Toxicon 68 (2013) 30–39

Contents lists available at SciVerse ScienceDirect

Toxicon

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

The pathological effects of Heminecrolysin, a dermonecrotic toxin from *Hemiscorpius lepturus* scorpion venom are mediated through its lysophospholipase D activity

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ARTICLE INFO

Article history:
Received 25 May 2012
Received in revised form 23 November 2012
Accepted 13 March 2013
Available online 2 April 2013

Keywords:
Heminecrolysin
Lysophospholipase D
Phosphatidylserine exposure
Hemolysis
Cytokines
Disseminated intravascular coagulation

ABSTRACT

We have previously identified Heminecrolysin, a sphingomyelinase D (SMaseD), as the major protein responsible for the main pathological effects observed following *Hemiscorpius* (*H.*) *lepturus* scorpion envenomation. We aimed herein to further investigate the kinetics and molecular mechanisms triggered by Heminecrolysin to initiate hematological disorders and inflammatory reaction. We show that Heminecrolysin highly hydrolyzes lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA) and choline, with a V_{max} = 1481 ± 51 μmol/min/mg and a K_m = 97 ± 16.78 μM, at a much lesser extend sphingomyelin but not phosphatidylcholine substrates. Its lysophospholipase D (lysoPLD) catalytic efficiency, up to three orders of magnitude higher, comparatively to spider's SMaseDs (newly referred as phospholipases D; PLDs), could explain its strong hemolytic capacity. Chelating agents such as EDTA, EGTA, and 1, 10-phenantroline blocked Heminecrolysin-induced LPC hydrolysis at 98, 48, and 70% respectively. Hemolysis blockade occurs only when the toxin is added to erythrocytes in the presence of serum, source of LPC and complement, indicating that the production of LPA and the presence of complement are mandatory for hemolysis. Moreover, we show that Heminecrolysin efficiently binds to erythrocyte's membrane and provokes phosphatidylserine (PS) translocation without cleavage of glycophorin A, suggesting that, unlike spider's PLDs, complement was activated only via the classical pathway. Interestingly, Heminecrolysin was found to induce PS exposure on human nucleated Jurkat T cells, to stimulate secretion of the pro-inflammatory (TNF-α, IL-6), and anti-inflammatory (IL-10) cytokines by human monocytes, and to provoke a disseminated intravascular coagulation on chick embryo chorioallantoic membrane model system.

Abbreviations: BSA-LPC, BSA bound-LPC; C1P, ceramide 1-phosphate; GP, glycophorin; HBS, HEPES-buffered saline; HPPA, 3-(4-hydroxy-phenyl propionic acid); IC50, half maximal inhibition concentration; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; lysoPLD, lysophospholipase D; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PC, phosphatidylcholine; PLD, phospholipase D; PS, phosphatidylserine; SM, sphingomyelin; SMaseD, sphingomyelinase D; VBS^{2+}, veronal-buffered saline.

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0041-0101/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2013.03.012

Taken together, our results indicate that Heminecrolysin evokes the major characteristic clinical features of *H. lepturus* envenomation by using mainly its lysoPLD, rather than its SMaseD's, activity.

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1. Introduction

In Iran, scorpion sting mortality is largely attributable to *Hemiscorpius lepturus* from *Hemiscorpiidae* family (Prendini, 2000). Venom of *H. lepturus* does not act as typical scorpion venom and elicits a complex pattern of clinical signs and symptoms that are dissimilar to other scorpions in terms of type, duration and severity (Pipelzadeh et al., 2007). Unlike venoms of *Buthidae* scorpions which are neurotoxic, venom of *H. lepturus* is cytotoxic and acts mainly on blood cells, kidneys and the liver function (reviewed in Jalali et al., 2010).

H. lepturus scorpion and *Loxosceles* brown spiders share several clinical and toxicological-induced manifestations such as hemolysis, local skin injury, vascular leakage, persistent inflammation, platelet aggregation, acute renal failure, cardiovascular disease and central nervous system disorders (Radmanesh, 1990; Jalali et al., 2010; Tambourgi et al., 2010). Venoms of both species seem to act mainly via sphingomyelinase D (SMaseD) proteins, also referred to as dermonecrotic toxins (Tambourgi et al., 1998; Borchani et al., 2011a).

