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Nitric oxide synthase is not expressed, nor up-regulated in response to cold acclimation in liver or muscle of threespine stickleback (*Gasterosteus aculeatus*)

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ABSTRACT

There are three isoforms of the enzyme nitric oxide synthase (NOS) in mammals: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). All three isoforms oxidize arginine to citrulline in a reaction producing nitric oxide (NO), which regulates multiple signaling pathways and physiological functions in mammals. Less is known about NOS in fishes, in which the existence of eNOS is controversial. Nevertheless, multiple adjustments occur during cold acclimation of fishes, several of which are known to be mediated by eNOS and NO in mammals, including mitochondrial biogenesis, vasodilation and angiogenesis. We hypothesized that if NOS was present, and NO stimulated these pathways in fishes, then the activity of NOS would increase during cold acclimation. To test this hypothesis, we measured the activity and mRNA levels of NOS in three tissues (liver, oxidative muscle, glycolytic muscle) known to undergo mitochondrial biogenesis and/or angiogenesis. Measurements were made in the threespine stickleback, *Gasterosteus aculeatus* acclimated to either warm (20 °C) or cold (8 °C) temperature for 9 weeks. Cold-acclimated fish were harvested on days 1–3, and at weeks 1, 4 and 9 at 8 °C, while warm-acclimated fish were harvested on day 0 and after 9 weeks at 20 °C. Transcript levels of NOS were quantified using quantitative real-time PCR, and NOS activity was measured using a radiochemical assay, which detected the rate of catabolism of ^{14}C-labeled arginine. Neither NOS activity nor transcripts were detected in oxidative muscle or glycolytic muscle of warm- or cold-acclimated stickleback, although transcript levels of nNOS and NOS activity were detected in brain. Arginine catabolism was detected in liver of animals held at 10 °C and 20 °C for 9 weeks, but was due to arginase activity, rather than NOS. Consistent with this, NOS transcripts were undetectable in liver. The absence of NOS in liver and muscles of stickleback indicates that signaling molecules other than NO likely mediate physiological changes during cold acclimation in stickleback.

1. Introduction

Nitric oxide (NO) is produced by three isoforms of the enzyme nitric oxide synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) [reviewed in 1]. Although originally named for the tissues and cells in which they were discovered, it is now recognized that all three isoforms are expressed in a variety of cell types in mammals [reviewed in 1]. All three isoforms of NOS have been detected in fish, and in a variety of tissues, including brain, liver, heart and muscle [2–11], although the presence of eNOS in fish is controversial. Endothelial NOS was detected in hearts of several fish species, including European eel (*Anguilla anguilla*), African lungfish (*Protopterus dolloi*), Atlantic bluefin tuna (*Thunnus thynnus thynnus)* and the Antarctic teleosts *Chionodraco hamatus*, *Chaenocephalus aceratus* and *Trematomus bernacchii*, but was identified using mammalian-derived antibodies [3,5,6]. To date, genomic evidence for the existence of eNOS in fishes is lacking. A recent phylogenetic analysis of NOS in metazoans suggests that the first putative eNOS gene in vertebrates appeared in African clawed frogs (*Xenopus tropicalis*) and chicken (*Gallus gallus*), subsequent to the divergence of teleosts from tetrapods [12]. Nonetheless, several aspects of fish physiology, including cardiac stroke volume, cardiac power output, angiogenesis and vasodilation, which are normally mediated by eNOS in mammals, seem to be regulated by NO in fishes [3,5,13–15], although the source of NO is unknown.

