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The inhibitory effect of saPLIγ, a snake sourced PLA_2 inhibitor on carrageenan-induced inflammation in mice

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ABSTRACT

SaPLIγ is a natural phospholipase A_2 (PLA_2) inhibitor, isolated from *Sinonatrix annularis,* that has been demonstrated to protect against envenomation by other venomous snakes. As snake venom PLA_2s and mammalian secretory PLA_2s are similar, saPLIγ is thought to have potential to alleviate inflammatory reactions in which PLA_2s act as a key enzyme for arachidonic acid release. The aim of this study was to investigate the anti-inflammatory effects and mechanisms of action of saPLIγ in an animal model of carrageenan-induced acute inflammation. The results indicated that saPLIγ inhibited PLA_2 subtypes extensively, especially IIA-PLA_2, in a dose-dependent manner. Paw swelling in mice was reduced markedly by intraperitoneal saPLIγ 2.5 mg/kg, and the effect was significantly better than observed with dexamethasone at the same dose. Lower neutrophil infiltration and tissue edema was observed in the paws of saPLIγ-treated mice. Additionally, carrageenan-induced cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines (TNFα and IL-1β) were also significantly down-regulated by saPLIγ in a dose-dependent manner. These results suggested that saPLIγ had effective anti-inflammatory effects *in vivo,* and these were produced by blocking mammalian IB, IIA, V and X sPLA2 subtypes.

1. Introduction

Phospholipase A_2 (PLA_2) is one of the most widely distributed enzyme families, and secreted PLA_2 ($sPLA_2$) represents the most abundant subfamily. $sPLA_2$ is a Ca^{2+}-dependent low molecular mass enzyme that catalyzes hydrolysis of membrane phospholipids (especially phosphatidylethanolamines, phosphatidylcholine and phosphatidylinositide) to produce free fatty acids and lysophospholipids. The reaction acts at the top of the arachidonic acid (AA) metabolic cascade, with released AA metabolized further to produce eicosanoids by cyclooxygenase (COX) and lipoxygenase (LOX) pathways. The products include prostaglandins, thromboxanes, leukotrienes, and lipoxins, which can regulate pain, edema, pyrexia, vasodilatation and other inflammatory responses (Balsinde et al., 2002; Laye and Gill, 2003).

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly employed drugs for treatment of inflammatory conditions. However, prolonged use of NSAIDs carries increased risk of side effects involving the gastrointestinal tract, liver and cardiovascular system. Reduction in beneficial effects may also occur because blockade of the COX pathway may potentiate LOX activity. Therefore, inhibition of PLA_2, the “master switch” of the AA inflammatory pathway, is a promising alternative. Upregulation of $sPLA_2$ has been identified to cause various inflammatory diseases such as rheumatoid arthritis, atherosclerosis (Oestvang and Johansen, 2006; Oorni and Kovanen, 2009; Pruzanski et al., 1995), sepsis (Huang et al., 2018; Tong et al., 1998) and cancer (Scott et al., 2010). An inflammatory reaction is also commonly observed in victims bitten or stung by venomous snakes, honeybees and scorpions (Echeverria et al., 2018; Petricevich, 2010; Prado et al., 2010). This suggests that inhibitors of animal venom PLA_2s also have potential against inflammation. The chemical structure of human and snake venom PLA_2 ($svPLA_2$) has very similar conformation indicating that they both likely interact with inhibitors at the same site and pattern. Recently, a proof-of-principle study using vanillic acid and its modified analogs verified that both human $sPLA_2$-IIA and $svPLA_2$ (BthTX-II) interacted with these inhibitors in a similar manner (Sales et al., 2017). Indeed, $svPLA_2$ has been used in experimental models for anti-inflammatory drug development (Sales et al., 2017).

