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Reactive oxygen species and anti-oxidant defenses in tail of tadpoles, *Xenopus laevis*

Jennifer Johnson [a,1], William Manzo [b], Eileen Gardner [b], Jaishri Menon [b,*]

[a] Brown University, Providence, RI 02912, USA
[b] Department of Biology, William Paterson University of New Jersey, Wayne, NJ, USA

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ABSTRACT

Tail regression in tadpoles is one of the most spectacular events in anuran metamorphosis. Reactive oxygen species and oxidative stress play an important role during this process. Presently, the cell- and tissue-specific localization of antioxidant enzymes such as superoxide dismutase (SOD) and catalase as well as neuronal and inducible nitric oxide synthase isoforms (nNOS and iNOS) responsible for production of nitric oxide (NO) were carried out during different stages of metamorphosis in tail of tadpole *Xenopus laevis*. NO also has profound effect on the mitochondrial function having its own nitric oxide NOS enzyme. Hence, *in situ* staining for NO and mitochondria also was investigated. The distribution of nNOS and iNOS was found to be stage specific, and the gene expression of nNOS was up-regulated by thyroxin treatment. *In situ* staining for NO and mitochondria shows co-localization, suggesting mitochondria being one of the sources of NO. SOD and catalase showed significant co-localization during earlier stages of metamorphosis, but before the tail regression begins, there was a significant decrease in activity as well as co-localization suggesting increased ROS accumulation. These findings are discussed in terms of putative functional importance of ROS and cytoplasmic as well as mitochondrial derived NO in programmed cell death in tail tissue.

1. Introduction

Anuran metamorphosis is a very complex process which converts the swimming tadpole to an adult under the influence of thyroid hormone as reviewed recently by Ishizuya-Oka, 2011; Shi et al., 2011). Anuran tadpoles have also been model systems to investigate effects of various xenobiotics/environmental contaminants (such as acetochlor, triclosan, etc.), which influence the gene expression of various enzymes of urea cycle as well as thyroid hormone receptor profile affecting the process of metamorphosis (Crump et al., 2002; Fort et al., 2010; Helbing, 2012). Environmental stressors such as nitrate and nitrite modify stress-associated gene expression in tadpole tissues (Hinther et al., 2012), and a major cause of cellular stress could be via formation of reactive oxygen species (ROS). Menon and Rozman (2007) have shown that oxidative stress plays an important role in the intestinal remodeling and tail regression in tadpoles of the African clawed toad, *Xenopus laevis*. ROS are a group of highly reactive molecular forms of oxygen containing unpaired electrons. ROS are continuously produced as a byproduct of the mitochondrial respiratory chain in normal, healthy cells, and 2% of oxygen consumed by mitochondria is converted to ROS (Balaban et al., 2005). Several enzyme systems are present in organisms that serve to detoxify the ROS and prevent or reduce oxidative damage. The major antioxidant enzyme systems responsible are SOD and catalase, which remove superoxide ($\cdot O_2^-$) and H_2O_2, respectively. Imbalance due to either increased ROS production or decrease in ROS degradation can cause ROS accumulation and cell damage. Interestingly, ROS, when tightly regulated, perform several functions in the cell including signal transduction and affect gene expression (Scandalios, 2005). The biological outcome of ROS signaling is intrinsically related to the nature of the ROS signal and is dose dependent—low doses of $\cdot O_2^-$ and H_2O_2 induce protective mechanisms and acclimation responses against oxidative stress, while high doses trigger cell death (Gechev et al., 2002).

