

Investigations

Effects of Recombinant Toxin Phospholipase D in Cardiac Muscle of Rats

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Article history

Received: 27-11-2014

Revised: 30-03-2015

Accepted: 16-05-2015

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Abstract: Loxoceles spiders gender are worldwide spread and its bites can cause dermonecrosis or even a systemic effect (hemolysis, kidney and liver injury). It is believed that phospholipase D, the main component present in the venom, could be responsible for the injury. In this study, we used a recombinant form of phospholipase D (rLiD1) and evaluated its direct and systemic effects on the contractility of papillary muscles and in the left intra ventricular pressure of isolated perfused hearts, respectively. In papillary muscle directly exposed to rLiD1 no effects on force, maximum speed of contraction (df/dt_{max}) or relaxation (df/dt_{min}) were observed. In isolated perfused heart, the peak of systolic pressure and the rate of relaxation (dP/dt_{min}) were reduced in animals treated with rLiD1. However, the maximum speed of pressure developed during contraction (dP/dt_{max}) was unaffected. These data suggest that rLiD1 did not affect directly the excitation contraction coupling or the contractility of the myocardium but its systemic effect can induce reduction in the cardiac performance.

Keywords: Cardiac Muscles, Excitation Contraction Coupling, Phospholipase D

Introduction

Loxoscelic accident or loxoscelism is the designation of the clinical outcome developed by individuals bitten by spiders of the genus *Loxosceles* (Futrell, 1992). This condition can manifest as dermonecrotic (84-97% of cases) or as a systemic outcome (3-16% of cases) (Barbaro *et al.*, 1992). The systemic effects are potentially fatal and should be medically addressed (Peterson, 2006).

In Brazil, the *Loxosceles* spider most closely related to accidents are the *Loxosceles gaucho*, *Loxosceles laeta* and *Loxosceles intermedia*. Regardless of the species, this poison can cause dermonecrotic and systemic outcome (Barbaro *et al.*, 2005; Chaim *et al.*, 2011; da Silva *et al.*, 2004).

The dermonecrosis is characterized by a rash in the bite region (Futrell, 1992). Clinical findings of the systemic effects are hemolytic anemia, thrombocytopenia resulting from platelet aggregation and intravascular coagulation, events that culminate in decreased hematocrit (Peterson, 2006). The ELISA method detected circulating antigens in kidney, heart,

lung and liver of poisoned rats (Dias-Lopes *et al.*, 2010). The main reason of death in cases of loxoscelism is due to acute renal failure (Futrell, 1992; Peterson, 2006).

Studies have shown that the component of the venom responsible for dermonecrotic effects is phospholipase D because this toxin hydrolyzes sphingomyelin and glicerophospholipids (Chaim *et al.*, 2011; Lee and Lynch, 2005).

The phospholipase D interacts with and degrades phospholipids, generating ceramide-1-phosphate and lysophosphatidic acid, initiating a cascade that allows the calcium to enter the cell by the L-type channels contributing to hemolysis (Chaves-Moreira *et al.*, 2011).

Hearts of mice poisoned by phospholipase D have increased the calcium current and L-type channels also increase the calcium transient, although the force of systolic contraction is decreased (Dias-Lopes *et al.*, 2010).

There is only a few data relating the effects of recombinant toxin phospholipase D in cardiac muscle. In this study, we investigated the direct and systemic effects of rLiD1 in cardiac muscle of the rat.

Materials and Methods

Animals

Thirty-four male Wistar rats, weighing 250-300 g were used. The animals were kept in cages under controlled conditions of temperature and light-dark cycle of 12 h, with free access to food and water. The Animal Experimentation Ethics Committee of the Biological Sciences Section at Federal University of Paraná approved all experimental protocols used in this study (AEEC-591). Animals were anesthetized (50 mg kg⁻¹ of ketamine and 10 mg kg⁻¹ of xylazine, injected intra peritoneally). After opening of chests the hearts were removed and transferred to a Becker containing 20 mL of Ringer's solution. This solution had the following composition (in mM): NaCl = 110; KCl = 4.0; CaCl₂ = 2.0; MgCl₂ = 2.0; TRIZMA = 10 and glucose 11, pH adjusted to 7.4 with NaOH or HCl and gassed with pure oxygen.

