Comparative Biochemistry and Physiology, Part A 204 (2017) 1–8

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Cortisol regulates nitric oxide synthase in freshwater and seawater acclimated rainbow trout, *Oncorhynchus mykiss*

Lucie Gerber *, Steffen S. Madsen, Frank B. Jensen

Department of Biology, University of Southern Denmark, DK-5230 Odense M, Denmark

ARTICLE INFO

Article history:
Received 7 July 2016
Received in revised form 2 November 2016
Accepted 7 November 2016
Available online 10 November 2016

Keywords:
Cortisol
Nitric oxide synthase
Osmoregulation
Rainbow trout

ABSTRACT

Cortisol and nitric oxide (NO) are regulators of ion transport and metabolic functions in fish. In the gill, they show opposite effects on Na^+/K^+-ATPase (NKA) activity: cortisol stimulates NKA activity while NO inhibits NKA activity. We hypothesized that cortisol may impact NO production in osmoregulatory tissues by regulating NO synthase (NOS) expression. We evaluated the influence of cortisol treatment on mRNA expression of *Nos1* and *Nos2* in gill, kidney and middle intestine of both freshwater (FW) and seawater (SW) acclimated rainbow trout and found both tissue- and salinity-dependent effects. *Nos2* expression was down-regulated in the gill by cortisol injection in both FW and SW trout. This was substantiated by incubating gill tissue with cortisol *ex vivo*. Similarly, cortisol injection significantly down-regulated *Nos2* expression in kidney of SW fish but not in FW fish. In the middle intestine, *Nos2* expression was up-regulated by cortisol injection in FW but unchanged in SW fish. *Nos1* expression was up-regulated by cortisol injection in FW kidney and down-regulated in SW kidney, whereas it was unaffected in gill and middle intestine of FW and SW fish. Our data provide the first evidence that cortisol may influence NO production in fish by regulating *Nos* expression. Indeed, the down-regulation of *Nos2* expression by cortisol in the gill may prevent the inhibitory effect of NO on NKA activity thereby furthering the stimulatory effect of cortisol on ion-transport.

1. Introduction

Cortisol is the major corticosteroid in fish and has a wide range of actions including ionoregulatory and metabolic functions (Mommsen et al., 1999). Interestingly, the gasotransmitter nitric oxide (NO) is also known to affect such processes (Cooper and Giulivi, 2007; Fago and Jensen, 2015; Perry et al., 2016) and it seems appropriate to expect that the two regulatory pathways may interact locally in various tissues. Whereas cortisol is a relatively slow-acting hormone, typically involved in transcriptional changes on a relatively long time scale (hours-days), NO is a gasotransmitter synthetized in various tissues that operates in seconds-minutes, characteristic of a paracrine/autocrine regulator. Even though they work on different time scales, the potential interaction between the two mediators remains to be elucidated. In salmonids, both *in vivo* and *ex vivo* studies have documented that cortisol mediates long-term osmoregulatory adjustments by stimulating branchial, renal and intestinal Na^+/K^+-ATPase (NKA) activity (Kiilerich et al., 2007, 2011b; Madsen, 1990; McCormick, 2001; McCormick et al., 1991, 2008; McCormick and Bern, 1989; Shrimpton and McCormick, 1999; Veillette and Young, 2005). Yet the recent discovery of NO as a rapid inhibitory modulator of ion transport and of NKA activity in fish challenges our understanding of how these regulatory processes are integrated. Evidence of NO involvement in ion transport in fish was first given by Tipsmark and Madsen (2003), who observed an inhibition of NKA activity by NO donors in gill and kidney of freshwater (FW)-acclimated brown trout. A similar inhibitory effect was subsequently reported in the gill of seawater (SW)-acclimated Atlantic salmon (Ebbesson et al., 2005). Furthermore, Evans et al. (2004) and Trischitta et al. (2007) observed a significant down-regulation of ion transport by NO in the opercular epithelium of SW-acclimated killifish and the middle intestine of SW eel, respectively. In addition, we recently showed a strong inhibition of Cl^- secretion by both endogenous NO production and NO donors in the opercular membrane of SW killifish, which was primarily mediated by activation of guanylyl cyclase and cGMP signalling (Gerber et al., 2016), but suggesting *S*-nitrosation of ion transporting proteins as an additional mechanism. Based on these former studies in fish, it seems that cortisol and NO may have antagonistic effects on epithelial ion transport. In mammals there is evidence of such interaction. Glucocorticoids inhibit NO production in various cell and tissue types, including lung, liver, kidney, cardiac and vascular endothelial cells and macrophages (Balligand et al., 1994; Di Rosa et al., 1990; Knowles et al., 1990; Lou et al., 2001; Radomski et al., 1990; Simmons et al., 1996) by mechanisms that include inhibition of the expression and activity of nitric oxide synthases and regulation of its cofactors and substrate (Whitworth et al., 2002). Thus, some of the physiological and pharmacological effects of cortisol may be due to interactions with NO synthesis. Nitric oxide synthase (NOS) is the enzyme that catalyses the production of NO. In fish, two distinct isoforms of the

