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Local inflammatory mediators alterations induced by *Daboia siamensis* venom

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ABSTRACT

The ability of Russell's viper (*Daboia siamensis*) venom (total RVV) and phospholipase A_2 (purified PLA_2) to induce the local pathological effects were investigated by the local inflammatory events and the release of inflammatory mediators. Both 0.5 μg of total RVV/mouse and 0.15 μg of purified PLA_2/mouse were administered via intra-peritoneal injection. After 30 min, 1 h, 2 h, and 4 h incubation time, the peritoneal cavity was flooded with normal saline and the total leukocytes were collected. The eicosanoids (lipid mediators) and the leukocyte expression of cyclooxygenase (COX-1 and COX-2) were investigated by ELISA assay and western blotting, respectively. The amounts of total leukocytes were increased from 30 min to 2 h, then decreased at 4 h, by both total RVV and purified PLA_2. Both treatments also induced the expression of COX-2 which was increased at 2 h and then decreased at 4 h, whereas only purified PLA_2 induced the expression level of a COX-1 protein which was increased at 30 min, then constantly expressed until 4 h. In addition, total RVV and purified PLA_2 caused the release of the eicosanoids; PGE_2, TXB_2, and LTB_4, which reached the peak after 2 h. The findings of this study indicate that purified PLA_2 has the potential effects to induce the local inflammation relating the amounts of leukocytes cells, lipid mediators and COX-2 more than the total RVV.

1. Introduction

Russell's viper (*Daboia siamensis*) is widely distributed across Southeast Asia. Its venom causes local and systemic symptoms to the victims (Chaisakul et al., 2019). The venom manifests the potent hematotoxic effects with inflammatory mediators, leading to inflammation reaction in their victims (de Carvalho et al., 2019). Although, Russell's viper venom (RVV) can be neutralized by anti-venom (Khaing et al., 2018; Tan et al., 2018), the local pathological changes are associated with inflammatory and immunological responses (Bernardes et al., 2015; Bickler, 2020). Envenoming symptoms at local tissue under pathological effects are rapidly incidents, but inefficiently healing response by the anti-venom. Therefore, understanding the pathological mechanisms resulting from RVV envenoming would enhance to a more effective therapy.

PLA_2 is the major component of RVV and cause various medical problems such as heamatotoxic tissue damage, edema, bleeding and pain (Menaldo et al., 2017). It has a main role to hydrolyze on membrane phospholipids to release the arachidonic acids (AA) and free fatty acids (Birts et al., 2009; Sales et al., 2017). Arachidonic acid is an important precursor of various bioactive molecules via COXs pathway. It is one of the most important substrates in the synthesis of eicosanoids, biologically active mediators of the inflammation (Mak et al., 2014).

Inflammation is one of the important processes of defense in animal cells against foreign invaders. The inflammatory response is a complex process which involves both cellular and vascular events with specific humoral secretions (Lordan et al., 2017). The processes include the infiltration of white blood cells, plasma, and fluid at inflamed site.

There are many chemical mediators of inflammation, such as the vasoactive amines and peptides, eicosanoids (e.g leukotrienes; LTB_4, prostaglandins; PGE_2 and thromboxane; TXB_2 (a stable degradation product of TXA_2)), proinflammatory cytokines, and acute-phase proteins. A few minutes after cell injury, inflammatory cells release chemical mediators which intermediate inflammatory process protecting the

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tissue and at last, restoring tissue function (Abdulkhaleq et al., 2018).

Eicosanoids are lipid signaling molecules derived from the enzymatic oxygenation of arachidonic acid (AA) which are generated by cyclooxygenase (COXs) and lipoxygenase (LOXs) pathways. These enzymes are well known mediators and regulators of inflammation, which the important role is to control key cellular processes (Zoccal et al., 2013). Many reports reveal that the lipid mediators induced by animal's venom are involved in inflammation and homeostatic biological functions. For example, *Tityus serrulatus* scorpion venom induced both PGE_2 and LTB_4, which has the opposite role each other from (Zoccal et al., 2016), and both also respond to PLA_2 from *Bothrop asper* venom in mice (Moreira et al., 2011). However, knowledge of these lipid mediators which respond to RVV and its components at the inflammation local site is limited.

