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Phenylhydrazine-induced anemia causes nitric-oxide-mediated upregulation of the angiogenic pathway in *Notothenia coriiceps*

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SUMMARY

Antarctic icefishes possess several cardiovascular characteristics that enable them to deliver oxygen adequately in the absence of hemoglobin (Hb). To gain insight into mechanisms driving development of these cardiovascular characteristics of icefish, we chemically induced severe anemia in a red-blooded notothenioid, *Notothenia coriiceps*. After 10 days of treatment with phenylhydrazine HCl, the hematocrit and Hb concentration of *N. coriiceps* decreased by >90% and >70%, respectively. Anemic fish exhibited a significantly higher concentration of nitric oxide (NO) metabolites in their plasma compared with that of control animals, indicating that corporeal levels of NO are higher in anemic animals than in control fish. The activity of nitric oxide synthase (NOS) was measured in brain, retina, pectoral muscle and ventricle of control and anemic animals. With the exception of retina, no significant differences in NOS activities were observed, indicating that the increase in plasma NO metabolites is due to loss of Hb, which normally plays a major role in the degradation of NO, and not due to an overall increase in the capacity for NO production. To determine whether loss of Hb can stimulate remodeling of the cardiovascular system, we measured expression of HIF-1α, PHD2 and VEGF mRNA in retinae of control and anemic fish. Expression of all three genes was higher in anemic animals compared with control *N. coriiceps*, suggesting a causative relationship between loss of Hb and induction of angiogenesis that probably is mediated through nitric oxide signaling.

Key words: Antarctic fish, icefish, hemoglobin, phenylhydrazine, angiogenesis, nitric oxide.

INTRODUCTION

It was once believed that hemoglobin (Hb) expression was a distinguishing characteristic of vertebrates. Antarctic icefishes (suborder: Notothenioidei; family: Channichthyidae) are the exception to this rule (Ruud, 1954). Channichthyids lost the ability to express Hb through a gene-deletion event that occurred when they diverged from red-blooded Antarctic notothenioids approximately 8.5 million years ago (Cocca et al., 1995; Near, 2004). In the absence of Hb, icefish blood carries oxygen in physical solution in plasma, resulting in an oxygen-carrying capacity that is less than 10% that of red-blooded notothenioids (Holeton, 1970). Loss of Hb expression would be a lethal mutation in most environments. However, the low water temperature of the Southern Ocean results in high oxygen solubility that undoubtedly contributed to the survival of icefish. Although decreased blood viscosity due to lack of red blood cells (RBCs) is energetically favorable, icefishes have a much higher cardiac output than red-blooded fish, resulting in an overall greater energetic cost of circulation (Wells, 1990; Sidell and O'Brien, 2006). Thus, loss of Hb is an energetically disadvantageous trait. Like all notothenioids, icefishes benefited from very low competition owing to a crash in species diversity that occurred sometime after the mid-tertiary period (Eastman, 1993). With greatly relaxed competition, negative selection will not operate on sublethal traits within a population, even if they result in a decrement in performance. The combination of a cold, well-oxygenated environment and low competition allowed early icefish to survive and persist even though they possessed a mutation that impaired their physiology (Sidell and O'Brien, 2006).

Today, 16 species of icefishes inhabit the Southern Ocean. Modern icefishes are genetically very closely related to their red-blooded relatives; however, red- and white-blooded fish have notably different cardiovascular systems. During the course of evolution, icefishes developed a unique cardiovascular system that appears to compensate for the loss of Hb. Channichthyids are characterized by increased blood volume, larger bore blood vessels, greater ventricular mass, higher cardiac output, denser vascularization and increased ventricular mitochondrial densities compared with red-blooded Antarctic notothenioids (Eastman, 1993; O'Brien and Sidell, 2000; Wujcik et al., 2007). Together, these cardiovascular characteristics facilitate delivery of oxygen throughout the body. Although these characteristics have been well described, the underlying mechanisms responsible for driving them have not been elucidated. Here, we describe how nitric-oxide-mediated signaling pathways might be triggered by the loss of Hb to stimulate angiogenesis in a red-blooded Antarctic notothenioid. Our observations provide insight into the evolutionary path that might have led to the cardiovascular characteristics of modern icefishes.

Nitric oxide (NO) is a pervasive signaling molecule produced from arginine and oxygen by nitric oxide synthases (NOS) (Alderton et al., 2001). In most vertebrates, the half-life of this potent molecule is very short *in vivo* because it is broken down rapidly through reactions with oxygenated Hb or myoglobin (Mb), resulting in the formation of nitrate (Gow et al., 1999; Flogel et al., 2001). NO degradation also occurs at a much slower rate through reactions with oxygen free radicals and thiols, resulting in the formation of nitrate and nitrite (Kelm, 1999).

In the absence of Hb, we predicted that steady-state levels of NO would be higher in icefish than red-blooded notothenioids owing to loss of the primary breakdown pathway for NO (Sidell and O'Brien, 2006). In previous work, we measured the concentration of nitrate plus nitrite (NO_x) in plasma of several species of notothenioids (Beers et al., 2010). Owing to technical difficulties surrounding accurate direct measurement of NO, the aggregate concentration of NO_x is often measured as a proxy for NO (Sun et al., 2003; Tsikas, 2005). Consistent with our hypothesis, we found that icefish species generally had higher concentrations of NO_x in their plasma than did red-blooded species (Beers et al., 2010). When the results are adjusted for the larger blood volume of fish lacking Hb expression, it is clear that corporeal content of NO_x is higher in icefishes than red-blooded notothenioids, strongly suggesting a corresponding difference in NO level.

