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Evidences of endocytosis via caveolae following blood–brain barrier breakdown by *Phoneutria nigriventer* spider venom

Edilene Siqueira Soares [a], Monique Culturato Padilha Mendonça [a,b], Silvia Pierre Irazusta [c], Andressa Coope [d], Leila Miguel Stávale [a], Maria Alice da Cruz-Höfling [a,b,*]

[a] Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas (UNICAMP), 13 083-970 Campinas, São Paulo, Brazil
[b] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), 13 083-970 Campinas, São Paulo, Brazil
[c] Faculty of Technology of Sorocaba, State Center of Paula Souza Technological Education (CEETPS), 18013-280 Sorocaba, São Paulo, Brazil
[d] Cell Signaling Laboratory, Faculty of Medical Sciences, State University of Campinas (UNICAMP), 13081-970 Campinas, São Paulo, Brazil

HIGHLIGHTS

- *Phoneutria nigriventer* venom (PNV), known to induce BBB breakdown, increases cav-1 expression.
- The cav-1-labeled capillaries number is higher in PNV-treated P14 rats than in adult rats.
- The number of Purkinje cells expressing cav-1 increases after PNV envenoming.
- The variable age affects cav-1 induction by PNV in the white matter and granular layer.

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ABSTRACT

Spider venoms contain neurotoxic peptides aimed at paralyzing prey or for defense against predators; that is why they represent valuable tools for studies in neuroscience field. The present study aimed at identifying the process of internalization that occurs during the increased trafficking of vesicles caused by *Phoneutria nigriventer* spider venom (PNV)-induced blood–brain barrier (BBB) breakdown. Herein, we found that caveolin-1α is up-regulated in the cerebellar capillaries and Purkinje neurons of PNV-administered P14 (neonate) and 8- to 10-week-old (adult) rats. The white matter and granular layers were regions where caveolin-1α showed major upregulation. The variable age played a role in this effect. Caveolin-1 is the central protein that controls caveolae formation. Caveolar-specialized cholesterol- and sphingolipid-rich membrane sub-domains are involved in endocytosis, transcytosis, mechano-sensing, synapse formation and stabilization, signal transduction, intercellular communication, apoptosis, and various signaling events, including those related to calcium handling. PNV is extremely rich in neurotoxic peptides that affect glutamate handling and interferes with ion channels physiology. We suggest that the PNV-induced BBB opening is associated with a high expression of caveolae frame-forming caveolin-1α, and therefore in the process of internalization and enhanced transcytosis. Caveolin-1α up-regulation in Purkinje neurons could be related to a way of neurons to preserve, restore, and enhance function following PNV-induced excitotoxicity. The findings disclose interesting perspectives for further molecular studies of the interaction between PNV and caveolar specialized membrane domains. It proves PNV to be excellent tool for studies of transcytosis, the most common form of BBB-enhanced permeability.

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1. Introduction

The blood–brain barrier (BBB) steady-state is crucial for preserving the homeostasis of the central nervous system (CNS) and normal neuronal and glial activity (Bradbury, 1993; Abbott

Abbreviations: BBB, blood–brain barrier; Cav-1, caveolin-1; CNS, central nervous system; GL, granular layer; ML, molecular layer; PC, Purkinje cells; PL, Purkinje layer; PNV, *Phoneutria nigriventer* venom.

* Corresponding author at: Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas (UNICAMP), Charles Darwin s/n, 13 083-970 Campinas, São Paulo, Brazil. Tel.: +55 19 3521 6224; fax: +55 19 3521 6185.
E-mail address: hofling@unicamp.br (M.A.d. Cruz-Höfling).

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et al., 2010). BBB disruption results from disarray of tight and adhesion endothelial cell proteins that prevent bi-directional paracellular diffusion and/or disturbance of the transport proteins and enzymes that restrict trans-cellular movement of solutes across the blood–brain border.

The armed-spider *Phoneutria nigriventer* (*Araneae*, *Ctenidae*), also known as the wandering spider or armed-spider, is responsible for a great number of accidents in the Southeast of Brazil. The majority of accidents only cause local pain and edema (89.8%). Less than 0.5% of cases are considered severe, involving systemic neurotoxic manifestations such as tremors, convulsions, spastic paralysis, priapism, cardiovascular arrhythmia, intense sudoresis and visual disturbances (Brazil and Vellard, 1925; Bucaretchi et al., 2008). These symptoms appear to be more severe in children (Bucaretchi et al., 2000). *P. nigriventer* venom (PNV) contains several types of simple peptides, capable of blocking Ca^{2+} and K^{+} channels and delaying inactivation of Na^{+} channels, consequently affecting neurotransmitter handling (Gomez et al., 2002; Love and Cruz-Höfling, 1986).

In the brain of rats, data have shown that increased trans-endothelial vesicle trafficking is one of the pathways affected after PNV-enhanced BBB breakdown (Le Sueur et al., 2004). Rows of omega-shaped indentations are formed in the adluminal and abluminal aspect of the endothelial plasma membrane followed by extravasation of the electron-opaque extracellular tracer lanthanum nitrate into the neuropil interstitial space. In addition, swollen peri-vascular end-feet processes of astrocytes and edematous synaptic contacts are observed (Le Sueur et al., 2003). However, it remains to be seen how transcytosis in PNV-induced BBB permeabilized vessels is intensified.