The peritoneal cavity is a potential space between the parietal peritoneum and visceral peritoneum. The peritoneal membrane, surrounds the peritoneal cavity, is a shiny layer of mesothelial cells, beneath which lies the supporting interstitium containing connective tissue fibers, blood, and lymphatics vessels. Thus, snake venom injection in the peritoneal cavity leads to diffusion into the surrounding tissue. The capillary pressure is later risen in the abdominal viscera and promotes rapid and significant increase in vascular permeability. Moreover, snake venom is able to induce leukocyte infiltration into the peritoneal cavity after intraperitoneal injection. The aim of this study was to investigate the mediators involved in the local inflammatory response to the direct action of the venom in the peritoneum. We evaluated the ability of total RVV and purified PLA_2 to activate leukocyte cells in the peritoneal cavity focusing on lipid mediators (PGE_2, LTB_4, and TXB_2) and expression of COXs. The results of this work may help to understand the inflammatory response by leukocyte cells at the site of total RVV exposure. These data could enhance our understanding of immunological consequences and promote a better understanding of future therapeutic strategies in *D. siamensis* envenoming.

2. Materials and methods

2.1. Reagents

The ELISA kits for the detection of PGE_2, TXB_2, and LTB_4 were purchased from R&D system, California, USA. Anti-COX-1, Anti-COX-2, Anti-Beta-actin, and goat anti-mouse polyclonal antibodies were also supplied by R&D system. The reagents and chemicals in all experiments were of analytical grades.

2.2. Venom preparation and PLA_2 purification

Daboia siamensis venom was provided by the snake farm of Queen Saovabha Memorial Institute (QSMI), The Thai Red Cross Society. PLA_2 was purified as previously described (Chaiyabutr et al., 2020).

2.3. Animal experimental design

Albino mice (ICR mice) weighing 20 g were enrolled in this study. The study protocols and experimental methods were approved by the Ethics and Animal Welfare Committee of Queen Saovabha Memorial Institute (No. 03/2017). All mice were housed under the specific pathogen-free condition and free access to food and water. Sixty mice were separated into 3 experimental groups to total RVV, purified PLA_2 and negative control. Each group consisted of 4 subgroups, 5 mice per subgroup, for 30 min, 1 h, 2 h and 4 h after venom exposure observation.

2.4. Induction of inflammatory reaction

For the experimental assays, final concentrations of 0.5 μg RVV/mouse or 0.15 μg PLA_2/mouse in sterile saline was administered through peritoneal injection (P.I), 1 ml per mouse. Normal saline was used as negative control. After 30 min, 1 h, 2 h and 4 h of injection, mice were euthanized, and the peritoneal fluid was harvested using sterile saline. The peritoneal exudates were centrifuged at 500 g, 25 °C for 10 min. Leukocytes were separated from exudates and counted under a light microscope.

2.5. Quantification of PGE_2, TXB_2 and LTB_4 concentrations

The eicosanoids were extracted from peritoneal cells-free fluid using Sep-Pak C18 columns (Waters Corporations, Milford, MA), and eluted with ethanol. After that, the concentrations of PGE_2, TXB_2 and LTB_4 was measured by specific enzyme immunoassay (ELISA) using commercial kits (R&D system, California, USA), following the manufacturer's instructions. In brief, 100 μl of each extracted sample was added to the 96 well pre-coated plates with a goat anti-mouse monoclonal antibody to PGE_2/or TXB_2, or rabbit anti-chicken polyclonal antibody to LTB_4. The secondary antibody, mouse monoclonal against PGE_2 or TXB_2, or chicken polyclonal against LTB_4, was added to the reaction plate. After the addition of eicosanoids conjugate and the substrate, the reactions were measured at 450 nm using an ELISA plate reader (Tecan Austria Gmbh 5082).

2.6. Western blotting

Leukocyte aliquots (2×10^6 cells) were lysed by 50 μl of sample buffer (20% SDS, 1% glycerol, 1 M β – mercaptoethanol, 0.1% bromophenol blue, 0.5 M Tris-HCl pH 6.8) and boiled for 10 min at 100 °C. The samples (5 μl) were loaded into 12.5% SDS-PAGE. After that protein was transferred to nitrocellulose membrane and blocked with 5% skim milk in Tris-buffer saline (TBS) pH 7.4 for 24 h, then washed three times with Tris-buffer saline tween (TBST) pH 7.4. The membrane was incubated with primary mouse antibody against COX-1(1:500 v/v)/or COX-2 (1:1500 v/v) for 3 h, and β-actin (1:10000) for 1 h. After washing with TBST, the membrane was incubated with a secondary anti-mouse antibody (1:2000) for 1 h. 4-chloro-1-naphthol was used as substrate for detection.