NO stimulates angiogenesis and mitochondrial biogenesis in mammals. Expression of genes from both signaling pathways increases in response to NO (Ziche and Morbidelli, 2000; Nisoli et al., 2003). Tissues of hemoglobinless icefishes display both dramatically greater mitochondrial densities (O'Brien and Sidell, 2000) and vascular densities (Wujcik et al., 2006) than those of their red-blooded relatives. Yet, despite the apparently higher level of NO in icefish, there is no significant difference in expression of mitochondrial biogenesis genes in ventricles and angiogenesis genes in retinae between red- and white-blooded adult notothenioids (Urschel and O'Brien, 2008; Beers et al., 2010). Feedback inhibition could be responsible for lack of upregulation in the genes, once stable well-oxygenated phenotypes are attained. Indeed, NOS activity is lower in icefish than red-blooded species, indicating that feedback inhibition might account for a decrease in the rate of NO production in adult icefish (Beers et al., 2010).

Although not evident in adults, where the anatomy has stabilized, NO might play a role in remodeling the cardiovascular system during early development of icefishes. However, owing to the inability to capture or manipulate early life-history stages of icefish, we cannot measure NO levels or gene expression in developing icefish. To test our hypothesis, we produced an icefish model by treating adults of the red-blooded notothenioid *N. coriiceps* for 10 days with phenylhydrazine HCl (PHZ). Phenylhydrazine is a hemolytic agent that lyses RBCs, leading to the degradation and clearance of Hb and drastically reducing the hematocrit (Hct) and Hb concentration (Smith et al., 1971; Gilmour and Perry, 1996; McClelland et al., 2005). We reasoned that treatment of a red-blooded notothenioid with PHZ would induce severe anemia that could provide insight into what happens when the primary breakdown pathway for NO is removed.

MATERIALS AND METHODS

Animals

Notothenia coriiceps Richardson 1844 were collected from Dallmann Bay in the Antarctic Peninsula region (64°08′S, 62°40′W) during the austral autumns of 2007 and 2009. Fish were caught from a depth of approximately 150 m by using Otter trawls and baited traps deployed from the ARSV *Laurence M. Gould*. Animals were held in flowing seawater tanks during transit to Palmer Station on Anvers Island. Fish were transferred to the Palmer Station aquarium and held in circular aerated covered flowing seawater tanks of *ca*. 3000 l volume at 0±0.5°C.

Experimentally induced anemia

Specimens of *N. coriiceps*, a red-blooded notothenioid with a normal hematocrit of 35–40%, were made anemic by treatment with phenylhydrazine HCl (PHZ), a hemolytic agent. PHZ was administered by an initial intraperitoneal injection, followed by continuous delivery of the drug by a surgically implanted osmotic pump, as described below. Moderately sized animals (39–43 cm total length; 1000–1400 g wet mass) were used for experimental treatments.

Fish were anesthetized before surgery using MS-222 (Finquel, Argent Chemical Laboratories, Redmond, WA, USA) at a dosage of 1:7500 w/v. Once unresponsive, animals were transferred to an inclined surgical table where their gills were irrigated continuously with chilled seawater containing anaesthetic at 1:12,000 w/v throughout the surgical procedure. A section of ventral abdominal surface, just anterior to the vent and lateral to the midline, was swabbed thoroughly with antiseptic (0.02% chlorhexidine) before making an incision of length 2 cm through the abdominal wall. An Alzet 2ML1 osmotic pump (Durect Corporation, Cupertino, CA, USA), containing 440 mmol l^{-1} PHZ in notothenioid Ringer solution, was inserted into the peritoneal cavity through the incision. Notothenioid Ringer solution comprised: 260 mmol l^{-1} NaCl, 2.5 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} KCl, 2.5 mmol l^{-1} $NaHCO_3$, 5.0 mmol l^{-1} NaH_2PO_4, pH 8.0 at 1°C. According to the algorithm provided by the manufacturer, this pump should deliver PHZ solution at a constant rate of ~4.5 µl h^{-1} for the duration of the experiment under these conditions. After implantation of the pump, the incision was closed by suturing with a 4/0 polypropylene monofilament. Animals then were injected intraperitoneally with PHZ at 10 mg kg^{-1} before initial transfer to a shallow holding tank for recovery. During the recovery period, a hose delivering flowing seawater at ambient temperature was held in the buccal cavity of the animal to ensure irrigation of the gills until the fish was able to resume autonomous ventilation. Animals then were transferred to circular 3000 l flowing seawater tanks, where they were held for 10 days.

Tissue preparation

All animals were anesthetized with MS-222 (1:7500 w/v). Blood was drawn from the caudal vein, and a small volume was drawn immediately into heparinized glass capillary tubes for Hct determination. The remainder of the whole blood was mixed with a 3.2% sodium citrate solution (9 parts blood to 1 part sodium citrate) to prevent clotting. Whole-blood samples were stored for hemoglobin determination. Plasma samples were obtained by centrifuging the blood at 5300 ***g*** for 10 min at 4°C; plasma was drawn off and frozen at –80°C for later NO_x determination. After drawing blood, anesthetized animals were killed by severing of the spinal cord, followed by rapid excision of the brain. All tissues collected for gene expression and enzyme activity measurements were flash-frozen with liquid nitrogen and stored at –80°C.

