Role of NO in arterial vascular function of intertidal fish (*Girella laevifrons*) and marine fish (*Isacia conceptionis*)

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Abstract

Previous studies performed in intertidal fish (*Girella laevifrons*),as well as marine fish (*Isacia conceptionis*), showed that acetylcholine (ACh) produced contractions mediated by cyclooxygenases that were dependent on the area and potency of contraction in several arterial vessels. Given that the role of nitric oxide is poorly understood in fish, the objective of our study was to evaluate the role of nitric oxide in branchial afferent (ABA), branchial efferent (ABE), dorsal (DA) and mesenteric (MA) arterial vessels from both *Girella laevifrons* and *Isacia conceptionis*. We studied afferent and efferent branchial, dorsal and mesenteric arteries that were dissected from 6 juvenile specimens. Isometric tension studies were done using dose response curves (DRC) for Ach (10^{-13} to 10^{-3} M) and blockade with L-NAME (10^{-5} M), and DRC for sodium nitroprusside (SNP, a donor of NO). L-NAME produced an attenuation of the contractile response in the dorsal, afferent and efferent branchial arteries and a potentiation of the contraction in the MA. SNP caused 70% dilation in the mesenteric artery and 40% in the dorsal artery. Our results suggest that Ach promotes precarious dilatation in MA mediated by NO; data that is supported by the use of sodium nitroprusside. In contrast, in the vessels DA, ABA and EBA our results support that the pathway Ach-NO-relaxation is absent in both species.

Keywords: intertidal fish, marine fish, vascular reactivity, NO-pathway.

Papel do NO na função vascular arterial de peixes entremarés (*Girella laevifrons*) e peixes marinhos (*Isacia conceptionis*)

Resumo

Estudos anteriores, realizados no peixe intertidal (*Girellalaevifrons*) no peixe marinho (*Isacia conceptionis*), mostram que a acetilcolina (Ach) provoca contrações mediadas por ciclooxigenases que eram dependentes da área e potencia da contração em vários vasos arteriais. Tendo em conta que o papel do óxido nítrico é mal compreendido em peixes, o objetivo do nosso estudo foi avaliar o papel do óxido nítrico em vasos arteriais de ambos os peixes *Girella laevifrons* e *Isacia conceptionis*. Nós estudamos os vasos aferente, branquial (ABA), eferente branquial (ABE), dorsal (DA) e mesentérica (MA), que foram dissecadas de seis espécimes juvenis. Estudos de tensão isométrica foram realizados utilizando as curvas de dose-resposta (DRC) para Ach (10^{-13} a 10^{-3}M) e bloqueio com L-NAME (10^{-5} M), e na DRC para o nitroprussiato de sódio (SNP, doador do NO). L- NAME produziu uma atenuação da resposta contrátil nas artérias dorsais, aferentes e eferentes branquial e uma potenciação da contração no MA. SNP causaram 70% da dilatação da artéria mesentérica e 40% na artéria dorsal. Nossos resultados sugerem que Ach promove dilatação precária em MA mediada por NO; dados que é suportada pela utlilização de nitroprussiato de sódio. Em contraste, nos vasos de DA, ABA e EBA nossos resultados suportam que a via de Ach-NO-relaxamento está ausente em ambas as espécies.

Palavras-chave: peixe entremarés, peixe marinho, reatividade vascular, NO-pathway.