In many species of fish, cold acclimation or acclimatization triggers mitochondrial biogenesis and angiogenesis to offset the

Abbreviations: 18s rRNA, 18S ribosomal RNA; ADC, arginine decarboxylase; AGAT, arginine:glycine amidinotransferase; ANOVA, analysis of variance; ARG1, arginase 1; ARG2, arginase 2; cDNA, complementary DNA; EF-1α, elongation factor-1alpha; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NAME, L-N$^{\omega}$-nitroarginine methyl ester; L-NMMA, N$^{\omega}$-mono methyl-L-arginine; mRNA, messenger RNA; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; nor-NOHA, N$^{\omega}$-hydroxy-nor-arginine; qRT-PCR, quantitative real-time PCR; SIN-1, 3-morpholino-sydnonimine; SNP, sodium nitropriusside.

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depressive effects of cold temperature on enzymatic reactions and oxygen diffusion [16–21]. Notably, both mitochondrial biogenesis and angiogenesis are stimulated by NO in mammals in an eNOS-dependent fashion [22–27]. Although these processes have been well-characterized in several fishes, including striped bass (*Morone saxatilis*) [17], crucian carp (*Carassius carassius)* [16,19], rainbow trout (*Oncorhynchus mykiss*) [21] and threespine stickleback (*Gasterosteus aculeatus*) [18], the molecular pathways governing cold-induced mitochondrial biogenesis and angiogenesis are not well understood. We hypothesized that if these processes were induced by NO in fishes during cold acclimation, then NOS mRNA levels and activity would be higher in cold-acclimated animals compared to warm-acclimated ones.

We tested this hypothesis using the threespine stickleback (*G. aculeatus)*. Stickleback were acclimated to either warm (20 °C) or cold (8 °C) temperature for 9 weeks and harvested throughout the acclimation period. Transcript levels of NOS and activity of NOS were quantified in liver, glycolytic muscle and oxidative pectoral adductor muscle harvested from animals throughout the acclimation period.

2. Material and methods

2.1 Animals

Threespine stickleback (*G. aculeatus*) were captured in Kashwitna Lake, Alaska (61°50′N, 150°00′W) in September 2007, using minnow traps. Animals were maintained in 3.5‰ seawater at 20 °C on a 10 h:14 h light:dark cycle, and fed twice daily *ad libitum* an alternating diet of brine shrimp and blood worms. Fish were maintained under these conditions for 12 weeks prior to acclimation to cold temperature. Acclimation temperatures reflect temperatures experienced by animals in their natural habit: water temperatures range between 4 °C and 20 °C throughout the year in lakes of central Alaska such as Kashwitna Lake. Fish were acclimated to cold temperature by decreasing the water temperature 5 °C per day for two consecutive days, and then by 2 °C on the third day to a final temperature of 8 °C. Cold-acclimated fish were maintained at this temperature for 9 weeks, and warm-acclimated fish were maintained at 20 °C for the duration of the cold acclimation period (9 weeks + 3 days). Fish were harvested prior to cold acclimation (20 °C), at day 1 (15 °C), day 2 (10 °C), day 3 (8 °C), week 1 (8 °C week 1), week 4 (8 °C week 4) and week 9 (8 °C week 9) of cold acclimation, and after 9 weeks + 3 days at 20 °C (20 °C week 9) (see [18] for detailed sampling scheme). Fish were killed by immersion in liquid nitrogen and stored at −80 °C. All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care Committee (135490-2).

