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Molecular characterization and hypoxia-induced upregulation of neuronal nitric oxide synthase in Atlantic croaker: Reversal by antioxidant and estrogen treatments

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ABSTRACT

Neuronal nitric oxide synthase (nNOS) catalyzes production of nitric oxide in vertebrate brains. Recent findings indicate that endothelial NOS and reactive oxygen species (ROS) are significantly increased during hypoxic stress and are modulated by antioxidants. However, the influence of antioxidants and steroids on nNOS upregulation by hypoxia is largely unknown. In this study, we characterized nNOS cDNA and examined the effects of hypoxia and antioxidant and steroid treatments on nNOS expression in Atlantic croaker hypothalamus. Hypoxia exposure (dissolved oxygen, DO: 1.7 mg/L for 2 and/or 4 weeks) caused significant increases in hypothalamic nNOS mRNA, protein and its neuronal expression. Hypothalamic nNOS expression and superoxide radical ($O_2\bullet^-$, an index of ROS) production were increased by pharmacological treatment of fish exposed to normoxic conditions with *N*-ethylmaleimide, an alkene drug which covalently modifies sulfhydryl groups and inhibits aromatase activity. In contrast, treatments with Nω-nitro-L-arginine methyl ester, a competitive NOS-inhibitor, or vitamin E, an antioxidant, prevented the upregulation of $O_2\bullet^-$ production and nNOS expression in hypoxia-exposed (DO: 1.7 mg/L for 4 weeks) fish. Moreover, treatment with 1,4,6-androstatrien-3,17-dione, an aromatase inhibitor, increased hypothalamic $O_2\bullet^-$ production and nNOS expression in normoxic control fish; whereas estradiol-17β treatment significantly reduced $O_2\bullet^-$ production and nNOS expression in hypoxia-exposed fish. Double-labeled immunohistochemical results showed that nNOS and aromatase proteins are co-expressed in the hypothalamus. Taken together, the results suggest that upregulation of nNOS and ROS in the croaker hypothalamus in response to hypoxia is influenced by antioxidant and overall estrogen status.

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1. Introduction

Low levels of oxygen (dissolved oxygen, DO: <2 mg/L called hypoxia) and insufficient antioxidant levels in the vertebrate brain results in the initiation of a complex series of pathological changes which leads to decreased energy levels and disruption of neuroendocrine functions (Janero, 1991; Lahiri et al., 2006; Traber and Stevens, 2011). A major pathological pathway leading to neuronal dysfunction such as apoptosis and neurodegeneration involves overproduction of cellular reactive oxygen species (ROS) and nitric oxide (NO) (Beckman and Koppenol, 1996; Rosselli et al., 1998; Estévez and Jordán, 2002; Brown, 2010). NO acts as a reactive free radical and reacts directly with superoxide anion ($O_2\bullet^-$) to form peroxynitrite ($ONOO^-$) (Andrew and Mayer, 1999; Guittet et al., 1999). $ONOO^-$ and other related reactive nitrogen species (RNS) cause nitration of DNA, proteins and lipids, leading to increased oxidative damage and irreversible modification of neuronal constituents (Cazevieille et al., 1993; Tagami et al., 1998; Yamagata et al., 2010).

Nitric oxide synthases (NOSs) are multienzyme complexes which act on L-arginine, molecular oxygen and NADPH to produce L-citrulline, $NADP^+$ and NO. This process requires several catalytic domains, cofactors, and cations (see Supplementary Fig. 1A, Griffith and Stuehr, 1995; Förstermann and Sessa, 2012). In mammals, three distinct NOS isofoms have been characterized based on their structure, localization, regulation and catalytic functions (Alderton et al., 2001). These isoforms are designated as neuronal NOS (nNOS) (Bredt et al., 1991), inducible NOS (iNOS) which is induced by stress or inflammation (Mungrue et al., 2003), and endothelial NOS (eNOS) which is present in the brain vasculature (Marsden et al., 1992; Stanarius et al., 1997).

