Comparative Biochemistry and Physiology, Part C 190 (2016) 24–31

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Effect of PMA-induced protein kinase C activation on development and apoptosis in early zebrafish embryos

Jelena Hrubik [a], Branka Glisic [a], Dragana Samardzija [a], Bojana Stanic [b], Kristina Pogrmic-Majkic [a], Svetlana Fa [a], Nebojsa Andric [a,*]

[a] University of Novi Sad, Faculty of Sciences, Department of Biology and Ecology, Laboratory for Ecotoxicology, Novi Sad, Serbia
[b] University of Novi Sad, Faculty of Technical Sciences, Department of Environmental Engineering and Occupational Safety and Health, Novi Sad, Serbia

ARTICLE INFO

Article history:
Received 8 June 2016
Received in revised form 5 August 2016
Accepted 8 August 2016
Available online 10 August 2016

Keywords:
Apoptosis
Pkc
PMA
Zebrafish embryos

ABSTRACT

Protein kinase C (PKC) isoforms have been implicated in several key steps during early development, but the consequences of xenobiotic-induced PKC activation during early embryogenesis are still unknown. In this study, zebrafish embryos were exposed to a range of phorbol 12-myristate 13-acetate (PMA) concentrations (0–200 μg/L) at different time points after fertilization. Results showed that 200 μg PMA/L caused development of yolk bags, cardiac edema, slow blood flow, pulsating blood flow, slow pulse, elongated heart, lack of tail fins, curved tail, and coagulation. PMA exposure decreased survival rate of the embryos starting within the first 24 h and becoming more pronounced after prolonged exposure (96 h). PMA increased the number of apoptotic cells in the brain region as demonstrated by acridine orange staining and caused up-regulation of caspase 9 (*casp9*) and p53 up-regulated modulator of apoptosis (*puma*) mRNA in whole embryos. PMA caused oxidative stress in the embryos as demonstrated by decreased mRNA expression of catalase and superoxide dismutase 2. Inhibition of Pkc with GF109203X improved overall survival rate, reduced apoptosis in the brain and decreased expression of *casp9* and *puma* in the PMA-exposed embryos. However, Pkc inhibition neither prevented development of deformities nor reversed oxidative stress in the PMA-exposed embryos. These data suggest that direct over-activation of Pkc during early embryogenesis of zebrafish is associated with apoptosis and decreased survival rate of the embryos.

1. Introduction

Protein kinase C (PKC) is a large family of kinases comprising approximately 11 different isoforms. In mammals, PKCs are separated into structurally and functionally different groups. The first group are classical PKCs (cPKCs: PKCα, PKCβ I and II, and PKCγ) that require diacylglycerol (DAG) and Ca^{++} for optimal activation. The second group, known as novel PKCs (nPKC: PKCδ, PKCε, PKCη, and PKCθ) are DAG-sensitive, but Ca^{++}-insensitive. The third group are atypical PKCs (aPKCs: PKCζ and PKCι, λ-murine) that are insensitive to both DAG and Ca^{++} (Ohno and Nishizuka, 2002; Rosse et al., 2010).

PKCs regulate diverse cellular processes, such as proliferation, differentiation (Poli et al., 2014), apoptosis (Li et al., 1999a, b; Musashi et al., 2000), inflammatory responses (Pierce et al., 1998; Pfeifhofer et al., 2006), synaptic plasticity (Perez et al., 2001), long term potentiation and learning (Saito and Shirai, 2002). Moreover, it has been shown that PKCs regulate embryogenesis in many organisms including fruit flies, sea urchins, nematodes, mice, and gastropods (Gallicano et al., 1997; Tabuse et al., 1998; Wu et al., 1998; Cox et al., 2001; Dickey-Sims et al., 2005; Zukaite et al., 2016). Several different Pkc isoforms have been identified in zebrafish embryos including Pkcα, Pkcγ, Pkcβ II, Pkcδ, Pkcε, Pkcθ, and Pkcζ. Pkcα, γ, ε, θ, and ζ are expressed in the zebrafish central nervous system (CNS) (Patten et al., 2007). Pkcγ is expressed in Rohon-Beard (RB) sensory neurons and Mauthner cells. Pkcε and ζ staining is widespread in the CNS, whereas Pkcθ and β II are expressed in the skeletal muscle, especially at the intersegmental boundaries (Slatter et al., 2005; Patten et al., 2007). Using genetic ablation of various Pkc isoforms in zebrafish, a number of studies implicated this kinase as an important player in regulation of several critical processes during embryogenesis. These studies showed that the loss of Pkc in zebrafish caused