Recombinant Toxin Phospholipase D(rLiD1)

The recombinant toxin was provided by the Laboratory of Extracellular Matrix and Poisons Biotechnology, division of Biological Science from The Federal University of Paraná. The toxin was produced as described previously (Chaim *et al.*, 2006; Chaves-Moreira *et al.*, 2011). The integrity, purity and quality of rLiD1 was checked by electrophoresis (Harlow and Lane, 1988) and its activity was measured using the Amplex Red Assay Kit (Molecular Probes, Eugene) as previously described (Appel *et al.*, 2008; Chaves-Moreira *et al.*, 2011).

Experimental Design

Rats were randomly separated into four groups, each containing a control group and an experimental group:

Group 1: Isolated perfused heart

Group 2: Isolated papillary muscle

Isolated Perfused Heart

The animals in this group were pretreated with rLiD1 (240 µg kg⁻¹) (Lucato *et al.*, 2011) administered intra peritoneally. Animals of the control group were injected with equivalent volume of PBS. The evaluation of cardiac performance of these animals was done 24 h after injection.

As previously described (Dianat *et al.*, 2014; Prendes *et al.*, 2014), the heart was quickly removed and transferred to a Langendorff perfusion system where steel cannula was secured to the aortic stump by suture, allowing immediate perfusion with Ringer's solution composition already described, continuously oxygenated, maintained 37°C and pH 7.4, immersed in Ringer's solution of identical composition. For measurement of left ventricular pressure, the left atrium was removed and a plastic-made balloon film connected

to a pressure transducer (WPI-rBPI) was inserted into the left ventricle through the mitral valve. The volume of the balloon was gradually adjusted to obtain the maximum value in the volume/pressure generated by myocardial contraction. The data were obtained using an acquisition system PowerLab 4/30, AD Instrument and subsequently analyzed using Lab Chart version 7.3.7 software.

Isolated Papillary Muscle

After thoracotomy, the heart was removed and transferred to a Petri dish containing Ringer's solution, as previously described (De Campos *et al.*, 2014; Szkudlarek *et al.*, 2014). The papillary muscles were immediately removed using ophthalmological scissors and mounted horizontally in a chamber with capacity for three milliliters containing Ringer's solution at a temperature of 37°C and pH 7.4, continuously oxygenated. The myocardial portion of the papillary muscle was fixed to a stationary clip while the tendinous portion was attached to a force transducer (Fort 10 WPI, Transduction Laboratories Co.). The papillary muscles were stimulated (0,5 Hz) with supra-threshold voltage pulses (10 to 15 V), with duration of a maximum of five milliseconds (ms) through a pair of platinum electrodes positioned along the entire length of the muscle. Under these conditions, the muscles were maintained for a stabilization period of 30 min and then the experimental protocols were performed. The data were collected using an acquisition system PowerLab 4/30, (AD Instrument) and subsequently analyzed using Lab Chart version 7.3.7 software.

To evaluate the possible effect of phospholipase D on the strength of isometric contraction was added to chamber increasing concentrations of phospholipase D (8.3 and 16.6 µg mL⁻¹). We evaluated the effects of toxin for 30 min. The experiments were performed in the control group with equivalent volume of PBS.

Statistical Analysis

Data are presented as mean ± standard error of the mean. Comparison in a single experimental condition between treated and control groups, will be done using Student's t-test. For multiple comparisons, in the same experimental condition ANOVA will be used. For data analysis and plotting GraphPad Prism 5 Software (San Diego, CA, USA) software was used. Statistically significant difference between groups will be accepted when the probability of the null hypothesis is less than or equal to 5% (p ≤ 0.05).

Results

The peak of left ventricle systolic pressure developed by isolated perfused hearts (LIVP) is shown in the Fig. 1-3. Significant differences between control and animals treated with rLiD1 were observed. The LIVP in control and treated groups were 137,3 ± 4,5 mmHg and 123,7 ± 3,4 mmHg (p = 0,031), respectively.

