked hypotension with nitroprusside establishes that NO relaxes vascular smooth muscle. Acetylcholine, at both doses, caused a fall in blood pressure that was rapid and associated with an expected fall in heart rate. The drop in blood pressure is consistent with possible NO-mediated vasodilation, however, the large drop in heart rate may have been responsible for sufficient cardiac output depression-related

hypotension independent of changes in vascular resistance. L-Arginine had little effect on blood pressure, although it is a substrate for NOS and should stimulate NO production. We attribute its lack of effect either to the fact that L-arginine may already be present in large enough quantities inside the vascular wall not to be limiting in the NOS reaction, or that NO is simply not be made by the normoxic vascular endothelium. L-NAME raised blood pressure slowly over a period of 20 minutes without altering heart rate. This would be consistent with either inhibition of vascular NO synthesis, or NO synthesis in the central cerebrovascular control centers of the brain, since there is ample demonstration of NO synthases in the brain and nervous tissue of many fish, including elasmobranchs[17]. Because cell-free hemoglobin, which binds NO avidly and irreversibly, and whose effect is largely intraluminal due to its large size, but causes no increase in blood pressure, we conclude that in normoxia there is no significant production of NO by the vascular endothelium. The data consistent with an apparent vascular NO production (acetylcholine and L-NAME) must be explained by other actions apart from the vascular endothelium, since cell-free hemoglobin was without effect on blood pressure.

Table 1: Changes in normoxic blood pressure and heart rate vs. baseline with NO-modifying agents ($n = 8$, * $p < 0.05$)

	Systolic (mmHg)	Diastolic	Heart rate (min^{-1})
Acetylcholine			
75 ug/kg	-3.7 *	-3.5 *	-6 *
750 ug/kg	-5.8 *	-4.8 *	-9 *
L-Arginine			
20 mg/kg	2.8	1.3	1
L-NAME			
25 mg/kg	3.2 *	2.4 *	0
100 mg/kg	5.3 *	2.8 *	-1
Nitroprusside			
12 mg/kg	-7.1 *	-5.2 *	-1
Cell-free hemoglobin			
1.5 umol/kg	0.4	0.0	2

The situation is different when fish are hypoxic (Table 2). With a fall in arterial PO_2 from 110 ± 12 to 30 ± 3 mmHg, blood pressure fell 4-5 mmHg along with a drop in heart rate of 10 min^{-1}. These changes did not occur immediately but developed over 40 minutes. The findings that L-NAME prevented the fall in blood pressure and cell-free hemoglobin raised blood pressure, both with similar reductions in heart rate as with hypoxia alone are consistent with hypoxic stimulation of vascular NO synthesis that leads to vasodilation. Plasma nitrite, a marker of NO production, rose from 4.5 uM to 5.7 uM with hypoxia, but did not when L-NAME was given. In contrast, plasma nitrite did not vary over 4 hours in normoxic fish and was not affected by L-NAME.

Table 2: Changes in blood pressure and heart rate from normoxia with hypoxia and NO-modifying agents ($n = 8$, * $p < 0.05$)

	Systolic (mmHg)	Diastolic	Heart Rate (min^{-1})	PaO_2 (mmHg)
Hypoxia	-4.9 *	-3.9 *	-10 *	27
Hypoxia + L-NAME 25 mg/kg	0.1	-0.1	-11 *	32
Hypoxia + Cell-free Hb 1.5 uml/kg	2.3*	1.3	-9 *	30

In conclusion, our data support a negligible role of endothelial NO formation in vasoregulation in normoxic elasmobranchs, although there may be neural NO production that may be involved in mediating central sympathetic control of blood pressure. The elements necessary for vascular NO production, nonetheless, are present and can be activated in elasmobranchs, since we show clearly that hypoxia causes hypotension and it can be prevented with either inhibition of NO synthesis or scavenging of NO by cell-free hemoglobin. We are presently analyzing various organ and vascular endothelia by immunocytochemistry to determine the presence and localization of NO synthases.