1. Introduction

Physiological barriers are important determinants for the distribution limits of species and populations. However, processes associated to environmental tolerance, differential habitat use, or species distribution patterns remain poorly understood. Latitude, altitude and depth all constitute gradients that can generate physiological diversity. Indeed, intertidal rock pools are characterized by their discrete nature in time and space, as well as by

500

Braz. J. Biol., 2016, vol. 76, no. 2, pp. 500-505

their daily and seasonal variability in temperature, salinity and oxygen concentration during low tide (Ojeda et al., 2000). In line with this, studies carried out in our laboratory and performed using several vessels (dorsal, mesenteric, afferent and efferent branchial arteries) in *G. laevifrons* support the presence of two muscarinic receptors of high and low sensitivity to acetylcholine and a coupled mechanism between muscarinic receptors and vasoconstriction mediated by cyclooxygenase (Moraga and Urriola-Urriola, 2014). In contrast, *Isacia conceptionis* is a fish that lives in the open coast and at a depth up to 50 m in rocky and sandy bottom environments. The habitat of this fish species is stable suggesting that they have less stress compared with pool fish. We studied the same vessels in *I. conceptionis* that were studied in *G. laevifrons* and observed that the acetylcholine response displayed a pattern of high sensitivity only in the afferent branchial artery and low sensitivity in the dorsal, mesenteric, and efferent branchial arteries (Moraga and Urriola-Urriola, 2015). Furthermore, similar to that described for *G. laevifrons*, the vasoconstriction induced by Ach was abolished with indomethacine (an inhibitor of COX). Higher vasoconstriction to acetylcholine was observed in ABA and EBA in *I. conceptionis* in comparison with *G. laevifrons*, and a minor effect of blockade with indomethacine in the maximum response to acetylcholine was observed in ABA and DA vessels in *I. conceptionis* as compared to *G. laevifrons*. In general, the results obtained in both of these species are partially in agreement with previous studies performed in species of great phylogenetic distance (agnathes, elasmobranches, teleost), that showed that acetylcholine induced contraction (Small et al., 1990; Olson and Villa, 1991; Miller and Vanhoutte, 1992; Evans and Gunderson, 1998a). However, the Ach-mediated vasoconstriction described in our study suggests that the coupled Ach-COX contraction produced when there is a decreased availability of nitric oxide (NO) is similar to that reported in human hypertension or hypertension models in rats (Vanhoutte et al., 2005; Shi et al., 2008). In mammals, NO is one of the most important regulators of vascular tone and consequently of global blood pressure. In general, NO is generated by nitric oxide synthase (NOS) located in the vascular endothelium, nitrinergic nerves and macrophages in endothelium (Furchgott and Zawadzki, 1980; Griffith et al., 1984; Toda and Okamura, 2003; Donald and Broughton, 2005), and its production is coupled with stimulation by the Ach-NOS-NO-relaxation pathway. In contrast, several studies have described that this pathway is uncoupled in elasmobranches and teleost fishes (Small et al., 1990; Olson and Villa, 1991; Miller and Vanhoutte, 1992; Evans and Gunderson, 1998b). Our objective was to evaluate the role of the acetylcholine-NO pathway by blockade with L-NAME, and the dilator role of NO using sodium nitroprusside (NO donor) in the arterial vessel of dorsal, mesenteric, branchial afferent and branchial efferent arteries from the intertidal fish *G. laevifrons* and marine fish *I. conceptionis*.

2. Material and Methods

2.1. Animals

Six juvenile *Girella laevifrons* and six juvenile *Isacia conceptionis* were extracted during low tide in the Totoralillo bay (30º 17' S, 71º 31' W) south of Coquimbo, Chile. All specimens were carried to our laboratory in the Universidad Católica del Norte and maintained for 3-5 days in filtered recirculation containers of fresh marine water at 15 °C. Prior to experimentation, *G. laevifrons* or *I. conceptionis* were anaesthetized with benzocaine (1:1000) and sacrificed by decapitation. Afterward, corporal mass (115±12 and 140±8 g) and longitude oral-tail (18±0.6 and 21±0.8 cm) were measured for each specimen, respectively. All animal care, maintenance, procedures, and experimentation were reviewed and approved by the Faculty of Medicine Ethics Committee of the Universidad Católica del Norte.

