Toxicon 119 (2016) 8–20

Contents lists available at ScienceDirect

Toxicon

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

An analysis of venom ontogeny and prey-specific toxicity in the Monocled Cobra (*Naja kaouthia*)

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ARTICLE INFO

Article history:
Received 23 March 2016
Received in revised form
25 April 2016
Accepted 28 April 2016
Available online 7 May 2016

Keywords:
Enzymes
Mass spectrometry
Phospholipases A_2
Three-finger toxins
Venom variation

ABSTRACT

Venoms of snakes of the family Elapidae (cobras, kraits, mambas, and relatives) are predominantly composed of numerous phospholipases A_2 (PLA_2s) and three-finger toxins (3FTxs), some of which are lethal while others are not significantly toxic. Currently, the only identified prey-specific toxins are several nonconventional 3FTxs, and given the large diversity of 3FTxs within Monocled Cobra (*Naja kaouthia*) venom, it was hypothesized that several 3FTxs, previously found to be non-toxic or weakly toxic 3FTxs in murine models, could potentially be toxic towards non-murine prey. Additionally, it was hypothesized that ontogenetic dietary shifts will be correlated with observable changes in specific 3FTx isoform abundance. Adult and juvenile *N. kaouthia* venom composition was investigated using ion-exchange FPLC, 1D and 2D SDS-PAGE, mass spectrometry, and various enzymatic and LD_{50} assays. Alpha-cobratoxin (α-elapitoxin) was the only significantly toxic (LD_{50} < 1 μg/g) 3FTx found in *N. kaouthia* venom and was equally toxic toward both lizard and mouse models. The abundance and diversity of 3FTxs and most enzyme activities did not vary between adult and juvenile cobra venoms; however, total venom PLA_2 activity and specific PLA_2 isoforms did vary, with juveniles lacking several of the least acidic PLA_2s, and these differences could have both biological (related to predation) and clinical (antivenom efficacy) implications. Nevertheless, the ubiquitous presence of α-cobratoxin in both adult and juvenile cobra venoms, with high toxicity toward both reptiles and mammals, represents a venom compositional strategy wherein a single potent toxin effectively immobilizes a variety of prey types encountered across life history stages.

1. Introduction

The Monocled Cobra (*Naja kaouthia*) is the most abundant species of Asian cobra, with a range that includes India, Bangladesh, Nepal, Myanmar, southwestern China, and Thailand (Mukherjee and Maity, 2002; Reali et al., 2003). In Thailand, snakebite envenomations by *N. kaouthia* account for the highest number of human fatalities among all venomous snake species (Kulkeaw et al., 2007). Patients who have systemic envenoming by *N. kaouthia* usually develop neurotoxic symptoms, including ptosis, dysphagia, and increased salivation, followed by coma and death from respiratory paralysis in severe cases (Sells et al., 1994; Reali et al., 2003).

Because *N. kaouthia* is very common and is responsible for human morbidity and mortality, there have been many studies published characterizing specific venom proteins and describing overall venom composition (Hamako et al., 1998; Sakurai et al., 2001; Meng et al., 2002; Mukherjee and Maity, 2002; Doley and Mukherjee, 2003; Kulkeaw et al., 2007; Mordvintsev et al., 2007, 2009; Debnath et al., 2010), including two recent publications on the complete venomics profile of *N. kaouthia* (Laustsen et al., 2015; Tan et al., 2015). The venom of *N. kaouthia* is primarily composed of three-finger toxins (3FTxs; neurotoxic and cardiotoxic/cytotoxic) and phospholipase A_2 (PLA_2) isoforms (Namiranian and Hider, 1992; Kulkeaw et al., 2007; Laustsen et al., 2015; Tan et al., 2015). Geographic venom variation was also recently documented for *N. kaouthia* (Tan et al., 2015), but because pooled venoms were used in these studies, there is still a lack of information on individual intraspecific venom variation or ontogenetic venom compositional changes in *N. kaouthia*.

Venom variability has been documented at the family, genus,

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http://dx.doi.org/10.1016/j.toxicon.2016.04.049
0041-0101/

species and intraspecific levels (Chippaux et al., 1991; Mackessy, 2010b). Intraspecific venom variation may occur between individuals of different geographic locations, dietary habits, genders and age (Minton and Weinstein, 1986; Mackessy, 1988, 1993; Chippaux et al., 1991; Daltry et al., 1996a; Daltry et al., 1996b; Mackessy et al., 2003; Menezes et al., 2006; Alape-Giron et al., 2008). Ontogenetic venom variation studies have largely focused on the subfamily Crotalinae (pit vipers), *Crotalus* and *Bothrops* species in particular (Meier, 1986; Minton and Weinstein, 1986; Mackessy, 1988, 1993; Gutiérrez et al., 1991; Saldarriaga et al., 2003; Alape-Giron et al., 2008; Zelanis et al., 2009). A few studies have analyzed ontogenetic venom variation in the Elapidae (cobras, kraits, mambas, and relatives), but the conclusions of these studies varied (see below).

Several studies of Australian elapids found that there was no significant difference in the venom composition of juvenile and adult Coastal Taipans (*Oxyuranus scutellatus*), Inland Taipans (*Oxyuranus microlepidotus*), and Tiger Snakes (*Notechis scutatus*) (Tan et al., 1992, 1993a, b). Minton (1967) found that venom toxicity, hemorrhagic activity, indirect hemolysin, direct hemolysin, and hemagglutinin for *Naja naja* ssp. increased with age. Unfortunately, since this study was conducted before the more recent revisions of the genus *Naja* that revealed at least ten species of *Naja* throughout India and southeast Asia (Wüster, 1996), it is unknown which species was actually studied. Meier and Freyvogel (1980) found a decrease in venom toxicity with age for the Black-necked Spitting Cobra (*Naja nigricollis*). A more recent study on ontogenetic venom variation in *Naja atra* found higher phosphomonoesterase and L-amino acid oxidase activity and lower nucleotidase, PLA_2, hyaluronidase, and fibrinolytic activity within neonate *Naja atra* venom (He et al., 2014).