* Corresponding author at: Department of Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark.
E-mail address: luciegerber@biology.sdu.dk (L. Gerber).

http://dx.doi.org/10.1016/j.cbpa.2016.11.002
1095-6433/

enzyme have been identified: NOS1 (also called neuronal NOS, nNOS) and NOS2 (also called inducible NOS, iNOS) (Andreakis et al., 2011). The two isoforms share common domains but also present distinctive features (in structures, expression/localisation, enzymatic functions and activities) that are well-described in mammals (Alderton et al., 2001; Andrew and Mayer, 1999; Michel and Feron, 1997). In fish, the characteristics of NOS1 and NOS2 are not clearly defined. Yet, the cofactor binding domains of mammalian and teleost NOSs are well conserved (Øyan et al., 2000; Hyndman et al., 2006; Andreakis et al., 2011) suggesting some common features. For instance, the NOS1 isoform requires Ca^{2+} for its enzymatic activity, whereas NOS2 is Ca^{2+}-independent and can be induced in various physiological conditions to produce NO (Nathan, 1997). Hence, the two isoforms can be activated and regulated differentially and may have distinct biological roles when expressed in different tissues.

In the present study, we hypothesized an interaction between cortisol and *Nos* expression in fish. First, we examined a comprehensive organ distribution of *Nos1* and NOS2 in FW rainbow trout. Then, we examined the influence of cortisol, the major teleost corticosteroid (Takahashi and Sakamoto, 2013), on mRNA expression of *Nos1* and *Nos2* in gill, kidney and middle intestine of cortisol-treated rainbow trout acclimated to FW and SW. This study is the first to report an interaction of cortisol with NOS expression in osmoregulatory tissues of a euryhaline teleost.

2. Materials and methods

2.1. Animals

Juvenile female rainbow trout of body mass ~40 g were obtained from a local fish farm (Lihme, Randbøl, Denmark). The fish were acclimated for two months to 15 °C and a 12 h:12 h light:dark cycle in aerated bio-filtered, recirculated freshwater or artificial seawater at 25 ppt (Red Sea Salts, Verneuil s/Avre, France). Fish were fed with commercial trout pellets every second day. Feeding was withheld three days prior the experiments. The experimental work followed the guidelines of the Danish Law on Animal Experiments.

2.2. Nos mRNA expression in different tissue types of FW rainbow trout

Samples of heart, muscle, gill, liver, kidney, ovary, middle intestine (section between the pyloric caeca and the ileorectal valve), brain and spleen from a separate group of six FW rainbow trout were used to determine expression levels of *Nos1* and *Nos2* mRNA in different organs. Sampling was carried out as described below. Samples were kept at −80 °C until analysis by qPCR.