2.2. Bath organ physiology

After decapitation, arterial vessels were carefully dissected from the following areas: branchial afferent (ABA), branchial efferent (ABE), dorsal (DA) and mesenteric (MA) and placed in cold (4 °C) physiological saline solution (PSS). Individual arterial ring segments having 2 mm length were mounted in a four channel small vessel wire myograph (model 510M Danish Myotech, Aarus, Denmark). The vessels were threaded onto two tungsten wires of 40 μm in diameter and attached to a force transducer and a micrometer for isometric measurements. All signals were acquired by a system acquisition (Powerlab 8sp, ADInstrument, Australia) and the data collected on a personal computer for further analysis. After mounting the rings, the arterial segments were incubated in PSS at 15°C and gassed with air for 30 min. Each vessel segment was stretched to its optimal diameter, i.e. the diameter at which it developed a contraction response to PSS-K+, using a diameter–tension protocol as previously described for mammalian small arteries (Stassen et al., 1997). In this way, the myograph permitted direct measurement of vessel wall tension while the internal diameter was controlled.

Following an equilibration period of at least 30 min, doses response curves (DRC) were performed for KCl (5.6-125 mM) and the cholinergic agonist acetylcholine (Ach) at concentrations ranging from 10^{-13} to 10^{-3} mol/L. Afterward, DRC for Ach were performed in vessels pre-incubated for 30 min with N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-5} M), a nitric oxide synthase inhibitor. Furthermore, DRC for sodium nitroprusside (a donor of NO) were obtained at concentrations ranging from 10^{-13} to 10^{-3} mol/L in arteries previously contracted with potassium chloride at 60 mM (maximum potassium contraction). The curve is expressed as dilation of maximum contraction to potassium and represents maximum dilation (Dmax). Between experiments, the arterial preparations were allowed to recover for at least 30-60 min to return to resting basal tension.

The PSS contained (in g/L): NaCl 7.37, KCl 0.31, KH_2PO_4 0.46, Na_2HPO_4 2.02, $MgSO_4$ 0.14, $CaCl_2$ 0.1, glucose 0.9 with pH adjusted to 7.8 (Olson and Villa,

Braz. J. Biol., 2016, vol. 76, no. 2, pp. 500-505

501

1991). For the PSS-K$^+$ solution (125 mM KCl), NaCl was replaced by an equimolar amount of KCl. All chemicals were reagent grade and purchased from Sigma Chemical (St Louis, MO, USA). The following drugs used in the study: acetylcholine (Ach), N^G-nitro-L-arginine methyl ester (L-NAME), and sodium nitroprusside (SNP) were purchased from Sigma Chemical (St Louis, MO, USA).

2.3. Data analysis and statistics

DRC were analyzed in terms of maximal response (Rmax), sensitivity (EC$_{50}$ or pEC$_{50}$) and maximum dilation (Dmax) to different contractile agents by fitting the individual data with a nonlinear sigmoid regression curve (Prism 4.0, Graphpad, San Diego, CA, USA). Rmax was expressed as (N/m) and Dmax was expressed as (%K$^+$max). Sensitivity was expressed as EC$_{50}$ (the concentration of agonist at which 50% of Rmax was obtained) or as pEC$_{50}$ (-logEC$_{50}$).

In order to evaluate the role of the Ach-NOS-NO-relaxation pathway, data were expressed as ratio response (RR) of Rmax Ach plus LNAME divided by Rmax of Ach, the evaluation of the ratio response (RR) should give values of RR>1 if there is an increase in wall tension due to decreased production of NO mediated by Ach, and values of RR=1 Ach-NOS-NO-relaxation is uncoupled and RR<1, Ach promote dilation don't mediated by NO.

All results were expressed as mean ± SEM. A two way ANOVA for repeated measurements was used for statistical analysis of physiological variables. Differences were considered significant when $p<0.05$ (Primer of Biostatistical v 3.0, Mc Graw Hill).

3. Results

The calculated internal diameter for all arteries is given in Table 1. The data show that not differences were observed in the optimal diameter determined for each of the arterial segments from the different territories studied in both species.