Snake blood phospholipase A2 inhibitors (sbPLI) are a category of proteins in snake blood that can neutralize the toxicities of svPLA2s, and prevent snakes from being damaged by their own or another animal's venom (Campos et al., 2016). The categories and activities of sbPLIs have been well-documented. A γ-type phospholipase A2 inhibitor (PLI), named γBjussuMIP, has been isolated from *Bothrops jararacussu* snake plasma and can inhibit the pharmacological effects (e.g. anticoagulant activity, myotoxicity and edema) of Asp49 PLA2s (Oliveira et al., 2011). It has been reported that NaPLI, isolated from

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Naja atra snake serum, has a strong inhibitory effect on sPLA2 in rheumatoid arthritis synovial fluid (Sadaria et al., 2011). These findings suggest that snake PLI can be used to treat sPLA2-related inflammatory responses in humans. We reported previously that a γ-type PLI (saPLIγ), isolated from the snake *Sinonatrix annularis*, was effective in alleviating hemorrhage and myonecrosis caused by the venom of *Deinagkistrodon acutus*, *Agkistrodon halys* and *Naja atra* (Xiong et al., 2017). A number of PLA2 isoenzymes are known to act at the top of AA inflammation cascade. Some of these, IB, II, V and X, are the common isoenzymes associated with preferred tissues and conditions (Kudo and Murakami, 2002). In this paper, the anti-inflammatory activity of saPLIγ was investigated experimentally, along with the interaction of saPLIγ with mammalian IB, IIA, V and X sPLA2 subtypes. We hope that this study will extend the indications of saPLIγ from envenomation to inflammatory diseases.

2. Materials and methods

2.1. Reagents and materials

Polyacrylamide gel reagents and Clarity™ ECL Western Blot Substrate kits were purchased from Bio-Rad (Hercules, CA, USA). Antibodies of $sPLA_2$ IB (ab103872), IIA (ab24498), V (ab97847) and X (ab166634) were obtained from Abcam (Cambridge, UK). Anti-saPLIγ mAb was prepared in our laboratory. Mouse IgG, IL-1β and TNFα ELISA kits were purchased from Boster Biotech (Wuhan, China). Protein G-Sepharose 4 Fast Flow (GE17-0618-01) was from GE Healthcare. The reverse transcription kit was obtained from Bioengineering Technology (Shanghai, China). Carrageenan was purchased from Solarbio Technology (Beijing, China), and dexamethasone sodium phosphate injection (DXM) from Sinopharm Group Rongsheng Pharmaceutical (Wuzhi, China).

2.2. Biological model

Kunming (KM) mice (18–22 g) were supplied by the Animal Center of Nanchang University. Mice were acclimatized for 3 days under standard laboratory conditions of chow, water, and light. All animal experiments were conducted in accordance with the Guide for the Management and Use of Experimental Animal Feeding in Nanchang University, and the principles of humanity were followed for laboratory animals.

All mice were divided randomly into six groups of five animals each. Carrageenan, DXM and saPLIγ were dissolved in saline.

Control (Ctrl) group: saline [30 μL, intramuscular (i.m.)];

Model group: 1% carrageenan (30 μL, i.m.) + saline [150 μL, intraperitoneal (i.p.)]

Dexamethasone group: 1% carrageenan (30 μL, i.m.) + DXM (2.5 mg/kg, i.p.)

saPLIγ groups: 1% carrageenan (30 μL, i.m.) + saPLIγ (i.p.) of 1.0, 2.5 and 5 mg/kg for low, medium and high-dose, respectively.

DXM and saPLIγ were administered 30 min before carrageenan injection as per published literature (Souza Lima et al., 2017; Toyama et al., 2009; Yang et al., 2015). The paw edema model was replicated on right hind limbs by inoculation of 30 μL carrageenan into the plantaris (Yang et al., 2015). The same saline volume was also injected into the contralateral paw to exclude an effect of the vehicle. Paw edema was carefully measured by vernier caliper.