The structure, characteristics, and catalytic functions of NOS enzymes have been well studied in tetrapods (Alderton et al., 2001),

Abbreviations: nNOS, neuronal nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species; NO, nitric oxide; NOx, nitrates and nitrites; Vit E, vitamin E; AOX, antioxidant; $O_2\bullet^-$, superoxide radical; $ONOO^-$, peroxynitrite; E2, estradiol-17β; ER, estrogen receptor; GRP30, G protein coupled receptor 30; ATD, 1,4,6-androstatrien-3,17-dione; CTK, cytokines; IL-1β, interleukin-1β; NEM, *N*-ethylmaleimide; SH, sulfhydryl; NAME, Nω-nitro-L-arginine methyl ester; RT-PCR, reverse-transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends; GSP, gene specific primer; UTR, untranslated region; ORF, open reading frame; FMN, flavin mononucleotide; FAD, flavin adeninedinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; DO, dissolved oxygen.

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whereas comparable information on the NOS enzymes is lacking in teleost fishes. At present, only two NOS isoforms have been fully characterized in several teleost fishes: nNOS in killifish (Hyndman et al., 2006), red drum (Zhou et al., 2009) and catfish (Yao et al., 2014); and iNOS in common carp (Saeij et al., 2000), rainbow trout (Wang et al., 2001) and catfish (Yao et al., 2014). There is currently no sequence information on fish eNOS mRNAs. Nevertheless, immunoreactive expression of eNOS protein has been detected in the heart of zebrafish (Fritsche et al., 2000), caudal peduncle of tilapia (Cioni et al., 2002), gill of salmon (Ebbesson et al., 2005), head kidney of rainbow trout (McNeill and Perry, 2006), liver tissues of Atlantic croaker (Rahman and Thomas, 2012) and ovary of catfish (Singh and Lal, in press). However, eNOS has not been detected in fish brains suggesting that eNOS may be not involved in NO production in the teleost brain. On the other hand, although nNOS is traditionally considered a neuronal-specific isoform; it has also been detected in the gill, head kidney, intestine, spleen as well as other peripheral tissues of fishes (Hyndman et al., 2006; McNeill and Perry, 2006; Zhou et al., 2009), suggesting that nNOS is probably the only constitutively expressed NOS gene in teleost fishes. Evidence also suggests that nNOS is highly regulated at both the transcriptional and translational levels, and is one of the most important contributors to NO production during hypoxic stress (Mauceri et al., 2002; McNeill and Perry, 2006). Moreover, nNOS is also capable of producing $O_2{\bullet}^-$ and $ONOO^-$ under certain stress conditions (Andrew and Mayer, 1999). However, despite its important role in pathological responses to hypoxia and other stressors, the regulation and functions of nNOS are not yet well understood in teleost brains during hypoxia exposure.

Hypoxia interferes with physiological functions in vertebrates by inducing ROS and RNS through activation of NOS enzymes (Li and Jackson, 2002). Hypoxia increases nNOS activity, mRNA and protein levels, and neuronal expression in rat and porcine brains (Prabhakar et al., 1996; Yamamotoa et al., 2003; Ward et al., 2005; Mishra et al., 2006; McLaren et al., 2007; Tsui et al., 2011). Hypoxia also increases iNOS and eNOS expression in tetrapod tissues (Grilli et al., 2003; Ducsay and Myers, 2011). Studies in teleosts have shown that hypoxia increases nNOS mRNA and protein expressions in the posterior cardinal vein, and these increases in nNOS expression are accompanied by increases in plasma levels of nitrates and nitrites (NOx, a metabolite of NO) in rainbow trout (McNeill and Perry, 2006). We have recently demonstrated that hypoxia increases plasma NOx levels, eNOS protein expression and $O_2{\bullet}^-$ production in croaker liver (Rahman and Thomas, 2011, 2012; unpub. obs.). Hypoxia drastically increases cellular ROS and RNS production and decreases antioxidant activity (Koskenkorva-Frank et al., 2013). Antioxidants exert protective effects against oxidative stress and prevent the propagation of ROS and RNS (Chow, 1991; Chow et al., 1999; Traber and Stevens, 2011). Therefore, it is likely that during hypoxia exposure, insufficient antioxidant capacity in the tissues to scavenge the increased formation of ROS and RNS may be an important contributing factor to neuronal dysfunction. However, there is a lack of information on the role of antioxidants in nNOS regulation and endogenous ROS/RNS generation in vertebrate brains during hypoxia exposure.