Supported by a New Investigator Award from the Salsbury Cove Research Fund to ERS.

1. Aksulu, H.E., Birgol, I., Karatas, F, Samanligil, H. and B. Ustundag. Changes in plasma ACE activity and noradrenaline responses to longterm NO inhibition in chickens. *Physiol. Res.* 49:175-182, 2000.
2. Evans, D.H. and M.P. Gunderson. A prostaglandin, not NO, mediates endothelium-dependent dilation in the ventral aorta of the shark *(S. acanthias)*. *Am. J. Physiol.* 274:R1050-1057, 1998.
3. Evans, D.H. Vasoactive receptors in abdominal blood vessels of the dogfish shark, *Squalus acanthias*. Physiol.Biochem. Zool. 74: 120-126, 2001.
4. Deem, S., Bishop, M.J., Kerr M.E. and E.R. Swenson. RBC augmentation of hypoxic pulmonary vasoconstriction: hematocrit dependence and importance of NO. *Am. J. Respir. Crit. Care Med.* 157:1181-1189, 1998.
5. Fritsche, R., Schwerte. T. and B. Pelster. NO and vascular reactivity in developing zebrafish. *Am. J. Physiol.* 279:R2220-2228, 1997.
6. Gladwin, M.T., Reiter, C.D., Yang, B.K., Bonaventura, C., and A.N. Schechter. S-Nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O_2/NO-linked allosteric function. *J. Biol. Chem.* 277:27818-27828, 2002.
7. Haverkamp, S, Kolb, H., and N. Cuenca. Endothelial NO synthase is localized to Muller cells in all vertebrate retinas. *Vision Res.* 39:2299-2304, 1999.
8. Hylland, P. and G.E. Nilsson. Evidence that acetylcholine mediates increased cerebral blood flow velocity in crucian carp through a NO-dependent mechanism. *J. Cereb. Blood Flow Metab.* 15:519-524, 1995.
9. Hylland, P., Nilsson, G.E. and P.L. Lutz. Role of nitric oxide in the elevation of cerebral blood flow induced by acetylcholine and anoxia in the turtle. *J. Cereb. Blood Flow Metab.* 16:290-296, 1996
10. McGeer, J.C. and F.B. Eddy. Effects of SNP on blood circulation and acid-base and ionic balance in rainbow trout: indications for NO induced vasodilation. *Can. J. Zool.* 74:1211-1218, 1996.
11. Miller, V.M. and P.M. Vanhoutte. Prostaglandins but not nitric oxide are endothelium-derived relaxing factors in the trout aorta. *Acta. Pharmacol. Sin.* 21: 871-876, 2000.
12. Mustafa, T. and C. Agnisola. Vasoactivity of adenosine in the trout coronary system: involvement of NO and interaction with noradrenaline. *J. Exp. Biol.* 201:3075-3083, 1998
13. Olson, K.R. Gill circulation: Regulation of perfusion distribution and metabolism of regulatory molecules. *J.Exp.Zool.* 293:320-329, 2002.
14. Olson, K.R. and J. Villa. Evidence against nonprostanoid endothelium –derived relaxing factor(s) in trout vessels. *Am. J. Physiol.* 260:R925-932, 1991.
15. Pellegrino, D, Sprovieri E., Mazza, R., Randall, D.J. and B. Tota. NO-cGMP-mediated vasoconstriction and effects of acetylcholine in the branchial circulation of the eel. *Comp. Biochem. Physiol. A.* 132:447-454, 2002.
16. Rea, M.S. and R.H. Parsons. Evidence of NO and angiotensin II regulation of circulation and cutaneous drinking in Bufo marinus. *Physiol. Biochem. Zool.* 74:12-20, 2001.
17. Renshaw, G.M. and S.E. Dyson. Increased NO synthase in the vasculature of the epaulette shark brain following hypoxia. *Neuro. Report* 10:1707-1715, 1999.