Nitric oxide (NO) is a pluripotent physiological messenger produced by the oxidation of L-arginine catalyzed by enzyme nitric oxide synthase (NOS). Nitric oxide synthases constitute a family of at least three isoforms, neuronal (nNOS, NOSI), inducible (iNOS, NOS2) and endothelial (eNOS, NOS3) (Wang and Marsden, 1995). nNOS and iNOS are soluble and found predominantly in the cytosol, while eNOS is membrane associated. Accumulating evidence suggests that NO, a versatile diffusible signaling molecule (Ignarro and Murad, 1995), may contribute to controlling the transition from cell proliferation to cell differentiation. NOS activity is also involved in the induction or suppression of apoptosis, depending on different factors such as cell type, NO concentration and presence of other radical species (Weller, 1999). The transient elevation of NOS expression arrests cell division and promotes differentiation (Bredt and Snyder, 1994). It has been proposed that ROS are key mediators in channeling NO

* Corresponding author. Tel.: +1 973 720 2795; fax: +1 973 720 2338.
E-mail address: menonj@wpunj.edu (J. Menon).
[1] Present address.

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into the death pathway in plant tissue as well (Gechev et al., 2006). Kashiwagi et al. (1999) have suggested the enhancement of oxidative stress elicited by NO by inhibiting the activity of an antioxidant enzyme, catalase, during tail regression of anuran tadpoles. It has been also shown by Heyland and Moroz (2006) that signaling mediated by hormones and NO can contribute to stress-related responses, and these molecules act as regulators of metamorphic transition. More recently Hinther et al. (2012)) documented that nitrogenous compounds such as nitrite and nitrate (which could act as NO donors) might be affecting anuran metamorphosis through their effect on thyroid hormone responsive as well as stress-associated gene expression.

Immunohistochemical studies have shown existence of mitochondrial NOS (mtNOS) in rat liver mitochondria, which play a role in respiration by regulating the activity of cytochrome *c* oxidase (Dedkova et al., 2004). mtNOS functionally couples with the mitochondrial respiratory chain to gain NOS activity and synthesize NO. Although mitochondria are essential for maintenance of aerobic life, they also trigger a sequence of events leading to apoptosis (Douglas and Reed, 1998).

In the present study, we have carried out (a) spatiotemporal distribution and Western blotting for nNOS and iNOS (in our studies, eNOS was not detected in the tail tissue, so present studies were focused on nNOS and iNOS), catalase and SOD1 (Cu/Zn); (b) *in situ* production of NO (using NO sensitive dye diamnofluorescein diacetate DAF-2DA) as well as immunostaining for mitochondria (using rhodamine 123 dye); and (c) gene expression for NOS(s) enzymes during different stages of metamorphosis as well as thyroxin-treated tadpoles.

2. Materials and Methods

2.1. Tadpoles

X. laevis tadpoles, purchased from *Xenopus I*, Ann Arbor, Michigan, were maintained in the laboratory in the aquaria and fed on the diet obtained from the same company. Water in the aquaria was changed on every alternate day. They were staged according to Nieuwkoop and Faber (1967). The metamorphic climax is during stages 59 through 64, when thyroid hormone is at the peak, and there is rapid metamorphic transition including tail regression at stage 64 (Shi, 1999). To investigate the effect of thyroid hormones on gene expression for NOS, tadpoles were treated with 8 nM 3,5,3′-L-triiodothyronine (T_3) for 5 days at stage 55—a concentration which is comparable with the peak concentration of 8 nM in the plasma of metamorphosing tadpoles (Leloup and Buscaglia, 1977).

2.2. Chemicals

The following chemicals were purchased from Santa Cruz Biotechnology: primary antibodies for nNOS and iNOS-EC 1.14.13.39 (rabbit polyclonal; catalog no. SC-648; SC-8310, respectively) and SOD 1-EC 1.15.1.1 (goat polyclonal, catalog no. SC- 8637), and secondary antibodies for iNOS (goat anti-rabbit, rhodamine conjugated, catalog no. SC-2091) and SOD (rabbit anti-goat rhodamine conjugated, SC-3945).