Ontogenetic changes in venom composition have been found to correlate with snake dietary shifts (Mackessy, 1988; Andrade and Abe, 1999; Mackessy et al., 2006; Zelanis et al., 2009). In Pacific Rattlesnakes (*Crotalus oreganus* [*viridis*]; Mackessy, 1988) and Jararacas (*Bothrops jararaca*; Andrade and Abe, 1999), an ontogenetic shift in diet from ectothermic prey (arthropods, lizards, and amphibians) as a juvenile to endothermic prey (mammals) as an adult was associated with a shift in venom toxicity, with juvenile venom more toxic to ectotherms and adult venom more toxic towards mammals. A similar pattern was noted for the Brown Treesnake (*Boiga irregularis*), with juvenile venoms more toxic than adult venoms towards geckos, suggestive that this trend is not just found in vipers (Mackessy et al., 2006).

In their first year, *N. kaouthia* feed primarily on frogs and newborn rats, and adult snakes feed on adult rats, snakes, lizards, fish, birds, and bird eggs (Chaitae, 2000). Therefore, as *N. kaouthia* ages, a wider diversity of prey is taken, most likely because juveniles are gape-limited to feed on smaller prey. Prey-specific toxins have not been identified in cobra venoms, even though 3FTxs that are weakly toxic towards murine models have been identified in cobra venoms, and 3FTxs are currently the only venom proteins to be directly linked to prey-specific toxicity (Pawlak et al., 2006, 2009; Heyborne and Mackessy, 2013). Non-conventional/weak 3FTxs exist in *N. kaouthia* venom, and this subclass of 3FTxs have been suggested to confer prey-specific toxicity in King Cobra (*Ophiophagus hannah*) venoms (Chang et al., 2013), but this hypothesis has not been tested.

Documentation of intraspecific variation in venoms also has important clinical and antiserum production applications. Intraspecific venom variation in the Spectacled Cobra (*Naja naja*) has resulted in the manifestation of different clinical envenomation symptoms and a lack of antivenom efficacy between India and Sri Lanka *N. naja* populations (Kularatne et al., 2009). The present study compares the biochemical composition of adult and juvenile *N. kaouthia* venoms and addresses the following questions: Are there differences in venom protein content and activity between adult and juvenile cobras? Is there a difference in the toxicity of adult and juvenile cobra venoms towards ectothermic (lizards) and endothermic (mammalian) prey? Are there components of *N. kaouthia* venom, such as nonconventional 3FTxs, that exhibit prey-specific toxicity? It was hypothesized that non-conventional 3FTxs in *N. kaouthia* venoms would exhibit selective toxicity toward non-mammalian prey.

2. Materials and methods

2.1. Reagents

Reagents for protein concentration assays were purchased from BioRad Inc. (San Diego, CA, U.S.A). Precast NuPAGE 12% Bis-Tris mini gels, Novex Mark 12 unstained molecular mass standards, LDS sample buffer and MES running buffer were purchased from Life Technologies (Grand Island, NY, U.S.A). Two-dimensional gel electrophoresis supplies, including DeStreak rehydration solution, IPG pH 3–11 buffer and Immobiline DryStrip pH 3–11, were purchased from GE Healthcare (Pittsburgh, PA, U.S.A). Phospholipase A_2 assay kit was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A). Azocasein, DTT, DTNB, acetylthiocholine iodide, bis-p-nitrophenylphosphate, L-kynurenine, human fibrinogen and all other reagents (analytical grade or better) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A).

2.2. Venoms and animals

Pooled and lyophilized adult (over three years in age) and 10 individual juvenile (less than three months in age) *N. kaouthia* venoms were donated by the Kentucky Reptile Zoo (Slade, KY, U.S.A). All *N. kaouthia* were of Thailand origin and kept under the same husbandry conditions. The pooled adult venom included parents of the juveniles to reduce the potential effects of venom variation due to genetic variability. Venoms were stored frozen at −20 °C until needed. Venom protein concentration was determined using bovine gamma globulin standard and the method of Bradford (1976) as modified by BioRad Inc. The calculated protein concentrations for each crude venom sample were used in all other analyses and enzymatic activity calculations. NSA mice (*Mus musculus*) were bred in the University of Northern Colorado (UNC) Animal Resource Facility and House Geckos (*Hemidactylus frenatus*) were obtained from Bushmaster Reptiles (Longmont, CO, USA); all procedures were reviewed and approved by the UNC IACUC (protocol 1504D-SM-SMLBirds-18).

2.3. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE on NuPage 12% Bis-Tris mini gels (Life Technologies, Inc., U.S.A) was performed to compare the relative molecular masses of venom components of adult and juvenile *N. kaouthia*. Samples and buffers were prepared under reducing conditions according to the manufacturer. Venom samples were run at 10 μg and 20 μg per lane, with 5 μL of Novex Mark 12 unstained mass standard in one lane for estimation of molecular masses. The gel was run at 180 V, stained with 0.1% Coomassie Brilliant Blue R250 overnight, destained (50/40/10, v/v, ddH_2O:methanol:glacial acetic acid) for two hours, and imaged.