2.3. In vivo experiments

Groups of FW ($N = 10$) and SW ($N = 14$) juvenile trout were injected intraperitoneally with 2 μg cortisol per gram of fish. Cortisol was administered as hydrocortisone hemisuccinate-sodium salt (H-4881, Sigma-Aldrich, Steinheim, Germany) dissolved in 0.9% NaCl solution at 2 mg ml^{-1}. Control fish were injected with 0.9% NaCl solution only. Fish were injected on day 0 and day 2 and sampled on day 3 (24 h after the last injection). In order to minimize handling stress, the fish were briefly anaesthetized with 2-phenoxy-ethanol (Sigma-Aldrich) in either FW or SW prior to each injection. For sampling on day 3, individual fish were netted, anaesthetized and then euthanized by cutting the spinal cord. The second gill arch on the left side, a piece of the posterior part of the trunk of kidney and the middle intestine (section between the pyloric caeca and the ileorectal valve) were dissected out and quickly frozen in liquid nitrogen (N_2) for subsequent storage at −80 °C.

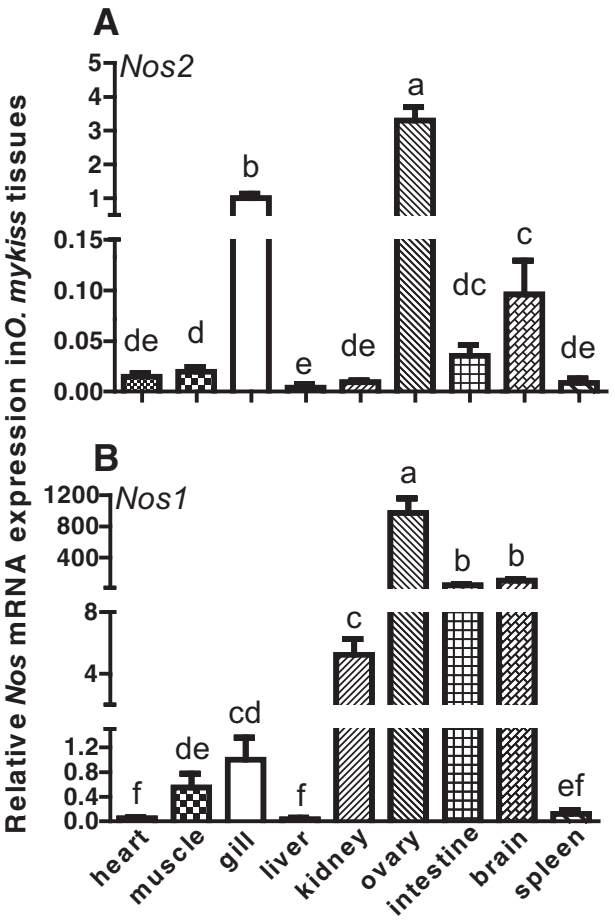

Fig. 1. *Nos2* (A) and *Nos1* (B) mRNA expression in different tissue types of FW-acclimated rainbow trout. For each target, the results are presented as mRNA level normalized to the level of *Ef1α* and relative to the gill mRNA expression ($N = 6$).

2.4. Ex vivo gill and kidney incubation

A tissue block incubation experiment was conducted on gill and kidney from FW-acclimated fish using a protocol modified from McCormick and Bern (1989). Freshly collected 2nd left side gill arches and posterior part of the trunk of kidney from six fish were first incubated for 1 h on ice in cold salmon Ringer's solution containing

Table 1
Target genes and primers sequences used to investigate mRNA levels of various genes in rainbow trout tissues.

Target sequence	Forward primer 5′–3′	Reverse primer 5′–3′	GenBank accession no.
O. mykiss Nos2	CCCAGCACTCCAGTCATTCT	ATGTCGTGTAGCCGTTGTTG	AJ300555
O. mykiss Nos1	TGTAGGACGCATTCAGTGGT	ACATTCCATGAGCCGTTGTG	DQ640498
O. mykiss Na^+/K^+-*ATPase α1a*	CCCAGGATCACTCAATGTCAC	CCAAAGGCAAATGGGTTTAAT	AY319391
O. mykiss Na^+/K^+-*ATPase α1b*	CTGCTACATCTCAAACCAACAACATT	CACCATCACAGTGTTCATTGGAT	AY319390
S. salar Ef1α	GAGAACCATTGAGAAGTTCGAGAAG	GCACCCAGGCATACTTGAAAG	AF321836