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No hemoglobin but NO: the icefish (*Chionodraco hamatus*) heart as a paradigm

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Summary

The role of nitric oxide (NO) in cardio-vascular homeostasis is now known to include allosteric redox modulation of cell respiration. An interesting animal for the study of this wide-ranging influence of NO is the cold-adapted Antarctic icefish *Chionodraco hamatus*, which is characterised by evolutionary loss of hemoglobin and multiple cardio-circulatory and subcellular compensations for efficient oxygen delivery. Using an isolated, perfused working heart preparation of *C. hamatus*, we show that both endogenous (L-arginine) and exogenous (SIN-1 in presence of SOD) NO-donors as well as the guanylate cyclase (GC) donor 8Br-cGMP elicit positive inotropism, while both nitric oxide synthase (NOS) and sGC inhibitors, i.e. L-NIO and ODQ, respectively, induce significant negative inotropic effects. These results therefore demonstrate that under basal working conditions the icefish heart is under the tonic influence of a NO-cGMP-mediated positive inotropism. We also show that the working heart, which has intracardiac NOS (shown by NADPH-diaphorase activity and immunolocalization), can produce and release NO, as measured by nitrite appearance in the cardiac effluent. These results indicate the presence of a functional NOS system in the icefish heart, possibly serving a paracrine/autocrine regulatory role.

Key words: nitric oxide, heart, Antarctic teleost, icefish, *Chionodraco hamatus*, myocardial performance, nitric oxide synthase (NOS), immunocytochemistry.

Introduction

Over the past years the universal signaling molecule nitric oxide (NO) has revealed itself to be a major modulator of cardio-circulatory function in mammals (Shah and MacCarthy, 2000). NO is generated by a family of enzymes called nitric oxide synthases (NOSs). The three NOS isoforms, i.e. the neuronal NOS (nNOS or NOS1), the endothelial NOS (eNOS or NOS3) and the inducible NOS (iNOS or NOS2), are the products of three distinct genes whose heterogeneous expression in different cell types is consistent with an independent functional role for each isoform. In the heart they are expressed, albeit in a cell-specific manner, in both myocardial and non-myocardial tissues, including coronary and endocardial endothelial cells (Brutsaert et al., 1998). A mitochondrial NOS (mtNOS), corresponding to a variant of nNOS, has been identified (Giulivi, 2003) and detected in isolated mitochondria of mouse cardiomyocytes (Kanai et al., 2001). Apart from non-cGMP-dependent actions, cardiac NO acts, in most cases, through activation of the heterodimeric soluble guanylate cyclase (sGC), thereby increasing intracellular cGMP levels (Shah and MacCarthy, 2000, and references therein). The NO-mediated physiological actions include heart rate, inotropism, excitation–contraction coupling, myocardial relaxation, diastolic function and the Frank–Starling response (Massion and Balligand, 2003).

While the pathway of NO formation is one of the oldest evolutionary signaling systems in animals (Feelish and Martin, 1995), in fish there is surprisingly scant information regarding the cardiocirculatory roles of NO. We have documented direct local cardiac and vascular actions of NO in various teleosts (*Anguilla anguilla*; heart, Imbrogno et al., 2001, 2003; branchial vasculature, Pellegrino et al., 2002; *Salmo salar*; heart, Gattuso et al., 2002; icefish *Chionodraco hamatus*; branchial vasculature, Pellegrino et al., 2003). The Antarctic teleost, the hemoglobinless *Chionodraco hamatus*, is an intriguing animal with which to explore aspects of unity and diversity regarding the physiological roles of NO. This species is member of the family Channichthyidae or icefish. Icefish belong to the cold-adapted diversification of the perciform suborder Nototheniodei, the dominant fish group, in terms of both abundance and number of species, of the Antarctic ichthyofauna. They are characterized by extreme stenothermia (Hofmann et al., 2000) and evolutionary loss of haemoglobin (Hb; Ruud, 1954) as well as, in some species, cardiac myoglobin (Moylan and Sidell, 2000). The absence of Hb appears not to be crucial for the animal's fitness in the highly

oxygenated and thermally stable Antarctic habitat (i.e. the 'blind cave fish phenomenon'; Somero et al., 1998). Multiple coupled compensations at different levels of organization allow for efficient oxygen delivery to icefish tissues. A notable example of compensation at the subcellular level is the dramatic proliferation of mitochondria in the myotomal (Egginton and Sidell, 1989) and cardiac (Johnston and Harrison, 1987; Tota et al., 1991a; O'Brien and Sidell, 2000) muscle. At the systemic level, the cardio-circulatory compensations appear to be crucial (Hemmingsen et al., 1972; Tota et al., 1991b), and include a low-resistance vascular tree, large blood volume (2–4 times greater than in red-blooded teleosts) coupled with remarkable large heart-to-body mass ratio (Johnston et al., 1983; Tota et al., 1991a), and a cardiac output (CO) greater than that of most fishes (Tota et al., 1991b). This large CO is attained with impressively large stroke volume (V_S), displaced at high flow rates, low heart rate (f_H) and low ventral aortic pressure (P_{VA}) (Hemmingsen et al., 1972; Tota et al., 1991b). Accordingly, the icefish heart works as a typical volume pump (Tota and Gattuso, 1996). Conceivably, in view of its modulation of myocardial inotropism, relaxation and energetics in the mammalian heart (Shah and McCarthy, 2000), intracardiac NO could have a paracrine/autocrine role for optimising such specialized volume pump function in the icefish. Furthermore, at the whole organismal level, it is of interest to explore NO functions in the icefish, since in this animal one of the major mechanisms for disarming NO bioactivity (the NO–Hb reaction) is lacking.