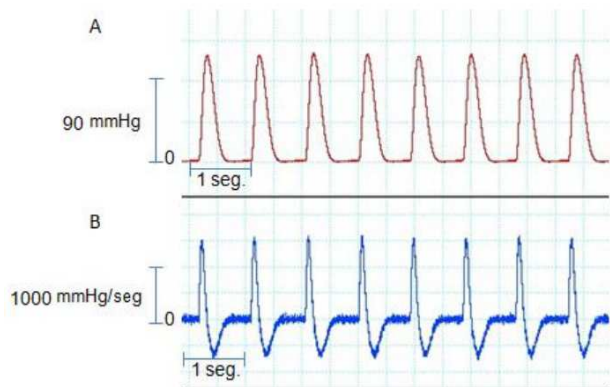


Fig. 1. Typical record of the left intra ventricular pressure of isolated perfused heart in (A) and the calculated first derivative in (B)

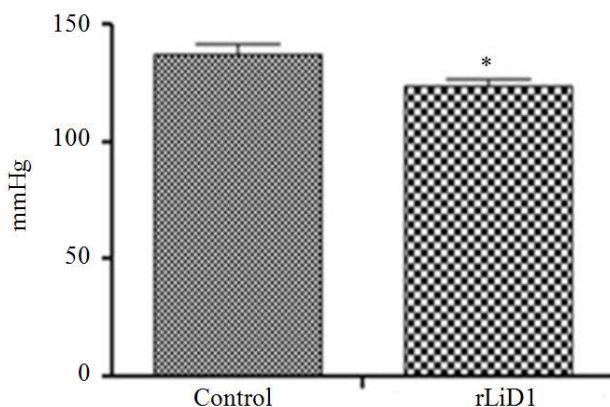


Fig. 2. Peak of left ventricle systolic pressure of isolated perfused heart from control and animals treated with rLiD1. The results are expressed as mean \pm sem. * represent statistical significant difference between groups. In the control and in treated animals the values were, respectively 137,3 \pm 4,5 and 123,7 \pm 3,4 mmHg. (p = 0.031)

However, no differences in the maximal speed of pressure development by the left ventricle (dp/dt_{max}) had been observed (1536,5 \pm 55,7 mmHg/sec in the control group vs 1392,3 \pm 37,6 mmHg/sec in rLiD1 treated group, Fig. 3A). Also, the maximal speed of pressure decrease in left ventricle (dp/dt_{min}) was significantly reduced in treated group (Fig. 3B). The dp/dt_{min} in the control and in the treated group were -819,1 \pm 29,0 mmHg/sec and -710,3 \pm 23,0 (p = 0,010), respectively.

The data obtained in isolated papillary is showed in Table 1. The maximal force developed, maximal speed of contraction (df/dt_{max}) and the maximal speed of relaxation (dp/dt_{min}) were unaffected by the presence of rLiD1 (8,3 or 16,6 μ g mL^{-1}).

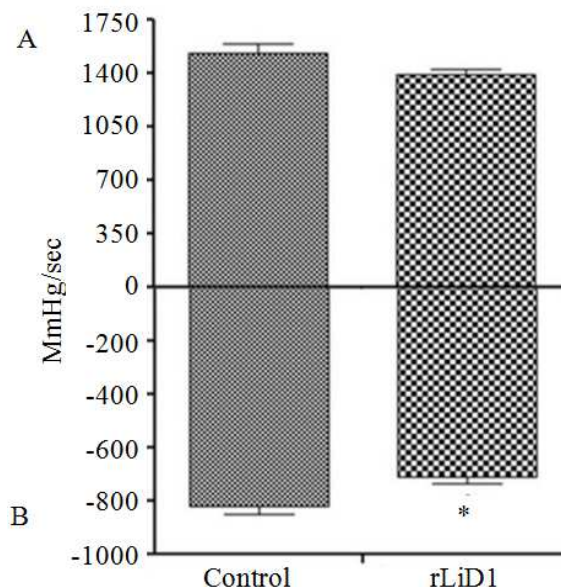


Fig. 3. Effects of rLiD1 on the maximal speed of the left intra ventricular pressure during the contraction (dp/dt_{max}) (A) and relaxation (dp/dt_{min}) (B). The results are expressed as mean \pm sem. *represent statistical significant difference between groups. In the control and in rLiD1 treated group the dp/dt_{max} and dp/dt_{min} were, respectively (in mmHg/sec) 1536,5 \pm 55,7 and 1392,8 \pm 37,6 (p = 0.0508); and -819,1 \pm 29,0 and -710,3 \pm 23,0 (p = 0,010)

