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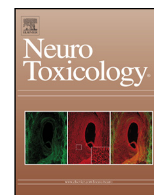

Full length article

Caveolae as a target for *Phoneutria nigriventer* spider venom

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A B S T R A C T

An important transcellular transport mechanism in the blood-brain barrier (BBB) involves caveolae, which are specialized delta-shaped domains of the endothelial plasma membrane that are rich in cholesterol, glycosphingolipids and the scaffolding protein Caveolina-1 (Cav-1). In this work, we investigated whether the increase in endocytosis and transendothelial vesicular trafficking in rat cerebellum after blood-brain barrier breakdown (BBBb) induced by *Phoneutria nigriventer* spider venom (PNV) was mediated by caveolae. The expression of Cav-1, phosphorylated Cav-1 (pCav-1), dynamin-2 (Dyn2), Src kinase family (SKF) and matrix-metalloproteinase-9 (MMP9), proteins involved in caveolar dynamics and BBB opening, was investigated. Immunofluorescence, western blotting (WB) and transmission electron microscopy were used to assess changes at 1, 2, 5, 24 and 72 h post-venom. WB showed upregulation of Cav-1, Dyn2 and MMP9 at 1, 5 and 72 h (corresponding, respectively, to intervals when intoxication was most evident, when signs of recovery were present, and when no intoxication was detectable). In contrast, pCav-1 and SKF, which are essential for internalization and transport, decreased when Cav-1 and Dyn2, proteins essential for caveolar formation, were increased. Overall, these changes indicated that vesicular trafficking across the endothelium (high pCav/SKF levels) coincided with lower numbers of caveolae (Cav-1/Dyn2 downregulation) and lower expression of MMP9. Thus, the internalization (disassembly) of caveolae alternates with caveolar neoformation (assembly), resulting in changes in caveolar density in the endothelium membrane. These caveolar dynamics imply tensional mechanical stress that is important in triggering key signaling mechanisms. We conclude that PNV-induced breakdown of transcellular transport in the BBB is caused by an increase in caveolae-mediated endocytosis; this effect was correlated with the progression of temporal signs of envenoming. Caveolar dynamics are probably involved in shear stress and BBBb regulatory mechanisms in this experimental model.

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1. Introduction

The venom of *Phoneutria nigriventer* (banana or wandering spider), a medically important spider in Brazil, contains a variety of pharmacologically-active neuropeptides, some of which activate or delay the inactivation of tetrodotoxin (TTX)-sensitive Na^+ channels; this modulation results in the depolarization of excitable membranes by increasing Na^+ influx and increase the stores of intracellular Ca^{2+}. *P. nigriventer* venom (PNV) also blocks K^+ channels and different types of Ca^{2+} channels (Fontana and Vital-Brazil, 1985; Love et al., 1986; Gomez et al., 2002). The ability of PNV to interfere with ion channel physiology adversely affects neurotransmission, as shown by the modulation of glutamate release and glutamate uptake (see Gomez et al., 2002). The clinical manifestations of severe envenomation by *P. nigriventer* include pain, edema, intense sweating, muscle weakness, arterial hypertension, cardiac arrhythmias, respiratory distress, agitation, blurred vision and sometimes convulsions (Bucaretchi et al., 2000, 2008).

Various studies have shown that PNV disrupts the blood-brain barrier (BBB) and alters the expression of proteins related directlyor indirectly to barrier function (Le Sueur et al., 2003, 2004, 2005; da Cruz-Höfling et al., 2009; Mendonça et al., 2012, 2013, 2014; Rapôso et al., 2007, 2012, 2014; Stávale et al., 2013). PNV-induced BBB opening involves a marked increase in transcellular vesicle transport (Le Sueur et al., 2004, 2005)

Abbreviations: BBB, blood-brain barrier; BBBb, blood brain-barrier breakdown; Cav-1, caveolin-1; CNS, central nervous system; Dyn2, Dynamin-2; IF, immunofluorescence; MMP9, metalloproteinase-9; pCav-1, phosphorylated caveolin-1; PNV, *Phoneutria nigriventer* venom; SKF, Src tyrosine kinase family; TEM, transmission electron microcopy; WB, western blotting.

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and redistribution of endothelial junction proteins (Rapôso et al., 2012; Mendonça et al., 2013). Augmented endothelial transcytosis in the brain microvessels of rats injected with PNV was seen as rows of flask-shaped indentations at the adluminal and abluminal surfaces of endothelial and smooth muscle cells of venules and arterioles. The finding that the indentations were loaded with lanthanum nitrate, a peripherally-injected extracellular tracer, indicated tracer uptake from the vessel lumen and vesicle trafficking across the endothelium with delivery of the vesicle to the interstitial spaces of the neuropil; cytotoxicity and vascular edema were also observed (Le Sueur et al., 2003, 2004). These flask-shaped indentations, known as caveolae, are cholesterol- and glycosphingolipid-rich membrane microdomains that compartmentalize many molecules, including caveolin, endothelial nitric oxide synthase, G proteins and phosphatases (Pattel et al., 2008).

Under physiological conditions, the BBB helps to maintain a stable microenvironment in the central nervous system (CNS) that ensures the proper functioning of neuronal and glia cells in brain homeostasis. Homeostasis of the CNS disrupts by many factors, including, but not limited to, endogenous neurodegenerative diseases and exogenous neurointoxication such as caused after envenomation by venomous animals.