Hemoglobin determination

The Hb concentration was determined using the cyanmethemoglobin method (Stadie, 1920). Briefly, 20 µl of whole blood containing sodium citrate was mixed with 5 ml of Drabkin's reagent (Sigma Aldrich, St Louis, MO, USA) and then incubated for 30 min at room temperature before spectrophotometric measurement at 540 nm. Total Hb concentration was calculated using a bovine Hb (Sigma Aldrich) standard curve. All samples were measured in triplicate, and mean values were computed for each individual.

Plasma nitrate plus nitrite (NO_x) determination

Plasma was deproteinated using an acetonitrile/chloroform treatment based on a protocol from Romitelli and colleagues (Romitelli et al., 2007). Plasma was mixed 1:1 with acetonitrile, vortexed for 60 s

and centrifuged at 21,000***g*** for 10 min at 4°C. The supernatant was transferred to a new tube, mixed with two volumes of chloroform and centrifuged at 12,000***g*** for 15 min at 4°C. The aqueous phase, containing the deproteinated plasma, was transferred to a new tube and frozen at –80°C.

Differences in NO concentration between species were inferred by measuring combined breakdown products – nitrate (NO_3^-) plus nitrite (NO_2^-) – according to the Griess method, as described by Grisham and colleagues (Grisham et al., 1995). First, NO_3^- was converted to NO_2^- by incubating 100 µl of deproteinated plasma with 0.1 units of nitrate reductase in 50 mmol l^{-1} HEPES, 5 µmol l^{-1} FAD and 0.1 mmol l^{-1} NADPH, pH 7.4 (final volume 500 µl) at 37°C for 30 min. To oxidize any remaining unreacted NADPH, 7.5 units of LDH and 50 µl of 100 mmol l^{-1} pyruvic acid were added next and incubated for an additional 10 min at 37°C. Finally, 1 ml of Griess reagent (0.1% napthyleneethylenediamine, 1% sulfanilamide and 2.5% phosphoric acid) was added to each sample and then incubated at 25°C for 10 min. The concentration of NO_2^- was determined by spectrophotometric measurement of diazonium salt formation at 543 nm. Samples were run in duplicate and compared with a NO_2^- standard curve to determine the total concentration of NO_x in the samples. Corrections for plasma volumes were performed to account for the addition of sodium citrate to the blood. The absorbance of PHZ-treated *N. coriiceps* plasma samples at 543 nm was measured before the addition of the Griess reagent. This baseline reading then was subtracted from the final reading to correct for the presence of trace PHZ.

Measurement of NOS activity

NOS activity was measured in brain, retina, ventricle and pectoral adductor muscle of control *N. coriiceps* and PHZ-treated *N. coriiceps* by quantifying conversion of [^{14}C]arginine to [^{14}C]citrulline. Crude extracts were prepared by homogenizing tissue in a 5% w/v ice-cold buffer solution (25 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA; pH 7.4) with a ground-glass homogenizer. Homogenates were centrifuged at 12,000***g*** for 5 min at 4°C to remove cellular debris. Supernatants were drawn off, transferred to fresh tubes and kept on ice.

Tissue extracts (10 µl per reaction) were incubated for 3 h at 5°C in reaction medium containing 25 mmol l^{-1} Tris-HCl, 3 µmol l^{-1} tetrahydrobiopterin, 1 µmol l^{-1} FAD, 1 µmol l^{-1} FMN, 10 mmol l^{-1} NADPH, 6 mmol l^{-1} $CaCl_2$ and [^{14}C]arginine (0.05 µCi per reaction) (40 µl final volume; pH 7.4). Reactions were terminated by adding to each tube 400 µl of 50 mmol l^{-1} HEPES stop buffer (pH 5.5) containing 5 mmol l^{-1} EDTA. [^{14}C]citrulline was separated from unreacted [^{14}C]arginine using batch ion-exchange chromatography. Briefly, 100 µl of Dowex 50WX8 resin (Na^+ form) was added to each reaction and mixed thoroughly. Samples then were transferred to 0.45 µmol l^{-1} cellulose acetate Spin-X columns (Costar, Corning Life Sciences, Lowell, MA, USA) and centrifuged at 16,000***g*** for 30 s. Finally, 450 µl of filtrate, containing the [^{14}C]citrulline, was transferred to a vial with 3 ml of scintillation cocktail and quantified by liquid scintillation spectrometry. All reactions were run in triplicate, and parallel controls were performed by adding 5 µl of the competitive NOS inhibitor L-NAME (N-ω-nitro-L-arginine methyl ester hydrochloride; 1 µmol l^{-1}) to the reaction mix. Enzyme activity is reported strictly as L-NAME-inhibitable activity.

Measurement of VEGF, HIF-1α and PHD2 mRNA expression in the retina

Total RNA was extracted from notothenioid retinae using an AllPrep DNA/RNA/Protein mini-kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were analyzed by spectral analysis with a Beckman DU540 spectrophotometer. The RNA then was analyzed using an Agilent 2100 Bioanalyzer to ensure that the samples were not degraded. DNA contamination was removed from RNA samples using Turbo DNA-free (Applied Biosystems/Ambion, Austin, TX, USA). First-strand cDNA was synthesized from total RNA using Superscript III Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) and oligo(dt) primer. DNAse-treated RNA was added to each reaction with a final concentration of 45 ng/µl.