In this study, we used an isolated and perfused working heart preparation of the icefish *C. hamatus* to demonstrate the responses to NO-donors and inhibitors, together with the release of NO (in terms of nitrite) detected by electrochemical assay in the cardiac effluent. Moreover, we have documented the presence of cardiac NOS by morphological methods (i.e. NADPH-diaphorase activity and immunolocalization). On the whole, these results demonstrate that NO, produced within the heart, directly modulates myocardial performance through a cGMP-mediated mechanism, thus emphasizing the potential for nitrergic autoregulation of cardiac function in icefish.

Materials and methods

Animals

The study was conducted on 38 specimens of the icefish *Chionodraco hamatus* Lönnberg of both sexes, weighing 355.47±14.4 g (mean ± S.E.M.). Fish were caught using nets in the Terranova Bay, Ross Sea, Antarctica during January 2002. Identification was based on descriptions of Fisher and Hureau (1985). After capture, the animals were maintained unfed for at least 5 days in aerated, running seawater at temperatures between 0–2°C. Only fish that appeared to be healthy were used in the experiments. All the experiments were done in the Italian Antarctic Base laboratories, Terranova Bay (74°42′S, 164°06′E), during the XVII Italian Antarctic Expedition (December 2001–February 2002).

European eels *Anguilla anguilla* L. (N=7) of both sexes, weighing 229±31 g, were provided by a local hatchery and kept at room temperature (18–20°C) without feeding for 5–6 days.

Isolated and perfused working heart preparation

The animals were anaesthetized in benzocaine (0.2 g l^{-1}) for 15 min. Each animal was opened ventrally behind the pectoral fins. The ventral aorta was cannulated and the heart removed without the pericardium and placed in an ice-chilled dish filled with saline for the atrium cannulation procedure. A polyethylene cannula was secured in the atrium at the junction with the sinus venosus. The cannulated heart was transferred to a perfusion chamber filled with saline and connected with a perfusion apparatus (as described by Tota et al., 1991b). Perfusion was immediately started; the heart received saline from an input reservoir pumped against an afterload pressure given by the height of an output reservoir. Isolation time was 5 min. In these experiments the chamber was designed to allow the development of subambient pressures during ventricle contraction, thus permitting suction filling of the atrium, which is an important mechanism operating in teleosts (Farrell et al., 1988). The chamber was completely filled with perfusate and covered with an unsecured Plexiglas lid (Fig. 1A). A thin layer of neoprene, placed between the large upper lid of the main part of the chamber and the lid, allowed slow capillary movements of the medium into and out of the chamber in response to the volume changes of the heart during its cycle. It is important to use this type of chamber for isolated heart preparations in those teleosts where it is difficult to isolate the heart with an intact pericardium in a reasonably short time. The parietal pericardium in the icefish is firmly connected to the perimisium of the muscles surrounding the heart (Tota et al., 1991a).

All the experiments were performed in a cold thermostatted cabinet (LKB 2021 Maxicoldlab, Malbo, Sweden), which allowed the heart and perfusion system to be maintained at near zero temperatures. The fish were weighed before each experiment and the blotted wet masses of whole hearts (atrium, ventricle and bulbus) and ventricles were determined at the end. For the detection of NO in the cardiac effluent (see below), we used for comparison a similar *in vitro* isolated and perfused working heart preparation from a temperate teleost counterpart, i.e. the European eel *Anguilla anguilla* (for details, see Imbrogno et al., 2001).

Saline

The icefish perfusate was a modified version of Cortland saline (Wolf, 1963), with an increased NaCl content to bring the ionic concentration up to the level found in channichthyid blood (Holeton, 1970). Its composition (in mmol l^{-1}) was: NaCl 252.4, KCl 5.0, $MgSO_4 \cdot 7H_2O$ 2.0, dextrose 5.56, $CaCl_2$ 2.3, $NaH_2PO_4 \cdot H_2O$ 0.2, $Na_2HPO_4 \cdot 2H_2O$ 2.3. The saline was aerated and the pH adjusted to 7.84 at 1°C, by using about 0.5 g of $NaHCO_3$.

The composition of the eel perfusate (in mmol l^{-1}) was:

NaCl 115.17, KCl 2.03, KH_2PO_4 0.37, $MgSO_4$ 2.92, $(NH_4)\cdot 2SO_4$ 50, $CaCl_2$ 1.27, glucose 5.55, Na_2HPO_4 1.90; pH was adjusted to 7.7–7.9 at room temperature (18–20°C), by using 1 g of $NaHCO_3$ (Imbrogno et al., 2001). The saline was equilibrated with a mixture of 99.5% O_2:0.5% CO_2.

Measurements and calculations

Hearts were stabilized under basal conditions for 15–20 min, before drug treatment. Pressure was measured through T-tubes placed immediately before the input cannula and after the output cannula, and connected to two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA, USA) in conjunction with a Unirecord 7050 (Ugo Basile, Comerio, Italy). Pressure measurements (input and output; expressed in kPa) were corrected for cannula resistance. Heart rate was calculated from pressure recordings. Cardiac output was collected over 1 min and weighed; values were corrected for fluid density and expressed as volume measurements. The afterload (mean aortic pressure) was calculated as 2/3 diastolic pressure + 1/3 maximum pressure. Stroke volume (*V*S = cardiac output/heart rate; ml kg^{-1}) was used as a measure of ventricular performance; changes in stroke volume were considered inotropic effects. Cardiac output and stroke volume were normalized per kg wet body mass. Ventricular stroke work (*W*S = afterload – preload × stroke volume/ventricle mass; mJ g^{-1}) served as an index of systolic functionality.

Experimental protocols

Basal conditions

Isolated perfused icefish hearts were allowed to equilibrate to conditions simulating an *in vivo* resting state for up to 15–20 min. Baseline hemodynamic parameters are listed in Table 1. In all experiments the control conditions were a mean output pressure of about 1.4 kPa, with a cardiac output set to 50 ml min^{-1} kg^{-1} wet body mass by appropriately adjusting the filling pressure. The heart generated its own rhythm. Cardiac parameters were simultaneously measured during experiments. Hearts that did not stabilize within 20 min of perfusion were discarded. The experiments were done at 3°C and were completed within 2 h; when perfused at constant temperature the isolated heart performance was stable for at least 3 h (Tota et al., 1991a).