2.2 Maximal activity of NOS (EC 1.14.13.39)

The activity of NOS was measured in brain, liver, glycolytic muscle and oxidative pectoral adductor muscle as described previously [28]. Tissues were homogenized in 10 volumes (w/v) ice-cold 25 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, pH 7.4. Tissue homogenates were pre-incubated with 5 mM of the NOS inhibitor L-N^{ω}-nitroarginine methyl ester (L-NAME) in 10 mM Tris-HCl pH 7.4 for 5 min prior to initiating the reaction by adding four volumes of reaction buffer (31.25 mM Tris–HCl pH 7.4, 3.75 μM tetrahydrobiopterin, 1.25 μM FAD and 1.25 μmol FMN, 75 μM $CaCl_2$, 1.25 mM NADPH) containing 0.05 μCi ^{14}C-L-arginine (Amersham, Buckinghamshire, UK). L-NAME was omitted when measuring total activity. The reaction was stopped after 30 min by adding eight volumes of 50 mM HEPES, 5 mM EDTA, pH 5.5. ^{14}C-L-arginine was separated from the reaction mixture by centrifuging homogenates for 30 s at 16,100*g* through Dowex 50Wx8 resin (pH 7.0–8.0) (Sigma Aldrich, MO, USA), packed into spin columns (Bio-Rad, CA, USA). Eluates were diluted in 10 mL Econo-safe scintillation fluid (Atlantic Nuclear, MA, USA) and ^{14}C activity was measured using a LS 6500 multi-purpose scintillation counter (Beckman Coulter, CA, USA). Background activity was determined in samples boiled for 5 min prior to incubating with ^{14}C-L-arginine. All measurements were made in triplicate at 21 °C in four to six animals harvested at each time point during warm and cold acclimation except boiled samples, in which background activity was measured once. ^{14}C activity of the untreated or the treated tissue homogenates, which will be referred to as ^{14}C-arginine catabolism (pmol min^{-1} g wet $tissue^{-1}$) was calculated using the following equation:

$$^{14}\text{C activity} = c^{*}\frac{\text{dpm tissue /vol tissue (ml)/time (min)}}{\text{Specific activity of } ^{14}\text{C-L-arginine (dpm pmol}^{-1})} \quad (1)$$

where *c* represents the concentration of the tissue homogenate and dpm the decay per minute counted in either the untreated or treated tissue homogenate. Specific activity of ^{14}C-L-arginine was 860.5 dpm $pmol^{-1}$.

NOS activity was defined as the difference in ^{14}C activity between untreated tissue homogenates and those treated with L-NAME.

2.3 Activity of arginase (EC 3.5.3.1)

The activity of arginase was quantified as described above, except that frozen liver homogenates were used and arginase activity was determined as the amount of activity inhibited by the arginase inhibitor N^{ω}-hydroxy-nor-arginine (nor-NOHA). Tissue homogenates were incubated with 20 μM nor-NOHA for 10 min prior to adding reaction buffer containing ^{14}C-L-arginine. Nor-NOHA was omitted for determining total activity. Measurements were made in triplicate in three to six animals harvested at 20 °C, 10 °C and after 9 weeks of warm or cold acclimation except in boiled samples, which were measured once. Arginase activity (pmol min^{-1} g wet $tissue^{-1}$) was calculated using Eq. (1).

We also quantified arginase activity using an arginase-specific enzyme assay to ensure that maximal rates of enzyme activity were measured [29]. Measuring maximal activity of arginase requires manganese to fully activate the enzyme and a higher pH than conditions used in the NOS assay [29]. Briefly, tissue homogenates were centrifuged for 10 min at 12,000*g* at 4 °C and protein concentration of the supernatant was determined using the Lowry assay [30]. 3.2 μg of protein was preincubated for 10 min at 14 °C with 5 mM $MnCl_2$ in 50 mM Tris-HCl, pH 7.5. The reaction was initiated by adding 250 mM arginine, pH 9.8. Arginine was replaced with 50 mM Tris-HCl, pH 7.5 to determine background activity. Reactions were stopped after 20 min by adding eight volumes of H_2SO_4:H_3PO_4:H_2O (1:3:7). A final concentration of 0.5% 1-phenyl-1,2-propanedione-2-oxime was added and the reaction mixture was incubated at 100 °C for 45 min. The reaction mixture was allowed to cool for 10 min in the dark and then absorbance was measured at 540 nm. Measurements were made at 14 °C in triplicate in four to six animals harvested throughout the acclimation period. Background activity was measured once in each individual. Arginase activity was quantified as the rate of production of urea (μmol urea min^{-1} mg $protein^{-1}$). The amount of urea in the reaction mixture was determined using a standard curve containing between 0 μg and 10 μg urea.