Sequencing of Antarctic notothenioid vascular endothelial growth factor (VEGF), hypoxia inducible factor-1α (HIF-1α) and the prolyl hydroxylase PHD2 has been described by Beers and colleagues (Beers et al., 2010). QPCR primers (Table 1) were designed in regions of the genes conserved among Antarctic notothenioids using Primer3 (Rozen and Skaletsky, 2000). All genes were amplified using SYBR GreenER (Invitrogen Life Technologies; 1 cycle of 5 min at 50°C, 1 cycle of 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C). Amplicon sizes for the genes examined were: 110 bp for VEGF, 216 bp for HIF-1α and 173 bp for PHD2. QPCR reactions had a final volume of 30 µl, with 2 µl of cDNA (diluted 1:10) and primer concentrations ranging from 20 to 40 nmol l^{-1}, depending on the gene. Each sample was run in triplicate. QPCR products were subjected to a melt-curve analysis and sequenced to ensure primer specificity.

Owing to the complex nature of retinal tissue and differences in tissue composition, as demonstrated by the increased vascular endothelial tissue in icefish compared with that of red-blooded species (Wujcik et al., 2007), samples were normalized to total RNA. This was completed by several rounds of careful quantification and dilution until all samples had the same RNA concentration. cDNA synthesis of all samples was done simultaneously with the same master mix to ensure the same efficiency of the reverse transcription reaction between samples. Finally, the same amount of cDNA was added to each QPCR reaction and all samples were run on the same plate for each primer set. A standard curve of linearized plasmid containing the gene of interest spanning five logs was run on each qPCR plate.

Statistical analyses

Comparisons among species for differences in NOS activity were performed in SigmaStat (Version 3.1; Systat Software, Chicago, IL, USA) using a Kruskal–Wallis one-way ANOVA on ranks for all tissue types assayed ($P \leq 0.05$). A one-way ANOVA followed by a

Table 1. QPCR primers

Gene product	Forward primer	Reverse primer
VEGF	5′ CAAGGGAGCGGAGAAGAGTA 3′	5′ TCCGCATACTCCGAGAAGAT 3′
HIF-1α	5′ TCTCTACAACGATGTAATGCTTCC 3′	5′ AATCTGATTTCATCTCCGAGTCC 3′
PHD2	5′ AAACGGGCAAGTTCACAGAC 3′	5′ TCCCAATTTGCCGTTACAAT 3′

Data from Beers et al. (Beers et al., 2010).

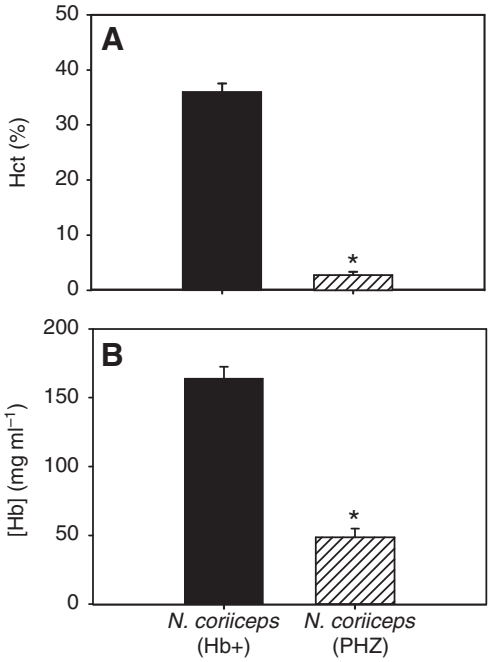

Fig. 1. Effect of phenylhydrazine HCl (PHZ) treatment upon hematocrit (Hct A) and hemoglobin (B) concentration in *N. coriiceps*. *Notothenia coriiceps* were treated for 10 days with the hemolytic agent PHZ (see Materials and methods). Asterisks denote significance between control and PHZ-treated *N. coriiceps* (P≤0.05). Values are means ± s.e.m.; N=8 for both control and treatment groups.

post-hoc Tukey's honest significant difference test was used to determine significant differences in gene expression and plasma NO_x (P≤0.05). The Hb concentration and Hct of treated and untreated *N. coriiceps* were analyzed using a two-sample *t*-test. Hct readings of <1% were conservatively considered 1% for statistical purposes. With the exception of NOS activity data, all statistics were performed in SYSTAT (Version 12; Systat Software, Chicago, IL, USA).

RESULTS

Phenylhydrazine treatment significantly reduces hematocrit and hemoglobin concentration

Blood samples from *N. coriiceps* treated for 10 days with PHZ were analyzed for Hct and Hb concentration. Compared with control animals, the Hct and Hb concentration measurements were significantly lower in PHZ-treated animals (P<0.001; Fig. 1). Hct values of PHZ-treated fish ranged from 4.8% to less than 1%, whereas the control fish had an average Hct of 36.1±1.5%. These numbers represent a greater than 90% decrease in Hct in the PHZ-treated *N. coriiceps*. The Hb concentration also was reduced dramatically in the PHZ-treated fish. Compared with the controls, the Hb content of PHZ-treated animals was reduced by >70%. Residual Hb in plasma of PHZ-treated animals is responsible for the less dramatic change seen in the concentration of Hb compared with the reduction in Hct (Fig. 2). These results demonstrate that we have successfully induced severe anemia in a red-blooded Antarctic notothenioid.