In the case of the eel heart preparation, the experiments were performed at 18°C.

Table 1. *Performance variables under basal conditions of isolated and perfused heart preparations of* C. hamatus

Heart rate (beats min^{-1})	31.2±0.79
Filling pressure (kPa)	0.117±0.011
Output pressure (kPa)	1.385±0.085
Cardiac output (ml min^{-1} kg^{-1})	50.5±3.62
Stroke volume (ml kg^{-1})	1.861±0.113
Stroke work (mJ g^{-1})	2.241±0.258

Values are means ± s.e.m. of 21 experiments.

Test condition

After the 15 min control period, the treated hearts were perfused for 20 min with Ringer's solution enriched with either L-arginine or 3-morpholinosydnonimine (SIN-1) or SIN-1 plus superoxide dismutase (SOD) or L-N^5-*N*-iminoethyl-L-ornithine (L-NIO) or 8-bromo-guanosine 3′5′-cyclic monophosphate (8Br-cGMP) or 1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one (ODQ). To construct a concentration–response curve (L-arg, SIN-1), the preparation was exposed for 20 min to physiological saline containing a given drug in increasing concentrations. Each preparation was tested for at most three concentrations of the drug. In the case of pre-treatment with superoxide dismutase, the preparations were exposed to this agent for 20 min before and during SIN-1 treatment. SIN-1 was used in a darkened perfusion apparatus to limit drug degradation.

Data analysis and statistics

The results are expressed as means ± S.E.M. Because each heart represented its own control, the statistical significance of differences was assessed on parameter changes using the paired Student's *t*-test ($P<0.05$). Percentage changes were evaluated as means ± s.e.m. of percent changes obtained from individual experiments.

Drugs and chemicals

All the solutions were prepared in double-distilled water (except ODQ, prepared in ethanol); dilutions were made in Ringer's solution just before use. L-arginine, SIN-1, SOD, L-NIO, ODQ and 8Br-cGMP were purchased from Sigma Chemical Company (St Louis, MO, USA).

Determination of nitrite by electrochemical assay

The electrochemical apparatus used in this work has been previously described (Palmerini et al., 1998, 2002). The assay measures NO and its derivatives (nitrosothiols and nitrite) in the nmolar range. Briefly, it consisted of a reaction vessel (5 ml) equipped with an injector, maintained at a constant temperature and supplied with a flow of N_2 at 5 ml min^{-1}. NO formed in the vessel was carried by the N_2 to an amperometric sensor (Fig. 1A), where NO was first transformed into NO_2 using a small trap filled with an acidic solution of permanganate (50 mmol l^{-1} $KMnO_4$, 0.5 mol l^{-1} $HClO_4$). The presence of NO in the reaction vessel was recorded *vs* time *t* as a peak of electric current *I*. The apparatus was calibrated by injecting known amounts of standard nitrite solution into the reaction vessel, which also contained 0.1 mol l^{-1} $CuCl_2$ and 0.1 mol l^{-1} cysteine in a final volume of 2 ml. The lowest detectable amount of NO was ~5–10 pmol. To measure nitrites, 100 µl aliquot samples of the specimen, i.e. either cardiac perfusate or plasma or hemolysate from icefish or eel, were injected into the reaction vessel of the electrochemical apparatus, which also contained 0.1 mol l^{-1} $CuCl_2$ and 0.1 mol l^{-1} cysteine. Specimens of plasma and hemolysate were obtained from heparinized blood collected from the caudal vein. Plasma was obtained following centrifugation at 800 ***g***; the pellets were suspended in hypotonic solution and

then centrifuged at 10 000 ***g*** to remove cell debris. Cardiac perfusates were obtained during perfusion experiments: after stabilization, cardiac perfusates of eight icefish and five eel hearts were collected (20 ml) and immediately stored at –80°C before use. Data are presented as mean ± S.E.M. (*N*=5 determinations).

NOS localization

NADPH-diaphorase

The protocol for the NADPH-diaphorase histochemical method for localizing NOS activity was modified by Hope et al. (1991). The hearts of three *C. hamatus* were removed immediately after sacrifice, flushed with ice-cold saline and fixed in 4% paraformaldehyde solution for 5–7 h. The tissues were cryoprotected by infiltration with 30% sucrose solution for 3–4 days. Then they were embedded in an optimal cutting temperature (OCT) compound, rapidly frozen in liquid nitrogen and cut at the level of the ventricle using a cryostat (Microm HM505E; Walldorf, Germany). The transverse ventricular sections (5 μm thick) were postfixed in phosphate