The rise of vesicles through the brain microvascular endothelium in cultures has been associated with a high expression of caveolae frame-forming proteins (Xia et al., 2009). Caveolae are flask-shaped invaginations of the plasma membrane described as playing important roles in endocytosis (Lajoie and Nabi, 2010), trans-cellular trafficking (Hansen and Nichols, 2010; Pelkmans and Helenius, 2002), compartmentalization of signaling molecules (Quest et al., 2004), regulation of the endothelium function during blood–brain barrier breakdown (Nag et al., 2009; Predescu et al., 2007), management of lipid homeostasis (Fielding and Fielding, 2001) and intracellular signaling (Stern and Mermelstein, 2010; Sowa, 2012; Luoma et al., 2008). Located in the endothelial cells and pericytes of all of the cortex micro-vessels (Virgintino et al., 2002), caveolin-1 (cav-1) appears to be the most abundant protein of its family and has been described as a selective marker for caveolae (see Razani et al., 2002 for review) in the BBB endothelium (see Zhao et al., 2014 for review). Cav-1 is formed by two structurally distinct isoforms (α and β) (Scherer et al., 1995), of which the alpha isoform is the most highly expressed in the brain (Ikezu et al., 1998).

The aim of the present study was to verify whether the increased trans-cellular trafficking of vesicles found following *P. nigriventer*-induced BBB breakdown involves caveolin-1α. The hypothesis is that omega-shaped indentations present in the capillary and venule endothelia of PNV-administered rats result from caveolae formation and subsequently the endocytotic mechanism used in enhanced transcytosis. In addition, a possible age-related difference in the expression of caveolin-1α was assessed in the white matter, granular layer and molecular layer of the cerebellum of neonate and adult rats at early (2 h), intermediate (5 h) and late (24 h) time periods after PNV envenoming. These periods were associated with severe intoxication, the onset of recovery and the absence of signs of intoxication, respectively. The present study contributes to an understanding of the molecular changes that accompany the PNV-induced BBB permeability and the toxic manifestations seen in victims of accidents involving the armed-spider. Understanding the functional endothelial alterations is likely to provide innovative ways to target the BBB in pathological conditions.

2. Materials and methods

2.1. Animals and venom

Male Wistar rats, aged 14 days (P14 - neonate group) and 8–10 weeks (adult group) were obtained from the Multidisciplinary Center for Biological Research (CEMIB) of the UNICAMP. The adult rats were maintained in a temperature-controlled room (25–28 °C), a 12–12 h light–dark cycle with ad libitum access to food and water for acclimatization. One batch of lyophilized *P. nigriventer* spider venom (PNV) was supplied by the Butantan Institute (São Paulo, SP, Brazil) and stored at −20 °C. It was dissolved in 0.9% sterile saline solution (0.5 mg PNV/ml in 0.9% sterile saline) immediately before use.

2.2. Exposure to P. nigriventer spider venom

The rats ($n = 45$ per age) were given an i.p. sub-lethal injection of PNV (170 μg/kg) while rats in the control group ($n = 45$ per age) received the same volume of 0.9% sterile solution. PNV dose was selected based on previous dose-response assessment (Mendonça et al., 2012). After 2 h (period of intense intoxication signs), 5 h (signs that recovery from intoxication is underway) and 24 h (no sign of intoxication is apparent) ($n = 5$ per time period), the animals were anesthetized with a lethal dose of a mixture (3:1) of ketamine chloride (Dopalen®, 100 mg/kg) and xylazine chloride (Anasedan®, 10 mg/kg) and the cerebellum was excised for the cav-1 immunohistochemistry assay. For qPCR and western blotting assay, the animals were euthanized by decapitation after CO_2 inhalation at 2 h, 5 h and 24 h after PNV injection ($n = 5$ per time period and per technique).

2.3. Imunohistochemistry (IHC)

After anesthesia and thoracotomy, the animals were perfused through the left ventricle with 0.9% sterile saline (150 ml for 8–10 weeks and 20 ml for neonates), followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), with a pH of 7.4 (250 ml for 8- to 10-week-old and 50 ml for neonate rats). The cerebellum was immediately removed and post-fixed in the same fixative overnight. Then, the organ was dehydrated in ethanol series, cleared in xylene and embedded in paraffin (Paraplast®, Sigma–Aldrich, St. Louis, MO, USA). Cav-1 immunohistochemistry was performed in 5 μm thick sections (2035 RM microtome, Reichert S, Leica), as described by Mendonça et al. (2012) using the rabbit polyclonal primary antibody for caveolin-1: isoform α (Santa Cruz, CA, USA – code: SC-894; dilution, 1:500). Negative control was performed with 1% PBS-bovine serum albumin (BSA) but without the primary antibody. The intensity of the cav-1 labeling was analyzed in the white matter and the granular and molecular layers of the cerebellum through the density of pixels obtained in images captured by an Olympus BX51 photomicroscope (Japan), equipped with Image-Pro Plus image analyzer software (Silver Spring, MD, USA). Ten pictures per period/treatment of each cerebellar region were taken using an objective of 40× and fixed illumination parameters. The expression of the protein was assessed using the free access program GIMP 2.6.4 software (GNU Image Manipulation Program, CNE, Free Software Foundation, Boston, MA, USA), which segments the immunochemical reaction by color (Solomon, 2009). Also see Mendonça et al. (2012) and Stávale et al. (2013).

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