Toxicon 112 (2016) 35–44

Contents lists available at ScienceDirect

Toxicon

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

Structural and evolutionary insights into endogenous alpha-phospholipase A_2 inhibitors of Latin American pit vipers

Maria Inácia Estevão-Costa [a, b, 1], Carlos Alexandre H. Fernandes [c, 1], Maurício de Alvarenga Mudadu [a], Glória Regina Franco [b], Marcos Roberto M. Fontes [c, **], Consuelo Latorre Fortes-Dias [a, *]

[a] Fundação Ezequiel Dias (FUNED), Belo Horizonte, MG, Brazil
[b] Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil
[c] Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil

ARTICLE INFO

Article history:
Received 3 December 2015
Received in revised form
16 January 2016
Accepted 20 January 2016
Available online 21 January 2016

Keywords:
Phospholipase A_2 inhibitor
PLI
Phospholipase A_2
PLA_2
Viperidae
Snake

ABSTRACT

Phospholipases A_2 are major components of snake venoms ($svPLA_2s$) and are able to induce multiple local and systemic deleterious effects upon envenomation. Several snake species are provided with $svPLA_2$ inhibitors (sbPLIs) in their circulating blood, which confer a natural resistance against the toxic components of homologous and heterologous venoms. The sbPLIs belong to any of three structural classes named α, β and γ. In the present study, we identified, characterized and performed structural and evolutionary analyses of sbαPLIs transcripts and the encoded proteins, in the most common Latin American pit vipers belonging to *Crotalus*, *Bothrops* and *Lachesis* genera. Mutation data indicated that sbαPLIs from Latin American snakes might have evolved in an accelerated manner, similarly to that reported for sbαPLIs from Asian snakes, and possibly co-evoluted with $svPLA_2s$ in response to the diversity of target enzymes. The importance of sbαPLI trimerization for the effective binding and inhibition of acidic $svPLA_2s$ is discussed and conserved cationic residues located at the central pore of the inhibitor trimer are suggested to be a significant part of the binding site of sbαPLIs to acidic $svPLA_2s$. Our data contribute to the current body of knowledge on the structural and evolutionary characteristics of sbPLIs, in general, and may assist in the future development of selective inhibitors for secretory PLA_2 from several sources.

1. Introduction

Secretory phospholipases A_2 from snake venoms ($svPLA_2s$) are very potent toxins that display a variety of biological activities including neurotoxic, myotoxic, haemolytic, edematogenic, hyperalgesic, pro-inflammatory, hypotensive, platelet-aggregation inhibitory, bactericidal, anticoagulant and cytotoxic actions (reviewed by Gutierrez and Lomonte, 2013). Acquisition of these various effects was achieved by gene duplication and accelerated exon evolution in the protein-coding region, while the mutation rates in cDNAs or genes ensured sufficient molecular plasticity to support the diverse physiological actions of those enzymes (Chuman et al., 2000; Nakashima et al., 1993, 1995; Nobuhisa et al., 1996; Ogawa et al., 1992; Ohno et al., 1998, 2003).

Several snake species possess circulating PLA_2 inhibitors (sbPLIs), which probably co-evolved with venom toxins, aiming at preventing any possible damage from toxins that might have found their way into the blood stream (Kochva et al., 1983). In *Gloydius brevicaudus* (formerly *Agkistrodon b. siniticus*), expression of a particular sbPLI has been shown to be up regulated by venom components (Kinkawa et al., 2010). Other physiological roles have been suggested for sbPLIs in the innate immune system and local regulation of other PLA_2s (Lizano et al., 2003; Ohkura et al., 1999; Okumura et al., 2003). Unique experimental data has been obtained for an sbPLI from *G. brevicaudus* (Shirai et al., 2009, 2010). This specific inhibitor binds to Cyt c and neutralizes the autologous Cyt c released from the dead cells. Hence, the authors suggested an

* Corresponding author.
** Corresponding author.
E-mail addresses: fontes@ibb.unesp.br (M.R.M. Fontes), consuelo.latorre@funed.mg.gov.br (C.L. Fortes-Dias).
[1] Estevão-Costa, M.I. and Fernandes, C.A.H. equally contributed to this study.

http://dx.doi.org/10.1016/j.toxicon.2016.01.058
0041-0101/

ancestral function for that svPLA$_2$ inhibitor as a Cyt c-binding protein, prior to the svPLA$_2$ inhibition.

Due to their inhibition selectivity against svPLA$_2$s and the sharing of structural similarities with human secretory PLA$_2$s, the sbPLIs are considered potential molecular models for the development of new inhibitors of the latter. However, to this moment, few studies structural studies were performed with these inhibitors (Fortes-Dias et al., 2014; Okumura et al., 2005; Santos-Filho et al., 2011).

The sbPLIs are grouped into three classes—α, β and γ—based on the presence of characteristic structural domains (Ohkura et al., 1997). Members of different classes can be concomitantly found in a single snake species (Lizano et al., 2000; Nobuhisa et al., 1998; Ohkura et al., 1997; Shirai et al., 2009; So et al., 2011) and even non-venomous snakes may possess this type of molecules (Okumura et al., 1999b, 2002, 2003; Shirai et al., 2009; Thwin et al., 2002). Presence of different sbPLIs in a single specimen appears to ensure effective inhibition of different target svPLA$_2$ whereas, in the non-venomous species, their function is yet to be understood. The typical structural domain of the sbαPLIs is a C-type lectin-like domain (CTLD) (Inoue et al., 1991), although they lack the carbohydrate-binding ability (Ohkura et al., 1993; Okumura et al., 2003, 2005; Santos-Filho et al., 2011). The first and most studied sbαPLIs were purified from the blood plasma of the Asian viperid *Protobothrops* (taxonomically renamed from the former *Trimeresurus*) and *Gloydius* (previously *Agkistrodon*) snakes (Inoue et al., 1991, 1997; Kinkawa et al., 2010; Kogaki et al., 1989; Nishida et al., 2010; Nobuhisa et al., 1997a, b; Ohkura et al., 1993; Okumura et al., 1999a, 2005; So et al., 2011). They are hetero or homotrimers of 20–25 kDa subunits and bind one PLA$_2$ molecule per trimer (Inoue et al., 1991, 1997; Kogaki et al., 1989; Ohkura et al., 1993, 1997; Okumura et al., 1999b).