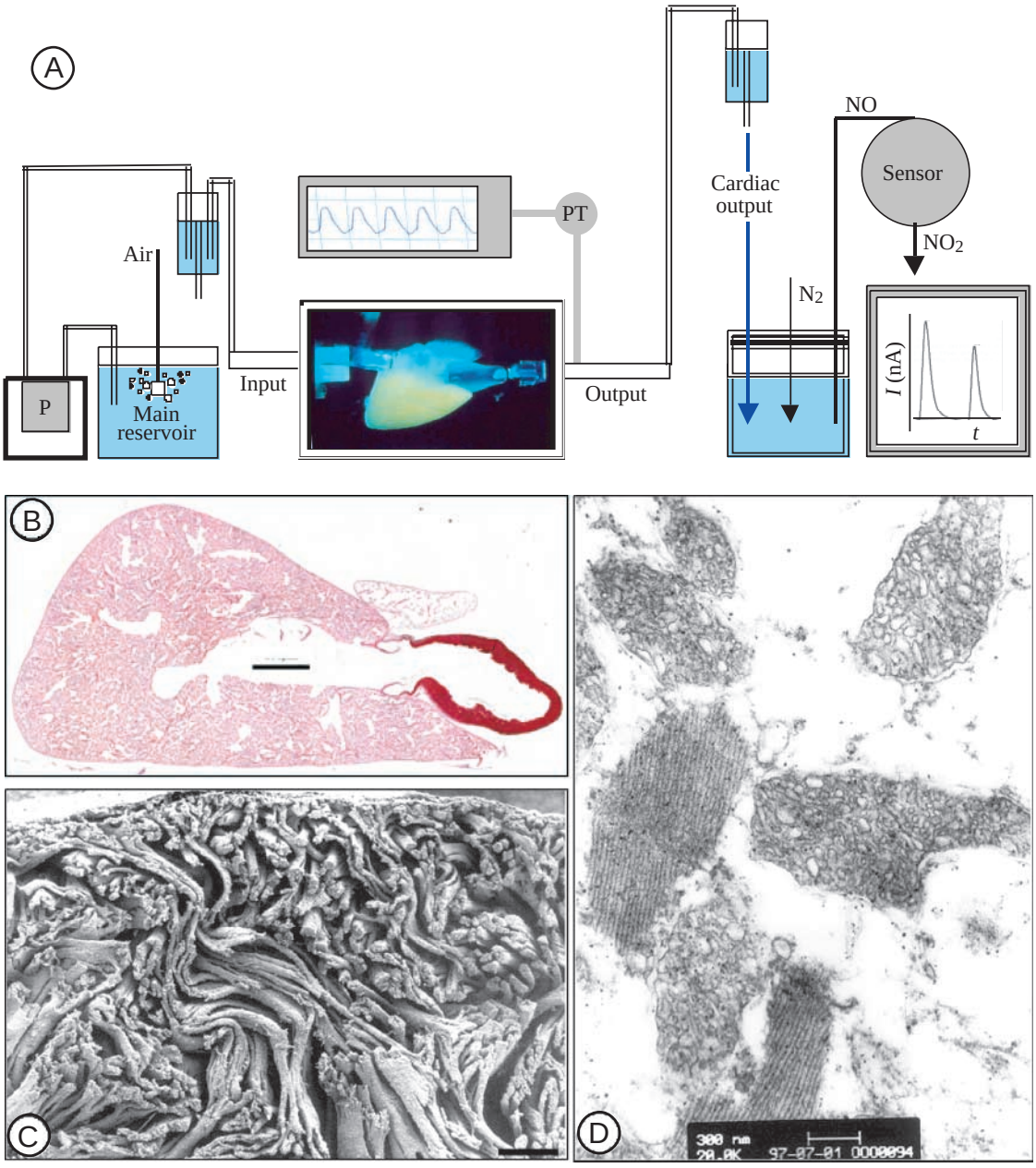

Fig. 1. (A) Scheme of the perfusion apparatus (left) connected with the amperometric gas sensor (right) used for nitrite detection in the cardiac effluent. (B,C) The myoarchitecture of the icefish heart showing the extensive and fully trabeculate ventricular wall. (B) Histological longitudinal section (Sirius Red staining) (bar, 0.25 cm); (C) scanning electron micrograph with details of the myocardial trabeculae and the intertrabecular spaces (lacunae) (bar, 100 μm). (D) Transmission electron micrograph showing mitocondria and myofibrils in ventricular myocytes. (J. M. Icardo and B. Tota, unpublished material). P, perfusate; PT, pressure transducer.

buffer (PB) enriched with 4% paraformaldehyde for 30 min. The sections were then incubated for 1 h at 37°C in Tris-HCl (0.1 mol l^{-1}, pH 7.5) containing Triton X-100 (0.3%), Nitro-blue Tetrazolium (NBT, 0.6 mmol l^{-1}), NADPH (1 mmol l^{-1}) and sodium azide (1 mmol l^{-1}). Parallel sections were incubated in the same buffer without NADPH as a control. After incubation, the ventricular tissue was observed under an optical photomicroscope (ZEISS Axioscope; Thornwood, NY, USA).

Immunofluorescence

Immediately after sacrifice, the hearts of three *C. hamatus* were removed, washed in ice-cold saline solution and blocked in diastole by perfusion with a high potassium hyperosmotic saline. The tissues were embedded in OCT, fixed in liquid nitrogen and stored at –80°C until use. Frozen sections (7 μm thick) were cut at the cryostat (Microm HM505E), postfixed with acetone for 10 min and stored at –20°C. Before immunostaining, the slides were washed with Tris-HCl buffered saline (TBS), and then incubated with 1:100 anti-eNOS or anti-iNOS antibodies (mouse monoclonal, FITC-conjugated; BD Transduction Laboratories, Lexington, KY, USA) for 24 h at 4°C. To stop the reaction the sections were washed with TBS then the slides mounted with mounting medium (Vector Laboratories, Burlingame, CA, USA) and observed under a confocal laser microscope (TCS-SP2, Leika Microsystem, Wetziar, Germany). Negative controls were obtained using parallel ventricular sections treated in the same manner, excluding antibodies (data not shown).

Results

Basal cardiac performance

The *in vitro* isolated and perfused heart preparation of *C. hamatus*, working under physiological loading conditions (Tota et al., 1991b), is able to generate comparable values of output pressure, cardiac output, ventricle work and power (Table 1) as *in vivo* (Hemmingsen et al., 1972).

NO–cGMP mechanism

Chronotropic effect

As shown in Table 2, in perfused icefish heart preparations, NO (L-arg, SIN-1, SIN-1+SOD) and cGMP (8Br-cGMP) donors, the NOS inhibitor L-NIO, and the sGC inhibitor ODQ did not affect the heart rate. These data suggest that in the icefish NO is not involved in the regulation of heart rate.