Phenylhydrazine treatment significantly increases the concentration of NO_x in the plasma of *N. coriiceps*

The aggregate concentration of plasma NO_x was measured in control and anemic (PHZ-treated) *N. coriiceps* as a proxy for NO (Fig. 3). The plasma concentration of NO_x in *N. coriiceps* increased by 33%

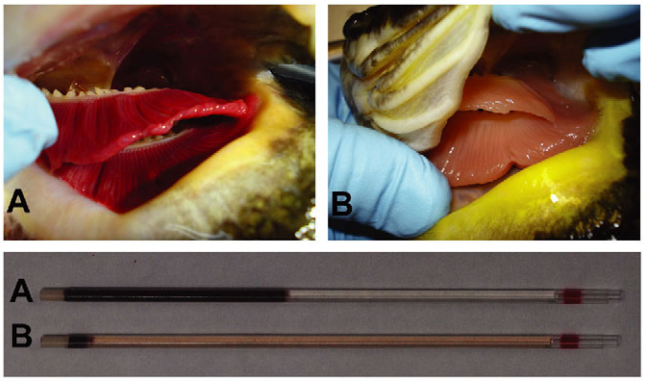

Fig. 2. Reduction in hematocrit of *N. coriiceps* by treatment with phenylhydrazine HCl (PHZ). Photographs of gills and Hct capillary tubes from control *N. coriiceps* (A) and *N. coriiceps* treated with PHZ for 10 days (B). Plasma of PHZ-treated *N. coriiceps* is red-tinted owing to the release of Hb when red blood cells are lysed by PHZ.

when animals were treated with PHZ (P≤0.01). While significantly different from untreated *N. coriiceps*, the concentration of plasma NO_x in anemic *N. coriiceps* is not significantly different from that of *C. aceratus* (Hb–) reported by Beers and colleagues (Beers et al., 2010). Thus, PHZ treatment results in a significant elevation in NO metabolites compared with control animals.

NOS activity in *N. coriiceps* treated with phenylhydrazine

Nitric oxide synthase (NOS) activity was determined in four different tissues of control and PHZ-treated *N. coriiceps* using a radiochemical method to measure the conversion of [^{14}C]arginine to [^{14}C]citrulline (Fig. 4). There was no significant difference in NOS activity in the brain, ventricle and pectoral muscle between control and PHZ-treated animals. Although no significant difference was found among estimates of pectoral muscle NOS activities, a strong trend towards elevation of NOS catalytic potential in PHZ-treated animals was observed (P=0.079). PHZ treatment also results in an approximately 2.5-fold increase in NOS activity in retinae compared with those of control animals (P<0.05). For both treated and

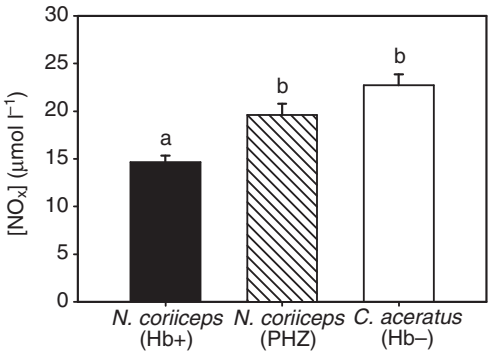

Fig. 3. The plasma concentration of nitrate plus nitrite (NO_x) is increased in *N. coriiceps* treated with phenylhydrazine HCl. *Notothenia coriiceps* (Hb+) is an Antarctic notothenioid species that expresses hemoglobin (Hb), whereas *C. aceratus* is a species that lacks Hb. *Notothenia coriiceps* treated with phenylhydrazine, a hemolytic agent, are labeled 'PHZ'. Values are means ± s.e.m.; N=8 for each species. Letters (a, b) signify that the samples are significantly different from one another at P≤0.05. The *C. aceratus* data, as reported by Beers and colleagues (Beers et al., 2010), are illustrated for comparison.

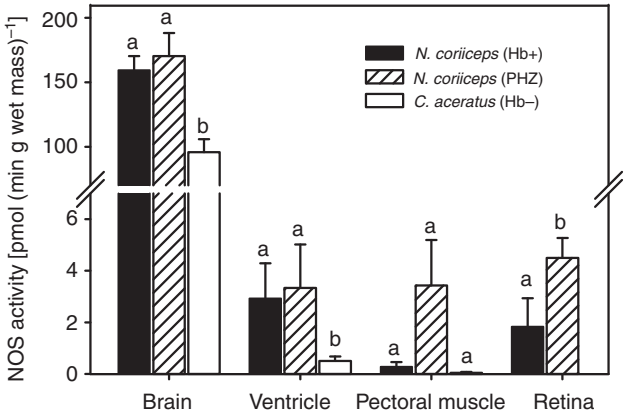

Fig. 4. Nitric oxide synthase (NOS) activity in tissues of control and PHZ-treated *N. coriiceps*. *Notothenia coriiceps* (Hb+) is an Antarctic notothenioid species that expresses hemoglobin (Hb), whereas *C. aceratus* is a species that lacks Hb (Hb–). *Notothenia coriiceps* treated with phenylhydrazine HCl, a hemolytic agent, are labeled 'PHZ'. Values are means ± s.e.m. (N=4 per group). Letters (a, b) signify that the samples are significantly different from one another at P≤0.05 within a tissue type. The *C. aceratus* data, as reported by Beers and colleagues (Beers et al., 2010), are illustrated for comparison. NOS activity was not detected in *C. aceratus* retina.