Hypoxia disrupts neuroendocrine functions by interfering with neuroenzyme activities and altering neurotransmitter levels in the vertebrate brain (Shang et al., 2006; Kumar, 2011; Thomas et al., 2007; Thomas and Rahman, 2012). Recent *in vivo* studies in several teleost fishes have shown that hypoxia suppresses the enzymatic activity of aromatase in the brain and decreases plasma estradiol-17β (E2) levels (Shang et al., 2006; Thomas et al., 2007; Thomas and Rahman, 2012). Our recent laboratory studies have shown that the hypoxia-induced marked inhibition of hypothalamic aromatase activity and reduction in plasma E2 levels is associated with a decrease in the gonadal development in croaker (Thomas and Rahman, 2012). We have also shown that hypoxia drastically decreases tryptophan hydroxylase (TPH) activity and causes a decline in serotonin levels in croaker hypothalamus (Rahman and Thomas, 2009, 2014). On the other hand, administration of E2 increases TPH activity and serotonin levels in croaker hypothalamus under hypoxic conditions (Rahman and Thomas, 2014), which suggests that sufficient endogenous E2 levels are essential for maintaining serotonergic functions. *In vivo* studies in tetrapods have shown that E2 decreases nNOS neuronal expression in the hypothalamic paraventricular nuclei and the anterior pituitary in rat brains under normoxic conditions (Qian et al., 1999; Gingerich and Krukoff, 2005). Treatment with E2 also decreases nNOS protein expression of human neutrophils *in vitro* (García-Durán et al., 1999; Molero et al., 2002). Other studies demonstrated that E2 treatment does not alter nNOS mRNA and protein levels and neuronal expression in the magnocellular, supraoptic and paraventricular nuclei (Ceccatelli et al., 1996; Wang and Morris, 1999) in rat brains, suggesting that the effects of E2 on nNOS expression in the vertebrate brain appear to be regionally specific.

The aims of the present study were four-fold. Our first aim was to characterize Atlantic croaker nNOS cDNA and examine the profiles of nNOS mRNA, protein and neuronal expression in response to different periods of exposure to hypoxia (DO: 1.7 mg/L for 2 and/or 4 weeks) in croaker hypothalamus. Atlantic croaker is a relatively hypoxia-tolerant marine teleost that inhabits estuarine and coastal regions along the US Atlantic and Gulf of Mexico coasts as well as the northern Gulf of Mexico that are often hypoxic during summer (Thomas et al., 2007; Thomas and Rahman, 2012). Severe and widespread impairment of reproductive and endocrine functions have been observed in croaker collected from hypoxic region in the northern Gulf of Mexico (Thomas et al., 2007; Thomas and Rahman, 2012). A second aim was to determine the role of vitamin-E, a potent antioxidant which regulates neuronal function(s) and maintains cellular integrity (Muller, 2010), on $O_2{\bullet}^-$ production and nNOS expression in croaker hypothalamus under both hypoxic and normoxic conditions. An alkylating drug, *N*-ethylmaleimide (NEM), which covalently modifies sulfhydryl groups to produce NO, and a NOS-inhibitor, *N*ω-nitro-L-arginine methyl ester (NAME), were used in normoxic and hypoxic conditions in order to determine whether $O_2{\bullet}^-$ is generated through a NOS-dependent pathway in response to hypoxia. The third aim was to compare the effects of chronic treatment with the aromatase-inhibitor, 1,4,6-androstatrien-3,17-dione (ATD) to those induced by hypoxia exposure on $O_2{\bullet}^-$ production and nNOS mRNA and protein expression in croaker hypothalamus. We also investigated whether chronic E2 treatment reverses these changes in hypoxia-exposed fish to levels observed under normoxic conditions. Finally, co-localization of nNOS and aromatase proteins in croaker hypothalamus were investigated to provide a potential neuroanatomical basis for any close interactions observed between these two neuroenzymes.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), TRI reagent, *N*-ethylmaleimide (NEM), *N*ω-nitro-L-arginine methyl ester (NAME), 1,4,6-androstatrien-3,17-dione (ATD), quinaldine, EGTA and EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibody against nNOS and rabbit anti-actin IgG horseradish peroxidase (HRP)-linked antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Novus Biologicals (Littleton, CO, USA), respectively. Rabbit polyclonal anti-aromatase antibody was a generous gift from Dr. Andrew H. Bass, Cornell University, Ithaca, NY, USA, and the specificity of antibody has been demonstrated in teleost brain previously (Forlano et al., 2006). Oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL, USA). Materials for molecular biology were purchased from Agilent Technologies (La Jolla, CA, USA), Promega (Madison, WI, USA), and Invitrogen (Carlsbad, CA, USA). All other chemicals were obtained from Sigma-Aldich and Fisher Scientific (Pittsburgh, PA, USA) unless noted otherwise.

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