Inotropic effect

The dose–response curve of L-arginine, the authentic NO-synthase substrate, at concentrations from 10^{-7} to 10^{-5} mol l^{-1}, shows a significant positive inotropism at 10^{-6} mol l^{-1} and 10^{-5} mol l^{-1} (Fig. 2). When L-lysine was used as control (Amrani et al., 1992), there was no effect (data not shown). Treatment with the NOS inhibitor L-NIO (10^{-5} mol l^{-1}) induced a significant negative inotropism (Fig. 4). Of the two NO donors, SNP and SIN-1, SNP (10^{-5} mol l^{-1}) induced a positive inotropic effect, similar to L-arginine (data not shown), while the dose–response curve of SIN-1 (10^{-7}–10^{-5} mol l^{-1}) (Fig. 3A) showed significant negative inotropism at 10^{-5} mol l^{-1}. It is known that SIN-1 simultaneously generates NO and superoxide anions, which react instantaneously to form peroxynitrite (Beckman and Koppenol, 1996). Therefore, to evaluate and clarify the SIN-1 negative inotropic effect, we tested the effects of SIN-1 (10^{-5} mol l^{-1}) in the presence of SOD (10 i.u. ml^{-1}), an antioxidant enzyme that competes with NO binding to the superoxide anions. The negative inotropic effect of SIN-1 was completely reversed in the presence of SOD (Fig. 3B), while SOD *per se* had no effect on cardiac contractility (data not shown). Consequently, we can state that the SIN-1 derived NO induces a positive inotropic effect, similar to SNP and L-arginine. To evaluate cGMP-related mechanisms, the cardiac preparations were exposed to either a stable and lipid-soluble analogue of cGMP, 8Br-cGMP (10^{-5} mol l^{-1}) or to a specific inhibitor of guanylate cyclase, ODQ (10^{-5} mol l^{-1}). Administration of 8Br-cGMP elicited a

Table 2. *Heart rate under basal condition (Control) and in the presence of either NO-donors (L-Arg, SIN-1, SIN-1+SOD), or NOS (L-NIO) and sGC (ODQ) inhibitors or 8Br-cGMP*

	Heart rate (beats min^{-1})	
Drug (10^{-5} mol l^{-1})	Control	Treatment
L-Arg	30.95±1.55	31.17±1.23
SIN-1	36.36±0.01	36.36±0.01
SIN-1+SOD (10 i.u. ml^{-1})	28.74±1.99	27.92±1.74
L-NIO	32.01±1.32	32.77±1.53
8Br-cGMP	31.09±0.622	30.47±0.485
ODQ	29.57±1.53	33.79±2.53

Neither donors nor inhibitors affected basal (Control) heart rate. Abbreviations are given in the List of symbols and abbreviations.

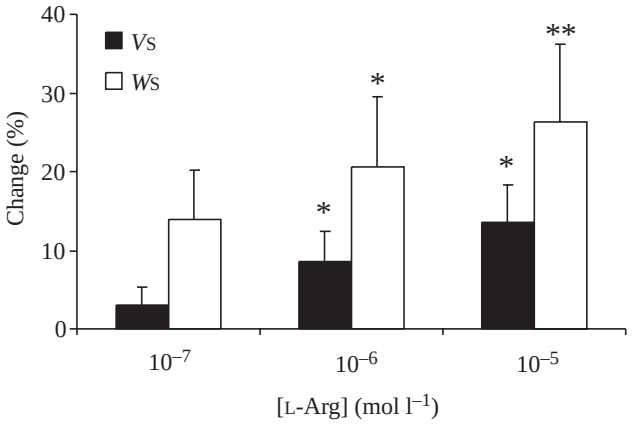

Fig. 2. Cumulative concentration-response curve showing the effect of L-arginine (L-Arg; from 10^{-7} to 10^{-5} mol l^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused icefish hearts. Percentage changes were evaluated as means ± S.E.M. of five experiments. Asterisks indicate values significantly different from the control value: **P*<0.05, ***P*<0.025.

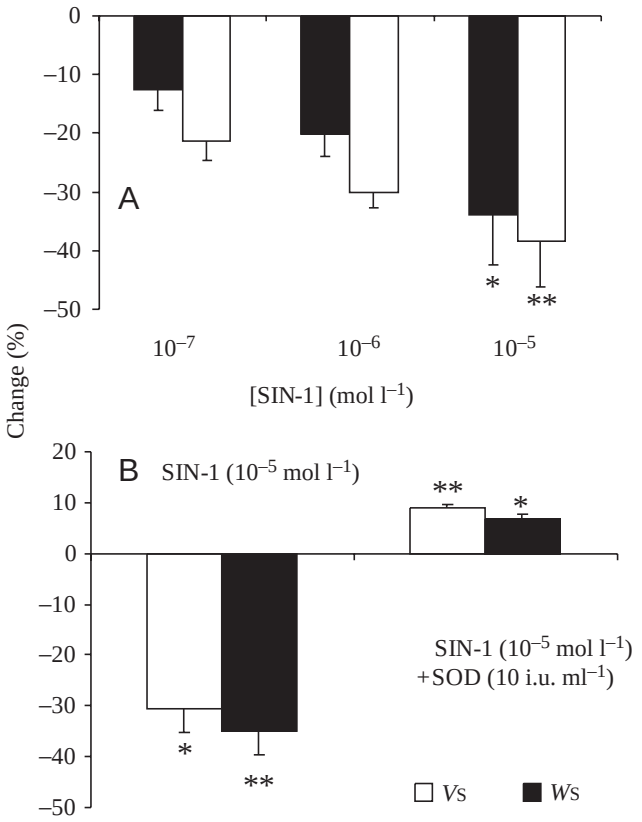

Fig. 3. (A) Cumulative concentration–response curve showing the effect of 3-morpholinosydnonimine (SIN-1; from 10^{-7} to 10^{-5} mol l^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused icefish hearts. (B) Effects of 3-morpholinosydnonimine (SIN-1; 10^{-5} mol l^{-1}) before and after treatment with superoxide dismutase (SOD; 10 i.u. ml^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused icefish hearts. Percentage changes were evaluated as means ± S.E.M. of five (A) and four (B) experiments. Asterisks indicate values significantly different from the control value: **P*<0.05; ***P*<0.025.