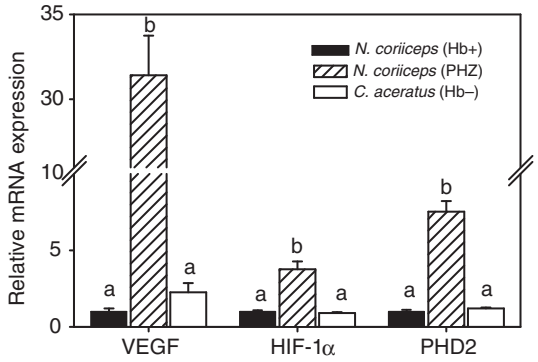

Fig. 5. Treatment with PHZ results in an increase in expression of genes associated with angiogenesis in retina. *Notothenia coriiceps* (Hb+) expresses hemoglobin (Hb), whereas *C. aceratus* lacks Hb (Hb–). *Notothenia coriiceps* treated with phenylhydrazine HCl, a hemolytic agent, are labeled 'PHZ'. Expression was normalized to total RNA and is expressed as relative to *N. coriiceps*. Values are expressed as means ± s.e.m. (N=4 per group). Letters (a, b) signify that the samples are significantly different from one another at P≤0.05 within a gene. The *C. aceratus* data, as reported by Beers and colleagues (Beers et al., 2010), are illustrated for comparison.

untreated *N. coriiceps*, the highest NOS activity was observed in brain tissue [approximately 160 and 170 pmol (min g wet mass)$^{-1}$, respectively]. Brain NOS activity was more than 50-fold higher than activities measured in ventricle, retina and pectoral muscle of control and treated *N. coriiceps*. Brain, retina and ventricle NOS activity of treated and untreated *N. coriiceps* is consistently higher than observed for *C. aceratus* (Hb–) (Beers et al., 2010).

Treatment with PHZ increases mRNA expression of VEGF, PHD2 and HIF-1α

Treatment of *N. coriiceps* with PHZ for 10 days resulted in an increase in the levels of retinal mRNA encoding PHD2, HIF-1α and VEGF (Fig. 5). Messenger RNA levels of PHD2, an oxygen-dependent regulator of HIF-1α, were approximately eight-fold higher in animals treated with PHZ than control animals (P<0.001). Expression of mRNA encoding HIF-1α, part of the HIF-1 transcription factor, was approximately four-fold higher in retinae of anemic than in control animals (P<0.01). VEGF, a growth factor regulated by HIF-1 that stimulates blood vessel growth, showed the largest increase in retinal mRNA levels with PHZ treatment. Retinal VEGF mRNA was approximately 30-fold higher in PHZ-treated animals than in control *N. coriiceps* (P<0.001). Increases in VEGF expression are an indication of ongoing angiogenesis. Retinae of PHZ-treated fish have mRNA levels of VEGF, HIF-1α and PHD2 that are significantly higher than levels measured in both control *N. coriiceps* and *C. aceratus* (Hb–) (Fig. 5). These data demonstrate that PHZ treatment stimulates transcription of VEGF, suggesting a corresponding stimulation of angiogenesis in the retina of *N. coriiceps*.

DISCUSSION

Our experiments were designed to provide insight into how icefishes have developed cardiovascular characteristics that are drastically different from those of red-blooded Antarctic notothenioids. We induced severe anemia in a red-blooded notothenioid to model conditions that icefish might experience early in development. Using this experimental model, we investigated a possible mechanism that could be responsible for some of the alterations to the cardiovascular system of the Antarctic icefish. We show that treatment of red-blooded *N. coriiceps* with PHZ results in increased levels of NO metabolites and elevates transcription of mRNA encoding a crucial factor in the angiogenesis pathway, VEGF.

Phenylhydrazine treatment results in increased levels of nitric oxide metabolites in a red-blooded Antarctic notothenioid

Adult icefish have higher levels of NO metabolites than red-blooded Antarctic notothenioids (Beers et al., 2010). To demonstrate that loss of Hb can induce an increase in markers of NO concentration, red-blooded *N. coriiceps* were treated with PHZ for 10 days. In previous studies that have utilized PHZ to induce anemia in salmonids – one intraperitoneal injection with PHZ (10 or 12.5 μg g^{-1}) was sufficient to decrease dramatically and rapidly the Hct (Smith et al., 1971; Gilmour and Perry, 1996; McClelland et al., 2005). By 1 day post injection (dpi), the Hct was significantly lower than that of control animals and continued to decrease for several days before starting to recover 8–10 dpi. Full recovery took ≥5 weeks, depending on the species. To ensure that the Hct would not recover during the ten day treatment employed in this study, we initially injected the animals with 10 μg PHZ per gram body mass and also surgically implanted into the animal an Alzet osmotic pump containing PHZ. Osmotic pumps are preferable to repeated injections because the animal receives a continual low dose of the chemical without the stress of repeated injections (Theeuwes and Yum, 1976). The fish responded remarkably well to the treatment with PHZ. All animals survived the surgery, injection and ten day treatment. Despite the fact that some animals had a Hct of <1% at the end of the treatment, there was no noticeable change in the health or behavior of the animals. The survival rate of PHZ-treated animals documents that, in a cold and well-oxygenated environment, loss of Hb is nonlethal.

