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Nanostructures as Robust Multimodal Anti-*Bothrops* Snake Envenoming Agents

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Abstract: Snake envenoming is a partly solved threat that constitutes a major concern for the public health of tropical and subtropical countries. Antisera based in polyclonal heterologous antibodies save the victims' life, but often are not efficient enough to avoid the amputation of the affected limbs. In this strategic approach we propose to complement the site-specific activity of the antisera by co administering special nanovesicles capable of adsorbing and thereby reducing the enzymatic activities responsible for neurotoxicity, pain, haemolysis, and fundamentally severe inflammation and myonecrosis, that end up in limbs 'amputation.

Keywords: nanovesicles, archaeolipids, envenoming, A-2 phospholipase

1. INTRODUCTION

In 2017, the snake envenoming was included as Tropical Orphan Diseases class A, between the conditions causing a major impact on worldwide health [WHO]. Annually nearly 2,5 million people-mostly women, child and rural farmers from low income countries- are victims of snake envenoming; nearly 120.000 die and 300.000 remain with some type of physical sequela (amputation). In Latin America, most snakebite is caused by species of the *Bothrops* genus (Gutiérrez, J.M., 2010). *Bothrops* venoms contain a range of molecules that may provoke local swelling, pain, renal and respiratory insufficiencies. Phospholipases A2 (PLA2s) is a common component of *Bothrops* venoms that hydrolyses glycerophospholipids at the sn-2 position of the glycerol backbone, upon binding by [protein-protein] interaction to sites different from the catalytic one, exerting pharmacological action in the target cells. As a result, the neuromuscular transmission is blocked, and acute muscle damage is caused, two activities responsible for key clinical manifestations in human envenomings (Warrell, D.A., 1996).

In this work we tested the effect of nanovesicles made of archaeolipids extracted from an hyperhalophilic archaebacteria (nanoarchaeosomes) as surrogate myocytes, on the activity of bee PLA2 as model enzyme for the more complex *Bothrops* venom. Different to nanoliposomes made of ordinary phospholipids, archaeolipids-containing nanoparticles remain structurally stable against a wide range of physical and chemical insults. The unique chemical structure of archaeolipids (ether linked glycerol backbone instead of ester, 2,3 sn stereoisomery and polyisoprenoi fully saturated chains) account for crucial difference between nanoliposomes bilayer and highly entropic but low fluid bilayer of nanoarchaeosomes (Caimi, A.T. et al, 2017; Higa, L.H. et al, 2017; Altube M.J. et al, 2017). The PLA2 activity was revealed employing an artificial substrate, consisting of a nanovesicle prepared with anionic lipids and a fluorescent hydrolysable derivative. In this first approach we showed that the nanoarchaeosomes acted as a trap for the PLA2 activity, reducing its activity on the artificial substrate.

2. MATERIALS AND METHODS

Hydrogenate soy phosphatidylcholine (HSPC) was from Northern LipidsInc, (Vancouver, Canada); Cholesterol was from Sigma Adrich (Missouri, USA). The remainder analytical grade reagents were from Anedra, Research AG (Buenos Aires, Argentina).

Halorubrum tebenquichense archaeas, isolated from soil samples of Salina Chica, Península de Valdés, Chubut, Argentina were grown in 15 L of basal medium supplemented with yeast extract and glucose at 37°C in a 25 L home-made stainless steel bioreactor. Cultures were monitored by absorbance at 660 nm, and harvested after 96 h growth. Total lipids were extracted from biomass using the Bligh and Dyer method modified for extreme halophiles and the total polar lipid fraction was collected by precipitation from cold acetone (Kates, M. et al, 1995). Between 400 and 700 mg of total polar lipids were isolated from each culture batch. The reproducibility of each total polar lipid extract´s composition was routinely screened by phosphate content (Böttcher, C. et al, 1961), and electrospray ionization mass spectrometry (ESI-MS) as described in Higa et al. 2012 (Higa, L.H. et al, 2012).

Nanoliposomes (L) made of HSPC:cholesterol 9:3 m:m and nanoarchaeosomes (ARC) were prepared by the thin film method. Briefly, the lipid mixtures were dissolved in chlorophorm: methanol 1:1 % v/v; the solvent was eliminated by N2 flush. The films were resuspended with magnetic agitation in 10 mM 0,9 % w/v NaCl pH 7,4 Tris buffer up to a total lipid concentration of 10 mg/mL. The resulting suspension was sonicated 1 h in bath sonicator at 80 W, 80 KHz and then extruded 21 folds across 2 apposed membranes of 200 nm pore diameter polycarbonate membrane (Merck Millipore Ltd. Cork, IRL) employing a manual extrusor (Avanti Polar Lipids, Alabama, USA). Finally, the

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nanovesicles were filtered across a sterile nylon membrane of 200 nm under laminar flow.