Table 1. The direct effects of rLiD1 (8,3 and 16,6 μ g mL^{-1}) on the twitch, maximal speed of contraction (df/dt_{max}) and relaxation (df/dt_{min}) of isolated papillary muscles. The twitch force is expressed in mN/mm^2 . df/dt_{max} and df/dt_{min} in $mN/mm^2/sec$. The data is expressed as mean \pm sem

		Ringer	8,3 μ g mL^{-1}	16,6 μ g mL^{-1}
Force	Control	13,1 \pm 2,0	12,3 \pm 1,3	10,6 \pm 0,6
	rLiD1	18,6 \pm 4,3	18,4 \pm 2,7	15,7 \pm 1,9
df/dt_{max}	Control	154,5 \pm 25,5	149,6 \pm 17,9	144,4 \pm 9,9
	rLiD1	240,0 \pm 43,8	243,6 \pm 38,4	232,2 \pm 34,4
df/dt_{min}	Control	-132,5 \pm 33,0	-110,0 \pm 11,5	-113,0 \pm 7,9
	rLiD1	-184,7 \pm 40,8	-204,0 \pm 39,8	-186,6 \pm 33,6

Discussion

Sphingomyelin is the main substrate of phospholipase D. Sphingomyelin products such as sphingosine and ceramide modulate potently cell activity by the activation of a G protein, triggering a cascade of events leading changes in intracellular Ca^{++} concentration which affects the activity of enzymes involved in the regulation of Ca^{++} and modulating intracellular Ca^{++} levels (Tornquist *et al.*, 2004). Ceramide can be phosphorylated by ceramide kinase originating ceramide-1-phosphate. Ceramide-1-phosphate can bind to sphingolipids receptors present

in the cell membranes activating PKC and in a dose-dependent manner, increases the influx of Ca^{++} (Hannun, 1996).

Cardiac troponin I can be phosphorylated by PKC (Roman *et al.*, 2004; Dong *et al.*, 2012). The calcium sensitivity of the myofilaments is decreased by the phosphorylation of Troponin I resulting in the reduction of the speed of cross bridge cycle. In other words, PKC phosphorylates troponin I and negatively regulates cardiac contraction (Roman *et al.*, 2004). This could explain the reduction in the peak of left ventricle systolic pressure observed in the present work. Also, mice envenomed with crude *Loxocles* venom showed a reduction in the force production (Dias-Lopes *et al.*, 2010). The reduction in the maximum speed of relaxation of the left ventricle (dP/dt_{min}) observed here suggest that rLiD1 can affect the mechanisms responsible for the reduction of intracellular calcium concentration during diastole (calcium uptake by the sarcoplasmic reticulum and/or the activity of sodium calcium exchange, calcium pump present in the sarcolemma).

The absence of direct effects rLiD1 on papillary muscle (peak of force developed during twitches, dF/dt_{max} and dF/dt_{min}) suggest that the excitation contraction coupling and the contractility are not affected by the toxin. One interesting possibility is that the amount of sub products formed by the action of the toxin is insufficient to induce the effects observed in isolated hearts.

Conclusion

Recombinant toxin phospholipase D (rLiD1) did not affect directly the excitation contraction coupling or the contractility of the myocardium but its systemic effect can induces reduction in the cardiac performance.

Acknowledgement

We would like to thank CAPES for the financial support of this work.

Author's Contributions

Joao Victor Capelli Peixoto: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Fernando Augusto Lavezzo Dias: Participated in all experiments.

Carlos Estevan Nolf Damiani: Contributed to the data-analysis and to the writing of the manuscript.

Ilana Kassouf Silva: Contributed to the writing of the manuscript.

Silvio Sanches Veiga: Coordinated the production and quality testing of phospholipase D.

Julio Cesar Francisco: Coordinated the mouse work.

Rosalvo Tadeu Hochmueller Fogaça: Designed the research plan, organized the study and contributed to the writing of the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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