PHZ-treated *N. coriiceps* have higher levels of NO_x than control *N. coriiceps*. In fact, the elevated concentration of NO_x in the plasma of anemic *N. coriiceps* is not significantly different from that of icefish (Hb–). Because the concentration of NO_x is often measured

as a proxy for NO, we infer that the treatment with PHZ results in an increase in NO concentration in red-blooded notothenioids. While our results strongly suggest that the increase in NO_x is due to the loss of Hb, they do not rule out a possible contribution of increased NO production. To examine more closely whether the loss of Hb is solely responsible for the increase in NO_x, we measured the catalytic capacity for NO production in several different tissues from control and PHZ-treated animals.

Does loss of hemoglobin affect the capacity for nitric oxide production?

Nitric oxide is produced by a family of NOS isoforms. At least one isoform of NOS is present across the phylogenetic spectrum of animals from insects to mammals. Mammals express three NOS isoforms. Endothelial (eNOS), inducible (iNOS) and neuronal NOS (nNOS) differ in how they are regulated and the tissues in which they are expressed (Alderton et al., 2001). Less is known about NOS in lower vertebrates. While mammals have three isoforms of NOS, the number of NOS isoforms in lower vertebrates is unresolved. The genomes of zebrafish and pufferfish contain a gene encoding nNOS, and iNOS is present in the zebrafish genome (www.ensembl.org). To date, a sequence encoding eNOS has not been identified in a fish genome. However, several laboratories have reported the presence of eNOS in different fish species based on cross-reactivity with mammalian antibodies (Fritsche et al., 2000; Ebbesson et al., 2005; Garofalo et al., 2009).

We measured NOS activity in untreated and PHZ-treated *N. coriiceps* by measuring conversion of radioactively labeled arginine to citrulline. This method does not discriminate among the different NOS isoforms. PHZ treatment resulted in an increase in NOS activity in retina, but not in brain, pectoral muscle or kidney. Lack of difference in NOS activity between PHZ-treated and control animals in three out of the four tissues measured indicates that NO production is generally not upregulated in anemic fish. These results suggest that the anemia-induced increase in NO_x is primarily due to loss of Hb and not due to a change in the rate of NO production.

Phenylhydrazine-induced anemia triggers an hypoxic response

We have focused on the role of Hb in NO metabolism; however, the primary role of fish Hb is to carry oxygen from the gills and deliver it throughout the body. Loss of Hb in PHZ-treated animals drastically reduces the oxygen-carrying capacity of blood. Decreases in oxygen availability at the cellular level trigger hypoxia-signaling pathways regulated by the transcription factor HIF-1 (Ke and Costa, 2006). Decreased oxygen results in a build-up of HIF-1α protein, which binds to constitutively expressed HIF-1β to form HIF-1. This transcription factor then stimulates expression of genes containing a hypoxia-response element, HRE, in their promoters. Through the HRE, HIF-1 regulates the expression of genes involved in erythropoiesis, angiogenesis, vascular tone, glucose metabolism, and cell survival.

We know that HIF acts as a global regulator of hypoxia-responsive genes in all vertebrates. While it is believed that regulation of HIF-1α by PHD2 is conserved throughout vertebrates, this has not been confirmed (Nikinmaa and Rees, 2005). PHD2 [also known as HPH2, HIF prolyl hydroxylase 2 or EGLN1 (egg laying abnormal nine homolog 1)] regulates the expression of HIF-1α protein in mammals. In the presence of oxygen, PHD2 hydroxylates HIF-1α on two proline residues in the oxygen-dependent degradation domain, targeting the protein for proteasomal degradation (Berra et al., 2003; Berra et al., 2006). Increased expression of PHD2 mRNA during hypoxia primes the system so that, when oxygen becomes available, PHD2 degrades HIF-1α rapidly (Metzen et al., 2005). Genes encoding PHD2 and other prolyl hydroxylases have been located in the genomes of multiple fish species, and the HIF-1α oxygen-dependent degradation domain is conserved in fish and mammals (Soitamo et al., 2001). Other than our own recent results (Beers et al., 2010), we are unaware of any studies that have examined the expression of PHD2 in fish species.

Expression of PHD2 mRNA in retina is not significantly different between adult red- and white-blooded Antarctic notothenioids (Beers et al., 2010). This suggests either that icefish are not hypoxic or that the transcription of the gene encoding PHD2 is not responsive to hypoxia in fish. Increased PHD2 mRNA expression in anemic *N. coriiceps* establishes that PHD2 is hypoxia responsive in Antarctic notothenioids. The demonstration that PHD2 is hypoxia responsive in notothenioids, but not upregulated in adult icefish, indicates that the cardiovascular characteristics of adult icefishes ensure normoxia of tissues despite the absence of Hb. Our results also support the view that regulation of HIF-1α by PHD2 is conserved in vertebrates.

In addition to PHD2, the expression of HIF-1α mRNA also was higher in retinae from PHZ-treated animals compared with untreated *N. coriiceps*. Expression of HIF-1α mRNA often is thought to be unaffected by hypoxia and solely regulated at the protein level by PHD2. However, changes in HIF-1α mRNA expression have been observed in hypoxic fish. HIF-1α mRNA expression in grass carp changes in response to length of hypoxia exposure and the tissue type, and HIF-1α mRNA expression increases in zebrafish embryos exposed to hypoxic conditions (Ton et al., 2003; Law et al., 2006). In the present paper, we have presented another example of hypoxia-induced HIF-1α mRNA expression. Many studies have examined only expression of HIF-1α protein. It is possible that regulation of HIF-1α expression at the mRNA level is more widespread than is currently appreciated.