The following chemicals were purchased from Sigma: primary monoclonal antibody for catalase EC 1.11.1.6 (catalog no. C0979) and secondary antibody for nNOS (goat anti-rabbit FITC conjugated, catalog no. F6005); NADPH-*d* (catalog no. N 1630) and *N*-monomethyl-L-arginine (L-NMMA, catalog no. 65825) for NOS histochemistry; and DAF-2DA (catalog no. D225) for NO staining and rhodamine 123 (R 8004) for mitochondria staining.

Alkaline phosphatase-conjugated secondary antibodies for the Western blots, goat anti-rabbit IgG (170–6518) for the nNOS and iNOS and goat anti-mouse IgG for catalase (170–6520) were purchased from Biorad.

For gene expression, RT-PCR kit and all required reagents were purchased from Invitrogen.

2.3. Tissue sampling

X. laevis tadpoles at different stages were euthanized in MS 222 (18 mg/100 mL) powder dissolved in water from housing tank and buffered with sodium bicarbonate and tails were removed.

2.4. Gel electrophoresis and Western blotting

2.4.1. Sample preparation

Tails from *X. laevis* tadpoles at the specified stages (stages 58, 60, 61 and 63; $n = 5$) were removed. For Western blot analysis, catalase bands were found to be consistent at stages 58, 60 and 63. Therefore, one more stage (61) was included in order to ascertain if there is change before the tail regression begins. Tissues were homogenized in 5× volume of SDS–PAGE sample buffer with 0.2 M dithiothreitol and 1 mM phenylmethyl sulfonyl fluoride (PMSF) as a protease inhibitor (from a 0.1-M stock in 100% ethanol).

2.4.2. Electrophoresis

Eight percent discontinuous polyacrylamide gels were run for the studies with the Laemmli buffer system (Laemmli, 1970) in Biorad minigel apparatus. The samples were applied, along with Biorad Precision Plus standards for molecular weight determination. Gels were transferred (see below) or stained with Zoion Fast Stain.

2.4.3. Transfer (electroblotting)

The electroblots were conducted by a modification of the Towbin procedure (Towbin et al., 1979), using a Biorad minigel transfer apparatus and Immobilon membrane.

2.4.4. Western blotting

Following an initial 1-h blocking step in 5% bovine serum albumin (BSA) in Tris-buffered saline (Tween-20 included), Western blots were conducted using the primary antibodies described above. Primary antibody was diluted (nNOS 1:50; iNOS 1:200; catalase 1:2000; SOD 1:100) in blocking buffer to which 0.1% aprotinin was added as a protease inhibitor. The primary incubation was conducted at room temperature overnight. The secondary incubation used alkaline phosphatase-conjugated secondary antibodies (dilution 1:3000 for all enzymes). The blots were reacted with the color developing substrates BCIP (5-bromo-4-chloro-3-indolyl phosphate) and nitroblue tetrazolium (NBT) in bicarbonate buffer as described (Menon et al., 2000). The blots were then rinsed in water and dried.

2.5. NADPH-diaphorase (NADPH-d) histochemistry

The formation of NO requires the presence of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for the NOS enzyme. A simple histochemical method for localizing NOS-containing cells in fixed tissue was performed according to Schober et al. (1993)); the selectivity of this method is presumably the result of resistance of NOS to aldehyde fixation. Tail tissues at stages 58, 60 and 63 were removed ($n = 3$ per stage). Fresh frozen sections of 10 to 12 μm were cut on cryostat maintained at −20 °C, fixed in 4% paraformaldehyde in PBS, washed in PBS and incubated in freshly prepared and filtered NADPH-*d* staining solution at room temperature, washed in PBS and photographed. Control sections were placed in incubation medium either without the substrate or by addition of an inhibitor of NADPH-*d*, *N*-mono-methyl-L-arginine (L-NMMA; 10^{-3} M) (Kashiwagi et al., 1999).

2.6. Immunohistochemistry for nNOS, iNOS, SOD and catalase

Tail tissues at stages 58, 60, 63 and 64 ($n = 3$ per stage) were removed. At stage 64, the tail is regressing, and to ascertain cell/tissue-specific role of these enzymes in production and/or removal