3.1. Response to potassium chloride (KCl)

Table 2 provides a summary for Rmax parameters obtained from analysis of the arterial response curves in both species. These data reveal that Rmax was significantly greater in the ABA and ABE in *I. conceptions* as compared to *G. laevifrons*. No differences were observed between DA and MA in both species. In addition, no differences were observed in the EC$_{50}$ in any of the arteries evaluated in both species.

3.2. Response blockade with L-NAME to acetylcholine

In Figure 1 we can observe an increase in the contractile response to Ach, only in MA in comparison with DA, ABA and EBA. On the other hand, in DA was observed such as maintenance. However, a dilation response was observed in

ABA and EBA to Ach. On other hand, significant differences were observed between species in ABA, EBA and MA, but not in DA. Table 2 summarizes the Rmax values obtained from analysis of the arterial curves. No differences were observed in the pEC$_{50}$ in any of the arteries evaluated.

### 3.3. Response to sodium nitroprusside (a NO donor)

In order to evaluate the role of NO, arteries were pre-contracted by Ach, and DRC for SNP (10^{-13}–10^{-10}M) were evaluated. Figure 2 reveals that the mesenteric artery exhibited dilation close to 70%, whereas the dorsal artery only had dilation close to 40%. No effect in dilation was found in ABA and ABE in the presence of SNP. Table 2 summarizes the pEC$_{50}$ and Rmax values obtained from analysis of the arterial curves. No differences were observed in the pEC$_{50}$ in any of the arteries evaluated.

4. Discussion

Our study compared vascular function of two fish with completely different habitats: *G. laevifrons* that lives and develops in an environment with extreme variation (intertide), and *I. conceptionis* that lives in an environment with little variation (open coast). Previously, we demonstrated that both species possess a vasoconstrictor mechanism mediated by acetylcholine supporting the presence of two muscarinic receptors: one having high sensitivity and a second one of lower sensitivity coupled to different mechanisms of activation (Moraga and Urriola-Urriola, 2014, 2015).

4.1. Role of L-NAME in the Ach-mediated contraction

Our results show that all vessels studied (ABA, EBA, MA and DA) exhibited a similar pattern of vasoconstriction mediated by Ach as previously described by Moraga and Urriola-Urriola (2014, 2015), in the same species. In addition,
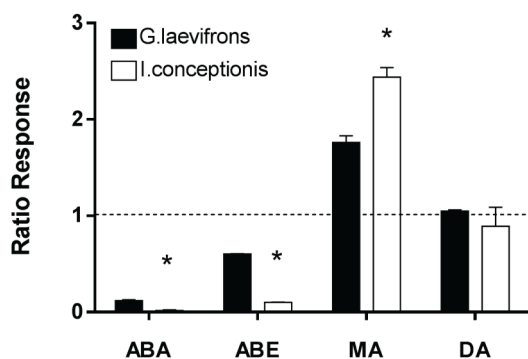
Figure 1. Ratio response in *G.laevifrons* (black) and *I.conceptionis* (open) from afferent branchial artery (ABA); efferent branchial artery (EBA); mesenteric artery (MA) and dorsal artery (DA). Each symbol represents the mean ± s.e.m. *p<0.05 *G.laevifrons vs I. conceptionis*.

Table 1. Optimal diameter (μm) in arterial vessel of marine fish.

|	ABA	EBA	MA	DA
G. laevifrons (6)	475 ± 103	413 ± 54	366 ± 58	488 ± 108
I. conceptionis (6)	425 ± 83	430 ± 44	408 ± 98	406 ± 88

Mean ± s.e.m. and number (n).

Table 2. Dose response curves to acetylcholine and L-NAME in several vascular vessels.