Typically, the endothelium of the brain microcirculation possesses few caveolae, as high electrical resistance and highly receptor- and carrier-regulated transcytosis restrict transport at the BBB. Therefore, an increase in the number of caveolae after exposure to PNV implies venom-induced permeabilization of the BBB through impairment of the transcellular pathway. We have recently shown that PNV upregulates the expression of caveolin-1α (Cav-1), the main caveolar scaffolding protein, and increases the number of vessels labeled with Cav-1 and the number of Cav-1 positive Purkinje neurons in rat cerebellum (Soares et al., 2014). These findings suggest a role for Cav-1 in the increased endocytosis and transcytosis reported in earlier studies of PNV-induced BBB breakdown (Le Sueur et al., 2003, 2004) and a possible role in neuronal signaling (Stern and Mermelstein, 2010).

Caveolin coated-caveolae contribute to the regulation of cell membrane dynamics (Nassoy and Lamaze, 2012) and caveolae mediate the endocytosis and trafficking of substances by sorting transporting vesicles into the cell cytoplasm (Pelkmans and Helenius, 2002; Shajahan et al., 2004b). Increased transcytosis of plasma protein by endothelial caveolae has also been linked to BBB breakdown and cerebral edema (Nag et al., 2009). Caveolae-derived vesicle trafficking requires phosphorylation of Cav-1 (pCav-1) by activation of the Src kinase family (SKF) (Shajahan et al., 2004b; Nag et al., 2009) and dynamin-2 (Dyn2), a caveolae neck-forming protein with a role in caveolar scission and internalization (Yao et al., 2005); the activation of these proteins results in plasma membrane flattening (Henley et al., 1998).

The purpose of the present study was to investigate two proteins that have pivotal importance in caveolar structure and function, namely caveolin-1 and dynamin-2; we also assessed possible mechanistic pathways triggered in response to circulating PNV in rats. This work was done at specific intervals of envenoming, namely, (a) when intense manifestations of acute toxicity were present (1–2 h post-PNV), (b) when recovery was in progress (5 h post-PNV) and (c) when there were no clinical manifestations and the rats showed normal behavior (24–72 h post-PNV).

2. Materials and methods

2.1. *P. nigriventer* venom (PNV)

Lyophilized venom from *P. nigriventer* (PNV) was kindly donated by Dr. Evanguedes Kalapothakis (UFMG, Belo Horizonte, MG, Brazil) and stored at −20 °C. The venom was dissolved in 0.9% sterile saline solution (0.5 mg PNV/ml sterile saline) immediately prior to use.

2.2. Animals and envenoming procedure

Male Wistar rats (*Rattus norvegicus*) 6–7 weeks old were obtained from the Multidisciplinary Center for Animal Investigation (CEMIB) at UNICAMP (Campinas, SP, Brazil). They were maintained at a 12 h light-dark cycle (25–28 °C) with free access to food and drinking water until the experimental procedure was performed. A single intravenous (i.v.) injection of a sub-lethal dose of PNV (0.5 mg/kg) (Soares et al., 2015) was administered to rats (n = 55 total) that were subsequently killed at 1, 2, 5, 24 and 72 h post-PNV (n = 11/interval, i.e., n = 5 for western blotting and n = 3 for immunofluorescence and transmission electron microscopy per time interval). A single control group received the same volume of vehicle and was killed 5 h later (n = 11, i.e., n = 5 for western blotting and n = 3 for immunofluorescence and transmission electron microscopy). The experiments were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol no. 3609-1) and were done in accordance with the general ethical guidelines of the Brazilian Society for Laboratory Animal Science (SBCAL).

2.3. Western blotting (WB)

After venom or saline treatment, the rats were killed in a CO_2 chamber and their brains removed and immediately frozen in liquid nitrogen. Brain proteins were subsequently separated in an extraction cocktail and run on 12% polyacrylamide gels in SDS-PAGE followed by electrotransfer to nitrocellulose membranes, as described elsewhere (Rapôso et al., 2012). The membranes were blocked overnight with 5% non-fat milk and incubated for 4 h with primary antibodies against caveolin-1 (1:1000), dynamin-2 (1:500), and metalloproteinase-9 (1:500), all from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Src tyrosine kinase family (SKF, 1:1000) and *p*-caveolin-1 (1:1000), both from Cell Signaling Technology (Danvers, MA, USA) and β-actin (1:1000; Sigma-Aldrich, St. Louis, MO, USA). The membranes were then incubated with secondary antibodies: anti-rabbit (1:1000) for caveolin-1, SKF and *p*-caveolin-1, anti-goat (1:1000) for dynamin-2 and metalloproteinase-9, and anti-mouse for β-actin (1:40,000; all from Sigma-Aldrich). Proteins bands were visualized by chemiluminescence (Thermo Scientific, Waltham, MA, USA) and band intensity was quantified by measuring the density of pixels of each band using Image J 1.45 s program (Wayne Rasband, NIH, Bethesda, MD, USA). The experiments were performed in a set of three replicates. The quantifications were normalized against the corresponding value for endogenous β-actin.

2.4. Immunofluorescence (IF)

One, 2, 5, 24 and 72 h after envenoming or saline administration the rats were killed with an overdose of anesthesia using a 3:1 mixture of ketamine chloride (Dopalen®, 100 mg/kg) and xylazine chloride (Anasedan®, 10 mg/kg). After that the rats were immediately perfused with 0.9% saline solution (100 ml) via the left ventricle followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (200 ml) as fixative. The cerebella were excised and immersed in 15% sucrose and then in 30% sucrose for cryoprotection (24 h each). Cerebellar samples were immersed in OCT-Tissue Tek (Sakura Finetek, Torrance, CA, USA) and frozen in *n*-hexane in liquid nitrogen (−70 °C). Frozen sections 5 μm thick were mounted on silanized glass slides and after rinsing with Trizma® buffer, pH 7.4, the slides were permeabilized with 0.1%

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