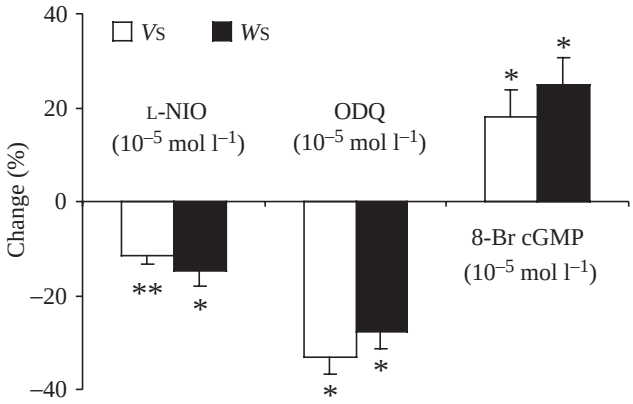

Fig. 4. Effects of L-N^5-*N*-iminoethyl-L-ornithine (L-NIO; 10^{-5} mol l^{-1}), 1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one (ODQ; 10^{-5} mol l^{-1}) and 8-bromo-guanosine 3′5′-cyclic monophosphate (8Br-cGMP; 10^{-5} mol l^{-1}) on stroke volume (*V*S) and stroke work (*W*S) in isolated and perfused icefish hearts. Percentage changes were evaluated as means ± S.E.M. of four experiments for each group. Asterisks indicate values significantly different from the control value: **P*<0.05; ***P*<0.025).

Table 3. *Concentration of nitrite in plasma, haemolysate and cardiac effluent from* C. hamatus *and* A. anguilla

	[Nitrite] (nmol ml^{-1})	
	C. hamatus	*A. anguilla*
Plasma	0.25±0.018	0.28±0.050
Haemolysate	0.30±0.022	0.25±0.030
Cardiac effluent	0.15±0.036	0.22±0.015

Values are means ± s.e.m. of 5 determinations; *N*=8 (C. hamatus); *N*=7 (*A. anguilla*).

significant positive inotropic effect while ODQ elicited negative inotropism (Fig. 4).

Determination of nitrite

Nitrite concentrations in plasma and blood cell hemolysates were higher than in the cardiac effluents and were comparable in both icefish and eel (Table 3). In all samples, nitrosothiols of both low and high mass were absent or undetectable.

NOS localization

NADPH-diaphorase

An intense dark blue staining resulting from NADPH-diaphorase activity was observed in the ventricular endocardial endothelium (EE) cells (Fig. 5A, arrowheads). A less intense reaction was observed in the cardiac muscle fibers (Fig. 5A, arrow). The specificity of this staining was confirmed by its complete absence in the control sections (data not shown).

Immunofluorescence

Morphological examination of *C. hamatus* ventricular sections incubated with anti-eNOS or with anti-iNOS antibodies revealed an intense iNOS labelling present throughout the ventricle. By contrast, eNOS immunoreactivity was not detectable in this cardiac region (data not shown). As shown in Fig. 5B, iNOS labelling was densely localized in the cytoplasm of the myocardiocytes while it was absent in the EE cells. The specificity of the binding was confirmed by staining of negative controls.

Discussion

Isolated working heart preparation

This kind of working heart preparation permits analysis of the mechanical performance free from extrinsic neuro-humoral stimuli and, at the same time, the controlled nature of the perfusion fluid may reduce the removal rate of NO and the production of peroxynitrites (Voelkel et al., 1995). It may also allow ‘physiological’ activation of a silencing eNOS, particularly at the EE level (Brutsaert, 2003), which might be

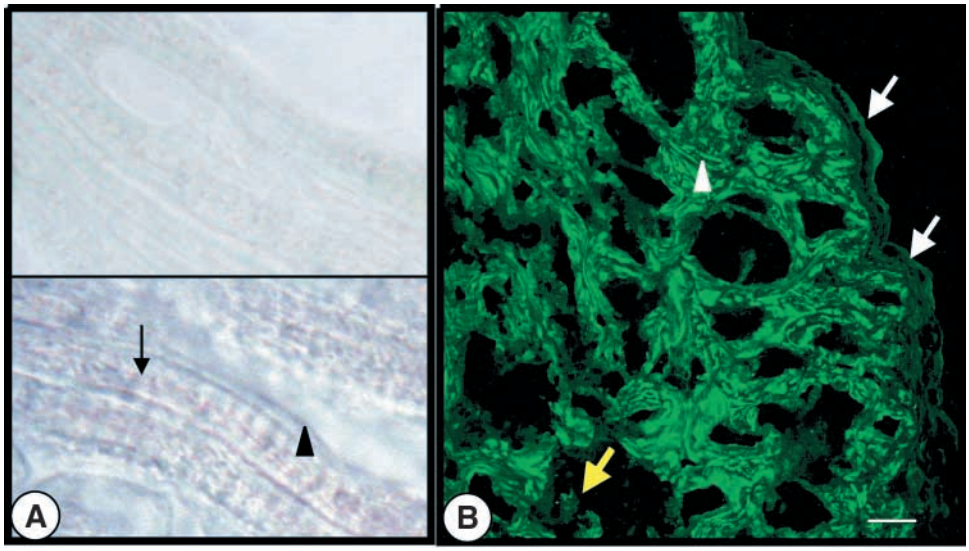

Fig. 5. (A) NADPH-diaphorase in the icefish ventricle. (Bottom) Representative image showing the localization of NOS in paraformaldehyde-fixed transverse ventricular sections (5 μm) processed as described in Materials and methods. Note the dark blue reaction product detecting NOS activity in endocardial endothelial (EE) cells (arrowhead) and cardiomyocytes (arrow) (100×). (Top) Control image obtained by incubating transverse ventricular sections in absence of NADPH (100×). (B) iNOS immunofluorescence. Frozen transverse ventricular sections (7 μm) were incubated with 1:100 anti-iNOS antibody as described in Materials and methods. Fluorescent immunolabeling of iNOS in the icefish ventricle is densely localized in the cytoplasm of the myocardiocytes (white arrowhead). Note the absence of iNOS in the subepicardial layer (white arrow) and in the EE cells (yellow arrow). Bar, 40 μm.