		ABA	EBA	MA	DA
CCR-K					
G. laevifrons.	EC_{50}(mM)	21.5±8.5	25.3±5.7	27.3±5.3	22.3±5.3
	Rmax (N/m)	2.4±0.4	1.8±0.4	1.5±0.3	2.2±0.2
I. conceptionis	EC_{50}(mM)	15,8±3.6	16.7±3.2*	19.0±3.2*	17.7±2.7
	Rmax (N/m)	7.2±0.6*¶	3.1±0.9 *	1.3±0.2	2.7±0.4
CCR-Ach					
G. laevifrons.	Rmax (N/m)	0.84±0.02	0.68±0.06	1.47±0.09†	3.87±0.16‡
I. conceptionis	Rmax (N/m)	5.98±0.38*¶	3.93±0.21*	1.46±0.02†	3.93±0.2
CCR- L-NAME					
G. laevifrons.	Rmax (N/m)	0.21±0.03	0.41±0.03	2.59±0.17†	4.06±0.22‡
I. conceptionis	Rmax (N/m)	0.16±0.05	0.40±0.04	3.56±0.5*	3.50±0.8§
CCR-SNP					
G. laevifrons.	pEC_{50} (mM)	9.34±1.20††	10.26±1.91††	7.20±0.39	7.30±0.35
	Dmax (%K⁺max)	2.0±0.88	1.0±0.89	70.0±5.4†	40.0±2.90§
I. conceptionis	pEC_{50} (mM)	8.94±1.20††	9.86±1.00††	6.58±0.59	7.31±0.83
	Dmax (%K⁺max)	12.0±4.8*	25.0±4.1*	61.0±0.7†	42.3±3.0§

Mean± s.e.m. * *G. laevifrons vs I. conceptionis*. ¶ ABA vs DA, EBA and MA. † MA vs DA, ABA and EBA. ‡ DA vs ABA, EBA and MA. § DA vs ABA and EBA. †† EBA vs DA, ABA and MA (P<0.05).
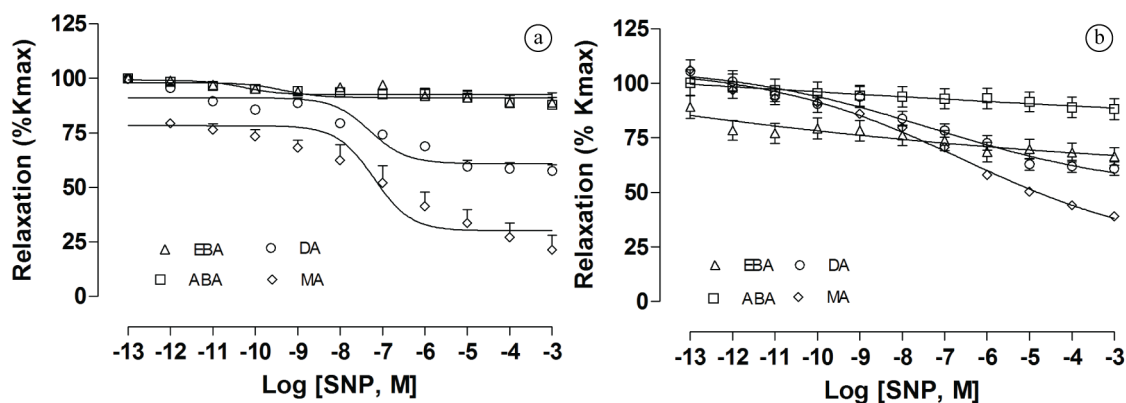
Figure 2. Sodium nitroprusside concentration-response curves in: (a) *G.laevifrons* (black) and (b) *I.conceptionis* (open) of isolated rings of arteries from afferent branchial artery (ABA, squares); efferent branchial artery (EBA, triangles); mesenteric artery (MA, rhombus) and dorsal artery (DA, circles) each symbol represents the mean ± s.e.m.

the same authors describe that this vasoconstriction was abolished by the use of atropine (10^{-5} M). This evidence has been corroborated by other studies in trout, shark and eel (Nilsson and Grove, 1974; Pellegrino et al., 2003; Pellegrino et al., 2002; Small et al., 1990), supporting the presence of muscarinic receptors in fish vasculature.