Abbreviations: AMPRA-R, alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor; *apaf1*, apoptotic peptidase activating factor 1; *bax*, bcl2-associated X protein; *bcl-2*, B-cell lymphoma 2; *casp3*, caspase 3; *casp9*, caspase 9; *cat*, catalase; CNS, central nervous system; DAG, diacylglycerol; *ef1α*, elongation factor 1α; *emx1*, empty spiracles homeobox 1; Erk, extracellular signal-regulated kinase; GFX, GF109203X, protein kinase C inhibitor; hpf, hours post fertilization; *mdm2*, mouse double minute 2 homolog (MDM2) oncogene, E3 ubiquitin protein ligase; NAC, N-Acetyl-L-cysteine; *ngn1*, neurogenin 1; *otx2*, orthodenticle homeobox 2; *p53*, tumor protein p53; *pax2a*, paired box 2a; Pkc (zebrafish)/PKC (rodents and human), protein kinase C; PMA, phorbol 12-myristate 13-acetate; *puma*, p53 up-regulated modulator of apoptosis; RB, Rohon-Beard sensory neurons; ROS, reactive oxygen species; *sod2*, superoxide dismutase 2; *sox2*, SRY (sex determining region Y)-box 2; *sox3*, SRY (sex determining region Y)-box 3; *syn2a*, synapsin IIa.

* Corresponding author at: Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, 2 Dositej Obradovic Square, 21000 Novi Sad, Serbia.
E-mail address: nebojsa.andric@dbe.uns.ac.rs (N. Andric).

http://dx.doi.org/10.1016/j.cbpc.2016.08.002
1532-0456/

abnormal thymosin beta-induced coronary development program (Bock-Marquette et al., 2009), angiogenesis (Oubaha et al., 2012), developmental speeding of alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPRA-R) in Mauthner cells (Patten et al., 2010), and claudin expression (Wu et al., 2012). However, only a limited number of studies have investigated the consequences of a direct PKC activation on embryogenesis. Direct activation of PKC in embryonic cell lines is an important mechanism in several physiological processes, such as epithelial to mesenchymal transition (Feng et al., 2012) and vasculogenesis (Bekhite et al., 2011). In addition, increase in the PKC activity was observed in certain pathological conditions including diabetic embryopathy (Gareskog and Wentzel, 2004; Zhiyong et al., 2008). One study performed on zebrafish embryos showed that indirect Pkc activation by cinnamon extract induced developmental arrest and apoptosis (Bansode et al., 2013). Moreover, over-activation of PKC by phorbol 12-myristate 13-acetate (PMA), an aromatic DAG analogue and a potent tumor promoter, caused disorganized and developmentally arrested snail embryos (Zukaite et al., 2016).

In this study, we aimed to investigate the consequences of xenobiotic-driven direct activation of the Pkc signaling pathway using developing zebrafish embryos. Zebrafish embryo model was chosen because it exerts a high developmental similarity to mammals in most aspects of embryonic development, including early embryonic processes involving cardiovascular, somite, muscular, skeletal, and neuronal systems (McCollum et al., 2011). In addition, this model is highly predictive of mammalian embryonic development and toxicity (Brannen et al., 2013). Therefore, investigations into a direct Pkc activation in zebrafish embryos will shed more light on the role of this signaling pathway in mammalian embryogenesis. To date, only a few studies have applied PMA in zebrafish embryos with the sole purpose of increasing the reactive oxygen species (ROS) production and establishment of the respiratory burst model (Hermann et al., 2004; Li et al., 2013). These studies have not analyzed the consequences of PMA exposure on development of zebrafish embryos. In this study, we have treated zebrafish embryos with PMA to provoke a direct over-activation of Pkc during embryogenesis. We have also applied a pharmacological inhibitor of the Pkc signaling GF109203X (GFX) to gain insight into the mechanisms of PMA action in zebrafish embryos. Pharmacological approach is attractive as small molecules/drugs can be applied and withdrawn at will, providing an alternative to expensive and time-consuming transgenic experiments. The use of the signaling pathway-modifying chemicals is particularly feasible in classical genetic model organisms, such as fruit fly and zebrafish, due to their relative low cost and the availability of a large number of fast developing embryos, which allows for testing of various concentrations and application time points (Hawkins et al., 2008). This approach, however, is associated with several potential problems. Pharmacological inhibitors exert strong selective action and their presence in high doses can alter several signaling pathways and not just the specific target molecules. It is also important to achieve such concentrations of the inhibitors that are not toxic to the embryos, but can effectively inhibit the activity of a target signaling pathway. Using pharmacological approach, we have demonstrated that over-activation of the Pkc signaling by PMA was responsible for increased apoptosis and decreased survival rate of zebrafish embryos.