otherwise inactive at the basal cellular level (Wu, 2002; Petroff et al., 2001). The EE is a relevant source of NO and appears to exert an important paracrine modulation on the subjacent myocardium performance in both mammals (Brutsaert et al., 1998) and fish (Imbrogno et al., 2001). This function can be of importance in the very large and fully trabeculated icefish heart, in which the extensive surface area of the EE lining the labyrinth of the lacunary spaces (see Fig. 1B,C) is particularly exposed to turbulent flow and relevant laminar fluid shear stress exerted by the superfusing blood.

NO–cGMP mechanism

Chronotropic effect

NO, tonically released in the heart, may be involved in the regulation of intrinsic heart rate f_H by different mechanisms, including cholinergic antagonism of the adrenergic-mediated positive chronotropism and direct NO-cGMP-mediated stimulation of the I_f pacemaker current (Musialek and Casadei, 2000, and references therein). Both NO-induced positive and negative chronotropic effects have been reported in several mammals including man (Musialek and Casadei, 2000). In the isolated working frog *Rana esculenta* heart, SNP elicited a negative chronotropic effect, while SIN-1 had no influence (Sys et al., 1997). In fish, McGeer and Eddy (1996) reported positive chronotropism in the trout *Oncorhynchus mykiss* exposed to SNP, while in Atlantic salmon embryos the same NO donor elicited a negative chronotropic effect (Eddy et al., 1999). In the isolated and spontaneously beating icefish heart, we have shown that both endogenous (L-arginine) and exogenous (SIN-1) NO have no effect on heart rate. The lack of significant chronotropic effects following exposure to either the NOS inhibitor L-NIO or the sGC inhibitor ODQ, or the analogue of cGMP, 8Br-cGMP, suggests that in *C. hamatus* the intrinsic f_H is not influenced by NO-cGMP signalling. In the same icefish heart preparation a negative chronotropism was induced by L-NMMA (N^G-monomethyl-L-arginine; Pellegrino et al., 2003). This discrepancy may be related to differences between the two NOS inhibitors, L-NIO being, in comparison with L-NMMA, more selective, potent and *per se* without effect on f_H (McCall et al., 1991). While the cholinergic control of f_H in several red-blooded Antarctic teleosts appears well developed compared to most other species (Axelsson et al., 1992), the situation in icefish may be different. For example, in the icefish *C. aceratus*, Hemmingsen et al. (1972) found that atropine produced only a slight increase in f_H, consistent with a relatively low vagal tone. Whether this intrinsic low f_H is related to the extreme stenothermia of this fish and/or to the constraints of a low-speed volume pump cardiac design (Tota and Gattuso, 1996) remains to be established.

Inotropic effect

Both the endogenous NOS substrate L-arginine and the exogenous NO-donor SIN-1 (in the presence of SOD) elicited a significant concentration-dependent positive inotropism. In particular, experiments where SOD competed with NO binding to superoxide anions (Beckman and Koppenol, 1996), unambiguously indicate that SIN-1-derived NO induces a positive inotropic effect, as with L-arginine. A similar positive inotropism elicited by the NO-donor SNP was reported in the same isolated working *C. hamatus* heart preparation (Pellegrino et al., 2003). The significant negative decrease of V_S and W_S following both NOS and sGC inhibition by their specific inhibitors L-NIO and ODQ, respectively, along with the increased V_S and W_S elicited by the GC donor 8Br-cGMP, are all consistent with the presence of a significant NO-cGMP-mediated positive inotropic influence on the basal (i.e. unstimulated) mechanical performance of the icefish heart.

The continuous generation of relatively low basal levels (nanomolar concentrations) of NO from the beating heart is considered critical for the functional integrity of the myocardial pump in mammals (Moncada et al., 1991; Pinsky et al., 1997). Both positive and negative inotropic actions of NO (and cGMP) at the cardiomyocyte level have been reported in various preparations, but the reasons for these opposite responses to NO, and the underlying mechanisms, remain to be identified (Massion and Balligand, 2003, and references therein). It is well acknowledged, however, that in any given situation, these direct nitrergic effects depend, among other factors, upon the amount of NO generated, the type and sub-localization of NOS isoenzyme involved, the target tissue (e.g. atrial or ventricular cardiocytes), the microenvironment (e.g. antioxidant status and prevailing redox balance) and the animal species examined (Shah and MacCarthy, 2000). Of note, in the icefish the NO–cGMP-mediated positive inotropism contrasts with the NO–cGMP-mediated negative inotropism shown in the isolated working heart preparations of teleost (*Anguilla anguilla*, Imbrogno et al., 2001; *Salmo salar*, Gattuso et al., 2002) and amphibian (*Rana esculenta*, Sys et al., 1997) hearts. These hearts exhibit the same trabeculated myoarchitecture and intracardiac blood supply as the icefish heart (Tota and Gattuso, 1996) and were studied under identical experimental conditions. Therefore, the opposite inotropic responses between the above mentioned species and icefish to NO and cGMP cannot be attributed to the experimental hierarchic level of investigation, nor to macroscopic differences in cardiac structure; but they may indicate species-specific differences at more subtle ultrastructural (e.g. mitochondria, intracellular NOS isoforms localization), biochemical (e.g. phosphodiesterase types and activities) or molecular (e.g. ion channel) levels. For example, we could speculate that the very expanded mitochondrial compartment in icefish cardiomyocytes (Fig. 1D) could reasonably be a target of NO. In fact, NO can act as a competitive inhibitor of cytochrome oxidase, with consequent decrease of mitochondrial respiration, while increased peroxynitrite formation in mitochondria may cause calcium release *via* the pyridine nucleotide dependent pathway (see Giulivi, 2003, and references therein).