Despite purification of a number of sbαPLIs from Latin American viperid snakes—*Bothrops asper* (Lizano et al., 1997), *Cerrophidion godmani* (Lizano et al., 2000) and *Atropoides mexicanus* (Quiros et al., 2007) from Costa Rica; *Bothrops moojeni* (Soares et al., 2003), *Bothrops jararacussu* (Oliveira et al., 2008) and *Bothrops alternatus* (Santos-Filho et al., 2011) from Brazil—few structural and evolutionary studies are currently available. Those inhibitors are generally named MIPs, an acronym for myotoxin inhibitor proteins, because they inhibit Lys^{49} homologues and Asp^{49} myotoxic svPLA$_2$s.

Aiming at a better comprehension of sbαPLs inhibitors and motivated by the rich biodiversity of the herpetological fauna in Latin America, in the present study, we identified sbαPLIs in the most common Brazilian pit vipers belonging to *Bothrops, Crotalus* and *Lachesis* genera. We performed evolutionary and structural analyses of isolated liver transcripts and the corresponding deduced proteins, in order to gain insights into the binding and inhibition of svPLA$_2$s by this class of inhibitors.

2. Materials and methods

2.1. Snake liver collection

Specimens of *Bothrops alternatus, Bothrops erythromelas, Bothrops jararaca, B. jararacussu, B. moojeni, Bothrops neuwiedi, Crotalus durissus terrificus* and *Lachesis muta muta* snakes were obtained alive from the Serpentarium of Fundação Ezequiel Dias in Brazil, with the exception of the latter, which died by natural causes. At the time of sampling, the snakes were euthanized with CO_2 atmosphere, according to the protocol approved by the Committee for Ethics in Animal Use (CEUA FUNED 022/2012). Liver samples were collected in DEPC-treated vials, rapidly frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA and cDNA were prepared as previously described for the sbγPLIs (Estevão-Costa et al., 2008). Briefly, total RNA was isolated from approximately 120 mg of frozen liver tissue with Trizol® (Invitrogen, USA). After integrity checking by electrophoresis on 1% agarose gel, cDNA was synthesized with oligo(dT)$_{12-18}$ primer using the First-Strand Synthesis kit (Invitrogen, USA). The cDNAs encoding for sbαPLIs were obtained by conventional polymerase chain reaction (PCR) in the presence of specific oligonucleotides designed on the basis of published nucleotide sequence encoding for sbαPLIs from Old World snakes—*Protobothrops flavoviridis* (g.i. D87549.1) and *G. brevicaudus* (g.i. AB026666.1) (Nobuhisa et al., 1997a; Okumura et al., 1999b): P1 forward (5′GGAAGGAAAGTACTTTCTCTGGAG3′, in the 5′UTR region), P2 reverse (5′TCATA AAATGAAATAAAACTCACACACGAC3′, in the C-terminal) and P3 forward (5′CATGAGACAGATCCTGACGGA3′), in the N-terminal of the mature protein. Amplification conditions comprised of 5 min at 94 °C; 35 cycles consisting of 3 min at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and an extension period of 7 min at 72 °C, in a Perkin Elmer 2400 Thermo cycler. The pair of primers used was P1/P2 and P1/P3, with expected amplification products of 730 bp and 420 bp, respectively. In the negative controls, DNA and reverse transcriptase were omitted. Aliquots of the amplification reactions were analysed by electrophoresis on 1.0% agarose gel in TBE buffer, in the presence of ethidium bromide.

2.3. DNA cloning and sequencing, primary structure deduction and alignments

DNAs encoding sbαPLIs were processed as previously described (Estevão-Costa et al., 2008). Briefly, fresh PCR products were cloned in a TApCR 2.1 plasmid according to manufacturer's instructions (TA Cloning kit, Invitrogen, USA) and the recombinant plasmids were used to transform *Escherichia coli* strain INVαF'competent cells. DNA from confirmed positive clones was purified using a commercial kit (Wizard Plus Miniprep, Promega, USA) and sequenced by the dideoxy chain termination method (Sanger et al., 1977) on an automated ABI Prism 3110 Genetic Analyser (Perkin Elmer Applied Biosystem, USA), with appropriate oligonucleotides and reagents. Consensus sequences for each clone were obtained from a minimum of four complete reads and all sequences were deposited at the GenBank (www.ncbi.nlm.nih.gov/genbank/).

We used the conventional algorithm of ClustalW for the alignments (Thompson et al., 1994) after similarity search with other sbαPLIs using the Blastn (www.ncbi.nlm.nih.gov/BLAST/). Inhibitors with incomplete primary structures, such as *Ba*MIP (g.i. P81077) from *Bothrops asper* (Lizano et al., 1997), were not included. We performed pairwise alignments of the primary sequences to obtain the percentages of identities and mutations using the BLASTp (NCBI). The sbαPLI from *G. brevicaudus* was used as reference in the sequence alignments. Mutation rates in the nucleotide (nt) sequences were evaluated by the dn/ds ratio, where dn and ds correspond to non-synonymous and synonymous nt mutations, respectively.