2. Material and methods

2.1. Chemicals

Phorbol 12-myristate 13-acetate (PMA), Pkc inhibitor (GF109203X, GFX), Erk inhibitor (U0126), N-Acetyl-L-cysteine (NAC), ethyl 3-aminobenzoate methanesulfonate salt (MS-222), and methylcellulose were purchased from Sigma (Steinheim, Germany). Acridine orange was purchased from Fisher Scientific (Leicestershire, UK). All other reagents were of analytical grade.

2.2. Zebrafish embryos

Zebrafish embryos were obtained after mating and spawning of adult male and female zebrafish in 3:1 ratio. Adult fish were kindly provided by Dr. Zsolt Csenki, Szent Istvan University, Godollo, Hungary. Conditions in zebrafish facility, mating and spawning of adult fish, collecting and staging of zebrafish embryos were described previously (Kimmel et al., 1995; OECD, 2006; Glisic et al., 2016). Since the embryos used in this study were no more than 5 days-old, the license was not required by the Council of Europe, Directive 86/609/EEC (EEC, 1986) or the Ethics Commission for Protection and Welfare of Experimental Animals of the University of Novi Sad.

2.3. Treatments

In order to determine the dose of PMA to be used in this study, zebrafish embryos were exposed to a range of concentrations (10, 50, 100, and 200 μg PMA/L) from the pharyngula stage (24 hpf) to the larval stage (120 hpf). Developmental phenotypes were recorded every 24 h. As the highest concentration of PMA showed the most prominent morphological malformations, this concentration was chosen for further experiments. In experiments with pharmacological inhibitors, the embryos were treated with GFX (20 μM), U0126 (10 μM) and NAC (50 μM). The concentration of 10 μM GFX was previously used in zebrafish embryos to prevent the effect of PMA, without causing adverse effects on embryonic development (Hermann et al., 2004). In this study, 20 μM GFX was well tolerated by the embryos. We have previously demonstrated that 10 μM U0126 was the tolerated concentration of the inhibitor that did not adversely impact zebrafish embryonic development after prolonged exposure (Hrubik et al., 2016). Moreover, it has been shown that 50–100 μM NAC exerts non-toxic effects in zebrafish embryos, while concentrations of 500 μM and above cause various developmental malformations (Timme-Laragy et al., 2009). All inhibitors were added at 5 hpf, followed by PMA (200 μg/L) addition at 24 hpf. Zebrafish embryos were collected after 24 h of incubation with PMA (48 hpf). The embryos were collected for gene expression analysis (5 embryos/group) and analysis of acridine orange staining (10 embryos/group). All treatments were prepared in reconstituted water and the concentration of dimethyl sulfoxide, a solvent used in this study, did not exceed 0.03% in any of the treatments.

2.4. Morphological analysis

Morphological characteristics of a particular developmental stage and malformations caused by treatments were monitored and imaged using Leica MZ16 magnifier (Heerbrugg, Switzerland) at 30× magnification. The main focus was on malformations that could be distinguished at this magnification, such as changes in the size and shape of the whole body, yolk and organs that were expected to develop in particular time point (e.g. blood flow, heartbeat). The embryos were observed for morphological defects daily and the number of embryos with at least one morphological defect in comparison to the control group was recorded.

2.5. Acridine orange staining

To detect the apoptotic cells, the embryos were imaged under the fluorescent microscope after staining with acridine orange. Acridine orange dye was diluted to a concentration of 2 mg/L in the buffer (50 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.4). Ten embryos in each experimental group were incubated with the dye for 30 min at 26 °C in the dark and washed twice afterwards with reconstituted water, followed by anaesthesia with 0.02%