The icefish heart as source of NO

Determination of free NO in cells and biological samples using conventional methods, normally within the nanomolar range, has been hampered by the chemical instability of the molecule in water solutions (half-life about 3–5 s), where traces of oxygen transform NO into nitrite, thus reflecting the amount of NO released by the tissues (Beckman and Koppenol, 1996; Palmerini et al., 2002, and references therein). Due to its low concentrations, however, measurement of nitrite requires high-sensitivity techniques. For this reason, we previously developed an electrochemical assay that allows the determination in biological fluids of nitrite and nitrosothiols in the nanomolar range (Palmerini et al., 2002). The method exploits a specific solid-state amperometric sensor for the rapid determination of NO in its gaseous phase based on the reduction of nitrite and/or nitrosothiols without any additional purification steps (Palmerini et al., 1998, 2002).

By using a temperate fish (*A. anguilla*) as the counterpart to *C. hamatus*, we found comparable concentrations of nitrite in plasma and hemolysate of both icefish and eel. Nitrite was also present in the cardiac effluents from both species (Table 3). This is the first demonstration that, like the mammalian heart (Pinsky et al., 1997), the contracting fish heart releases a substantial amount of NO under basal conditions, challenging studies regarding alternative NO carriers in the absence of Hb.

Morphological evidence of NOS

Using two different morphological methods (i.e. NADPH-diaphorase and immuno-fluorescence) we have demonstrated NOS expression in the heart of *C. hamatus*.

The NADPH-diaphorase histochemical method revealed an intense NOS activity in the EE cells lining the ventricular trabeculum and a less intense reaction in the ventricular myocardiocytes.

NOS immunolocalization in icefish was conducted using two mouse monoclonal antibodies, anti-eNOS and anti-iNOS. In spite of the remarkable phylogenetic distance between mouse and icefish, mouse anti-iNOS specifically bound the icefish antigen, thus allow identification of the myocardial NOS as iNOS. The intense staining observed in the ventricular EE cells with NADPH-diaphorase was not confirmed by the application of anti eNOS or iNOS antibodies. Since EE cells mainly express the eNOS isoform (Brutsaert et al., 1998), we can hypothesise that the lack of eNOS immunostaining is due to the low specificity of the mouse antibody toward the icefish endocardial NOS isoform. On the other hand, the absence of iNOS labelling in the EE cells may suggest that the EE isoform is indeed eNOS. Due to the elevated binding specificity of monoclonal antibody, the particular epitope of interest of the eNOS molecule may have undertaken minor amino acid sequence modifications so as to be unavailable for reaction (i.e. negative staining). Further studies should explore the possibility that in the icefish the iNOS isoform could have been conserved during evolution while the eNOS isoform could have encountered structural modifications.

It is well known that iNOS is expressed under inflammatory and immune stimuli (Vallance et al., 2000). In the absence of any induced stimulation, our data suggest a basal expression of this isoform in icefish ventricular myocytes. Recent data have demonstrated cross-reactivity between antibodies directed against different NOS isoforms, including that localized in the mitochondria (mtNOS; Ghafourifar and Richter, 1997). Since icefish ventricular myocardiocytes contain a notably large mitochondrial compartment, it is possible that mtNOS contributes to the observed high iNOS immunostaining. Taken together with the physiological evidence for a tonic NO–cGMP-mediated inotropism, these immunofluorescence results suggest that a ventricular myocardial NOS isoform (either iNOS and/or mtNOS) is involved in the basal release of NO in the icefish heart effluent.

It has been suggested that mitochondrial NOS, as the source of NO acting as competitive inhibitor of cytochrome *c* oxidase, can play an important 'local' role by helping average oxygen utilization between cells at different distances from the capillaries (i.e. allowing the oxygen supply to diffuse further into the tissue; Thomas et al., 2001; Giulivi, 2003). In icefish cardiomyocytes the increased mitochondria, often arranged in clusters, have been viewed as oxygen conduits and are particularly important for maintaining oxygen fluxes in species lacking Hb (O'Brien and Sidell, 2000). Therefore, it is possible that in *C. hamatus* the presence of mtNOS in the myocardiocytes could be an important molecular strategy for expanding the mechanism by which these cells consume oxygen.

In conclusion, in the icefish, the nitrergic regulation of cardiac performance, the detection of nitrite as marker of NO released in the cardiac effluent, along with the morphological localization of cardiac NOS, all indicate an important role of NO in modulating heart function and, putatively, oxygen gradients in the absence of hemoglobin. These data are also of evolutionary interest since they suggest that the genetic retention of the NOS system in the cold-adapted Notothenioids has permitted retention of nitrergic effector functions located at the interface of organismal and molecular physiology, including molecular lines of defence against the hemoglobinless condition.

List of symbols and abbreviations

8Br-cGMP	8-bromo-guanosine 3′5′-cyclic monophosphate
CO	cardiac output
EE	endocardial endothelium
f_H	heart rate
GC	guanylate cyclase
Hb	haemoglobin
L-NIO	L-N^5-*N*-iminoethyl-L-ornithine
NBT	Nitro-blue tetrazolium
NO	nitric oxide
NOS	NO synthase
OCT	optimal cutting temperature
ODQ	1H-[1,2,4]oxadiazole-[4,3-a]quinoxalin-1-one
P_{VA}	ventral aortic pressure
SIN-1	3-morpholinosydnonimine
SOD	superoxide dismutase
V_S	stroke volume
W_S	stroke work

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