In mammalian vascular endothelium, several extracellular signals can stimulate NOS, increasing the production of NO, including Ach, shear stress, etc. (Furchgott and Zawadzki, 1980). The nitric oxide produced by endothelium rapidly diffuses into the smooth muscle cell where it activates a soluble guanylyl cyclase (sGC), which generates cyclic guanosine monophosphate (cGMP) that mediates vasodilation (Mondaca et al., 1991). A traditional approach to evaluate coupled Ach-dilation is by the use of blocking agents that inhibit NOS, decreasing NO production, such as L-NAME or others (Buxton et al., 1993). The expected effect of blocking NO production would be an increase in the basal tension, or an increase in the Ach-mediated tension. Therefore, evaluation of the ratio response (RR) should give values of RR >1 if there is an increase in wall tension due to decreased production of NO mediated by Ach, and values of RR=1 or RR<1 if Ach-NOS-NO-relaxation is uncoupled. Indeed, studies show that there is an absence of muscarinic receptors coupled to the NOS-NO dilation pathway in fish (Donald and Broughton, 2005). In contrast, our results revealed a different effect when blocking with L-NAME in the arterial vessel. For example, in ABA and ABE there was a reduction in the Ach-mediated vasoconstriction suggesting

activation of other mediators that promote vasodilation, such as prostacyclin, H_2S or CO (Olson and Villa, 1991; Miller and Vanhoutte, 1986; Evans and Gunderson, 1998b; Feng et al., 2007; Dombkowski et al., 2008; Jennings and Donald., 2010). On the other hand, DA did not modify arterial tension in the presence or absence of L-NAME, supporting the lack of Ach-NOS-NO-relaxation coupling. In MA, we described an increase in Ach-mediated vasoconstriction indicating the presence of a coupled Ach-NOS-NO dilation. This evidence suggests that there is Ach-NOS-NO-relaxation coupling in MA in both species, such as that shown in *Danio rerio* in the same vascular area (Fritsche et al., 2000; Holmberg et al., 2006).

4.2. Role of SNP in vascular dilation

In order to corroborate the role of NO, we studied the effect of SNP, a NO donor, in all the vessels. We found that ABA and ABE in *G. laevifrons* were insensitive to stimulation with SNP, and a poor response in the same vessel was observed in *I. conceptionis*. The lack or poor response to SNP in these areas suggests a different mechanism of activation for vessel dilation, independent of NO. This lack of dilation in the fish vessels studied was also described in the isolated ventral aorta of *O. mykiss* (Miller and Vanhoutte, 1992). In addition, an eel contraction response in the presence of SNP was described by Pellegrino et al. (2002) suggesting that NO could be mediated via reaction with superoxide ions to produce the very reactive peroxynitrite that promotes vasoconstriction (Beckman and Koppenol, 1996) by a reduction in the bioavailability of NO and a stimulation of COX that promotes the production of vasoconstrictors. In contrast, our results show dilatators response of 70%in *Girella laevifrons* and 60% were observed in *Isacia conceptionis*, in MA, and dilation close to 40% in DA in both species. This evidence supports a coupled mechanism of dilation in these vessels mediated by the NO-GMPc-relaxation pathway. Furthermore, our results are in agreement with the presence of SNP-mediated dilation described in arteries of the trout (*O. mykiss*): coronary, celiacomesenteric and efferent branchial (Olson and Villa, 1991; Small et al. 1990; Olson et al., 1991, Olson et al., 1997).

In conclusion, our results suggest differences in the vascular function between *I. conceptions* and. *G. laevifrons*, could be explained by ontogenic, habitat and/or phylogenetic components Furthermore, our results suggest that the MA expresses a vascular response when the Ach-NOS-NO relaxation pathway is coupled, whereas the results in DA, ABA and EBA suggest the presence of another mechanism. Future studies are needed to further evaluate the vascular response in these species.

Acknowledgements

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