The ability of L and ARC to reduce the hydrolytic activity of PLA2 was determined. To that aim, the EnzChek® Phospholipase A2 Assay from Molecular Probes, Invitrogen (Oregon, USA) kit was used. This assay allows following the activity of bee PLA2, employing a fluorescent specific substrate (RED/GREEN BODIPY PC-A2) included in the structure of DOPG/ DOPC nanoliposomes (LS). The hydrolisis of the fluorescent phospholipid is monitored by monitoring the fluorescence intensity (FI) at 515 nm λemission: the higher the FI, the higher the enzymatic activity. The cleavage of BIODIPY FL pentanoic acid at the sn-2 position generates a diminished of the quenching generated by the BIODIPY linked at the sn-1 position.

To estimate the ability of L and ARC to inhibit the PLA2 hydrolytic activity, L and ARC were co-incubated with PLA2 and its LS substrate. Briefly, 0,25 U/mL PLA2 was poured on each of a 96 wells plate. The PLA2 was co-incubated with LS and ARC or L at 2,5; 25 or 75 µg/mL ARQ and L final concentration and 25 µg/mL LS. The maximal activity of PLA2 was determined by incubating with only LS. All incubations were carried out at room temperature. The FI was measured at λemission 515 nm and 460 nm λ excitation every 15 minutes in a *Cytation™ 5* Cell Imaging Multi-Mode Reader.

3. RESULTS

Table 1. Structural features of nanoliposomes

Sample	Mean Diameter (nm)	pdi	ζ Potential (mV)	Total Lipids (mg/ml)
L	157 ± 29	0.12 ± 0.1	-7 ± 1	5.7 ± 1.2
ARC	185 ± 6	0.23 ± 0.1	-39 ± 7	7.2 ± 2.2

Overall, the results showed that both L and ARC reduced the PLA2 activity. Nonetheless, ARC made it in a dose dependent manner, to achieve a reduction of 30 % of the enzymatic activity at 75 µg/mL. On the other hand, identical concentration of L had no effect on PLA2 activity; only at 150 µg/mL a reduction of 15 % activity was achieved. These results suggest that ARC were at least 4 folds more efficient in reducing the PLA2 activity than L.

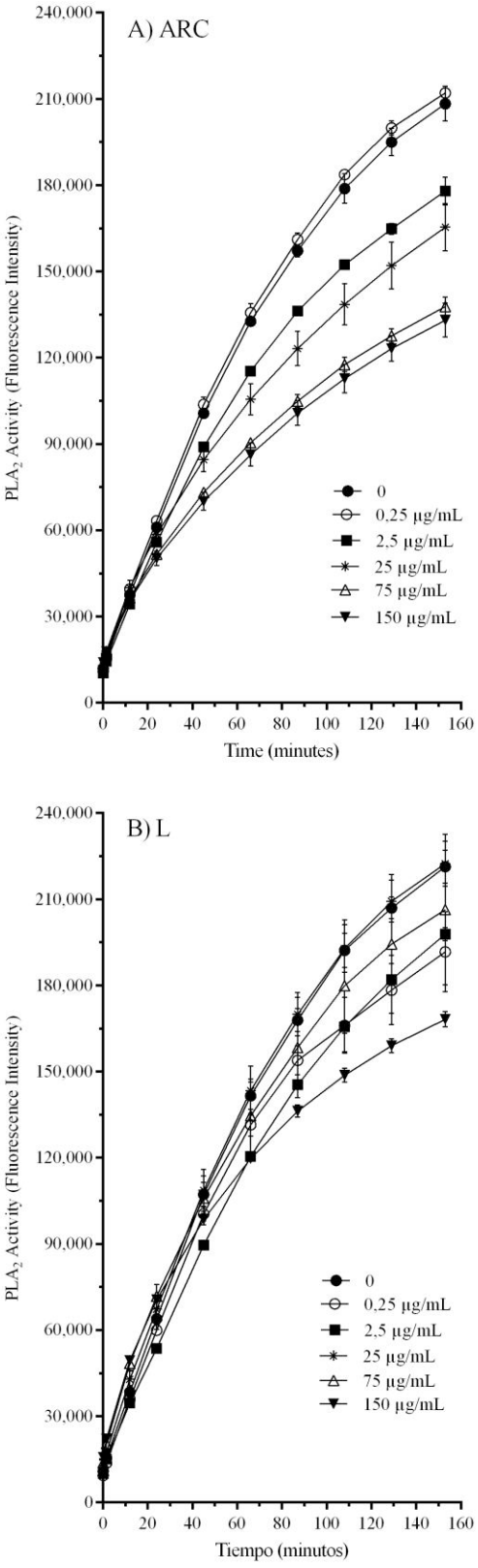

Fig. 1. Inhibition of PLA_2 activity at different concentrations of A) ARC and B) L.

4. CONCLUSIONS

The toxicity of PLA2 is not limited to the hydrolysis of phospholipids, since also a pharmacological activity is exerted by means of interaction with tissue specific protein receptors. In this context, our preliminary results suggested that nanoarchaeosomes made of lipids extracted from *H. tebenquichense*, probably because of the unique structure of nanoarchaeosomes bilayers, may act as sinks to trap interfacial enzymes such as PLA2; by stablishing a competence between nanoarchaeosomes and target cells, the chances of binding to tissues would be reduced. The nanoarchaeosomes thus, can be proposed as aids to supress the PLA2-dependent deleterious activity of snake venoms.

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