Spider's dermonecrotic toxins are very characteristic and conserved molecules. They were characterized as SMaseDs molecules based on their ability to hydrolyze the phospholipid SM, a major constituent in the outer leaflet of the lipid bilayer of plasma membrane, yielding to the production of choline and ceramide 1-phosphate (C1P) (Kurpiewski et al., 1981). Several studies have reported that SMaseDs differ in their catalytic efficiency, substrate specificity or their biological effects intensity. These molecules have been grouped into two classes based on sequence, structural and biochemical data (Murakami et al., 2006). The class I, represented by SMaseD1 from *Loxosceles laeta*, is characterized by the presence of a single disulphide bridge and an extended hydrophobic loop. Class II comprises SMaseDs that contain an additional intra-chain disulphide bridge linking the flexible loop to the catalytic loop. Depending on their ability to hydrolyze SM, they were further subdivided into class IIa, more active, and IIb less active or inactive. Based on lipid biochemical analysis, these proteins were later found to hydrolyze not only sphingophospholipids but also lysoglycerophospholipids in order to generate LPA. They were therefore renamed phospholipases D (PLD) to represent a more accurate and broader denomination (van Meeteren et al., 2004; Lee and Lynch, 2005). It has been postulated that by hydrolyzing phospholipids generating C1P or LPA, spiders PLDs activate signaling pathways in different cell types causing pathophysiological changes among which complement dependent-hemolysis, inflammatory response, platelet aggregation, and an increase of blood vessel permeability (for review, Chaim et al., 2011).

LPA has attracted much interest due to its involvement in physiological and pathological conditions. LPA is produced by activated platelets, endothelial cells, or by enzymatic cleavage of membrane phospholipids (Eichholtz et al., 1993; Aoki et al., 2002). It is typically present in micromolar concentration, in biological fluids and in higher concentration at sites of inflammation (Chen et al., 2003). LPA has been shown to induce its various biological and pathological responses by signaling through five specific G-protein-coupled receptors, referred to as LPA_1–LPA_5, (Anliker and Chun, 2004). It was often associated with platelet aggregation during coagulation, endothelial hyperpermeability, and with various immunomodulatory responses including prevention of apoptosis, chemotaxis, cytokines (IL-1β, TNFα, IL-6) and chemokines (IL-8, MCP-1) secretion (Moolenaar et al., 2004). The production of LPA from albumin-bound LPC was found to stimulate an influx of micromolar extracellular Ca^{2+} into fresh red blood cells (Yang et al., 2000). This results in a loss of membrane asymmetry and an exposure of PS on the cell surface, facilitating therefore the binding of C1q, the initiator of the complement classical pathway activation (Tambourgi et al., 2007). Several previous reports have shown that cell stimulation with LPA can activate NF-κB (Palmetshofer et al., 1999; Fang et al., 2004). LPA-induced NF-κB signaling controls the production of pro-inflammatory cytokines and chemokines in tumor cells, and contributes directly or indirectly to the survival of the malignant cells, indicating important pathophysiological functions for this pathway (Fang et al., 2004; Mills and Moolenaar, 2003).

Following a scorpion envenomation, a variety of pro-inflammatory cytokines (IL-1, IL-6, TNF-α), and chemokines (Il-8) are released along with counter-regulatory anti-inflammatory cytokines (IL-10) (Meki and Mohey El-Dean, 1998; Jalali et al., 2011). The outcome of an inflammatory response is controlled by a variety of factors among which the balance between the pro-inflammatory and anti-inflammatory responses (Petricevich, 2006). Jalali et al. (2011) have observed that in comparison to *Mesobuthus eupeus* scorpion which causes primarily neurotoxic manifestations, *H. lepturus*-stung patients presented, significantly and in a severity-related manner, higher mean values of serum IL-1β, IL-6, IL-10, IL-8 and TNF-α.

In a previous study, we reported that Heminecrolysin, a 33 kDa protein of *H. lepturus*, is able to hydrolyze the SM and presents dermonecrotic and hemolytic activities, which are the characteristics of the whole venom pathological effects (Borchani et al., 2011a). We also noticed that its low SMaseD activity, comparatively to that of spider's dermonecrotic toxins, cannot account alone for its high hemolytic activity. The aim of this study was to check whether this unique scorpion SMaseD hydrolyzes, like spiders SMaseDs, LPC substrate, allowing us to classify this

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