Hypoxia might induce angiogenesis through a nitric-oxide-mediated pathway

Our data suggest that PHZ treatment of red-blooded *N. coriiceps* results in both low oxygen levels and high NO levels. These conditions might mimic those experienced by developing icefishes. We hypothesize that this unique set of circumstances stimulates remodeling of the icefish cardiovascular system early in development by stimulating angiogenesis. To test whether loss of Hb stimulates angiogenesis, we measured mRNA expression of the angiogenic growth factor VEGF. Our gene-expression studies were confined to retinal tissue because of the documented relationship between retinal vascular densities and the circulating concentration of hemoglobin in notothenioids (Wujcik et al., 2006). We suggest that our results might be representative of a more widely spread pattern in the cardiovascular system of these animals.

Angiogenesis, the growth of new blood vessels from pre-existing blood vessels, is stimulated by hypoxia and/or nitric oxide (Ziche and Morbidelli, 2000; Pugh and Ratcliffe, 2003). During hypoxia, HIF-1α and HIF-1β bind in concert to an HRE in the promoter of the gene encoding VEGF, stimulating transcription. NO also is known to stimulate transcription of VEGF through an increase in HIF-1α protein expression. NO can block the ability of PHD2 to bind to oxygen, inhibiting the enzyme from hydroxylating HIF-1α. This inhibition of PHD2 by NO results in accumulation of HIF-1α protein (Kimura et al., 2000; Kimura et al., 2001; Metzen et al., 2003).

What happens to HIF-1α protein levels, and thus the expression of genes downstream of HIF-1α in the angiogenic pathway (e.g.

the gene encoding VEGF), when oxygen is low and NO is high is a matter of debate. Conflicting evidence for the effect of NO upon PHD2 activity during hypoxia also has been reported. Several studies have indicated that NO increases the activity of PHD2 during hypoxia and that treatment of cultured cells with NO donors inhibits accumulation of HIF-1α (Brune and Zhou, 2003). Hagen and colleagues (Hagen et al., 2003) suggested that NO binds to cytochromes in the electron-transport chain at low oxygen levels, making oxygen available to other oxygen-binding proteins, such as PHD2. Such a mechanism would enable PHD2 to hydroxylate HIF-1α, thus targeting the protein for degradation, even when oxygen is in short supply. This effect of NO upon PHD2 activity might, however, be dose dependent with respect to NO. Hypoxic HIF-1α protein expression in human liver HepG2 cells is diminished by low levels of NO, but higher levels of NO stabilized hypoxic HIF-1α protein (Callapina et al., 2005). High levels of NO during hypoxia inhibit PHD2 activity by blocking the ability of the protein to bind to oxygen. This results in the accumulation of HIF-1α, stimulating the transcription of genes, such as that encoding VEGF, downstream in the angiogenic pathway. We believe this to be the situation observed during the PHZ-induced anemia of *N. coriiceps*.

Our results indicate that PHZ-induced anemia results in elevation of NO metabolites, presumably reflecting increased NO levels. Concomitant with these effects, we observe in retinae an increase in HIF-1α gene expression and an approximately 30-fold increase in expression of mRNA encoding VEGF in PHZ-treated animals compared with untreated fish. We conclude that inhibition of PHD2 in the retina by the presence of NO and by reduced oxygen availability results in accumulation of HIF-1α protein, stimulating transcription of VEGF. This is a possible mechanism that could be activated early in the development of icefish, resulting in the higher density of vasculature present in retinae of adult animals. This scenario also might be extrapolated to the vasculature supplying other aerobically poised tissues.

Loss of Hb triggers endogenous signaling pathways

Since the loss of Hb by their progenitor, the family Channichthyidae has radiated to contain 16 species of fish that have exploited different niches in the Southern Ocean. While there certainly have been changes in the genome of icefishes as the family has evolved, we present a mechanism that might account for the inception of their cardiovascular adaptations and might still contribute to ontogenetic development of these traits in modern species. Here, we have demonstrated that removal of Hb in an adult red-blooded Antarctic notothenioid stimulates transcription in the retina of the gene encoding VEGF, a potent angiogenic growth factor. Severe anemia in the adult fish resulted in high levels of NO_x and presumed hypoxia. The presence of this homeostatic system would have helped ancestral icefishes to compensate immediately for the lower oxygen-carrying capacity of blood due to the loss of Hb expression.

LIST OF SYMBOLS AND ABBREVIATIONS

dpi	days post injection
eNOS	endothelial nitric oxide synthase
Hb	hemoglobin
Hb–	hemoglobinless channichthyid icefishes
Hb+	red-blooded notothenioid fishes
Hct	hematocrit
HIF-1	hypoxia-inducible factor 1
HRE	hypoxia-response element
iNOS	inducible nitric oxide synthase
L-NAME	N^{ω}-nitro-L-arginine methyl ester hydrochloride
Mb	myoglobin
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
NO_x	nitrate plus nitrite
PHD2	prolyl hydroxylase domain containing protein 2
PHZ	phenylhydrazine HCl
QPCR	quantitative real-time PCR
VEGF	vascular endothelial growth factor

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