Paw thickness was measured at 0 (before injection), 1, 2, 3 and 4 h after carrageenan administration. Paw swelling rate was defined as the increase in paw thickness compared to the normal state. ΔE was calculated as follows: ΔE = ER-EL, where EL was edema thickness of the left paw, and ER was edema thickness of the right paw. Edema inhibitory activity was calculated according to the following formula. Percentage inhibition (%) = (ΔE model – ΔE treated)/ΔE model × 100. Pre-experimental observations indicated that mouse toe swelling reached a maximum at 4 h after carrageenan administration. At 4 h, both hind paws were photographed and the mice sacrificed by cervical dislocation. The right hind limb paws of all the mice were then cut and placed on ice. The muscles were removed carefully from the paws, and divided into several parts for further use.

2.3. Histopathological sections and hematoxylin-eosin staining

Small muscle parts (3 × 3 mm) were fixed in 4% paraformaldehyde at 4 °C for at least 24 h. The materials were then dehydrated using a graded ethanol series, vitrified in xylene and embedded in paraffin. Sections (5 μm thick) were prepared using a microtome (Leica RM2235, German), and stained according to hematoxylin-eosin staining methods. The number of neutrophils was observed under an optical microscope.

2.4. Western blot analysis

Paw muscle tissue was homogenized in RIPA lysis buffer. The supernatant was collected after centrifugation at 14,000 rpm for 10 min at 4 °C. Protein lysates in each group were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Target proteins were transferred onto polyvinylidene fluoride (PVDF) membranes according to standard western blot protocols. Membranes were treated with blocking solution (5% skimmed milk in 0.05% tween-20 in Tris-buffered saline) for 1 h and then incubated at 4 °C overnight with the primary rabbit monoclonal antibodies (glyceraldehyde-3-phosphate dehydrogenase, 1:5000; $sPLA_2$ IB, 1:500; $sPLA_2$ V, 1:50000; $sPLA_2$ X, 1:5000) and mouse monoclonal antibodies ($sPLA_2$ IIA, 1:1000), respectively. After three washes in 0.05% tween-20 in Tris-buffered saline, secondary incubations were performed with horseradish peroxidase-conjugated goat anti-rabbit or -mouse antibody (1:1000). Immunoreactive proteins were visualized using chemiluminescence. Target signals were quantified by Image software (version 5.1, National Institutes of Health) with a western blotting detection system (Bio-Rad, Hercules, CA, USA).

2.5. Interaction of saPLIγ with murine PLA_2s

The co-immunoprecipitation method was used to evaluate the interaction between saPLIγ and murine PLA_2s. Anti-saPLIγ monoclonal antibody (mAb) 2 μL and normal mice IgG were mixed with 50 μL of protein G resin in a 2 mL EP tube and incubated at 4 °C overnight. Centrifugation was performed to remove the supernatant and the coated beads were retained for use. Samples were prepared by mixing 300 μL purified saPLIγ with 600 μL toe lysate and then incubated with "mAb-Protein G resin" at 4 °C overnight. The liquid was discarded by centrifugation at 500*g* for 5 min and the complex then washed three times using RIPA lysis buffer. The co-immunoprecipitates were subjected to 12% SDS-PAGE separation and electro-transferred to PVDF membranes. The membranes were immune-detected using anti-PLA_2 IB (dilution 1:500), $sPLA_2$ IIA (dilution 1:1000) and anti-saPLIγ (1:1000) antibodies after blocking in 5% non-fat milk solution. Positive control (input) was prepared by mixing purified saPLIγ and toe lysates.

2.6. Gene expression of TNFα, IL-1β and COX2

Mice were killed 4 h after carrageenan administration. Total RNA was extracted from the carrageenan-induced paws using TRIzol according to the manufacturer's instructions. cDNA was obtained by reverse transcription using TAKAR reverse transcription kit (RR047A). The relative expression of TNFα, IL-1β and COX-2 was determined by real-time PCR with the primers listed in Table 1.