2.7. Statistical analysis

Data are represented as the mean ± standard deviation (SD) from five animals. The comparisons among the groups were performed with *t*-test facilitated by software in Biostatistics version 3.02, PRIMER (The McGraw-Hill Companies, Inc, San Francisco, California, USA). Probabilities of less than 5% ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Total leucocytes in the peritoneal cavity induced by total RVV or purified PLA_2

After venom injection total leukocytes in the mouse peritoneal cavity were counted and compared with the control group. The result showed that the number of these cells increased from 30 min to 4 h compared with the control group. The purified PLA_2 induced more significant leukocyte infiltration than total RVV at all observation times. The highest amount of leukocyte cells was at 2 h after exposure of both treatments (Fig. 1).

3.2. COX-1 and COX-2 expression in the leukocyte induced by total RVV or purified PLA_2

The expression of COX-1 and COX-2 in peritoneal leukocytes was determined by western blotting. The result showed that the maximum expression of COX-2 protein occurred at 2 h, and decreased at 4 h after injection of total RVV or PLA_2, compared with the control group. For the

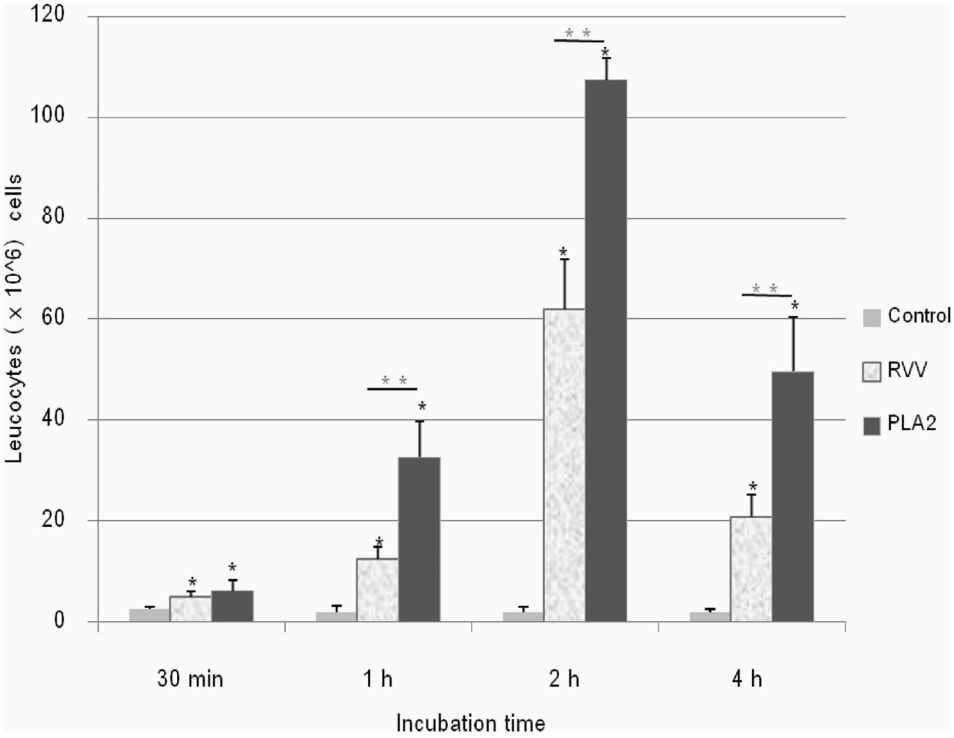

Fig. 1. Total leukocyte counts in the peritoneal cavity induced by 0.5 μg total RVV/mouse and 0.15 μg purified PLA_2/mouse, compared with the control group at 30 min, 1 h, 2 h, and 4 h. Each point represented as the mean ± SD of 5 mice. *$p < 0.05$ when compared with the control group, meanwhile, **$p < 0.05$ differences between total RVV and purified PLA_2.

expression of COX -1 protein, the results were not different to the control when the cells were treated with total RVV even it reached 4 h after exposure. In contrast, COX-1 protein was increased at 30 min by purified PLA_2 then constantly expressed until 4 h (Fig. 2).

3.3. Total RVV and purified PLA_2 induce the release of PGE_2, LTB_4, and TXB_2 from leukocytes

The ability of 0.5 μg total RVV/mouse and 0.15 μg purified PLA_2/mouse to induce lipid mediators release in the peritoneal cells-free fluids of mice was detected after 30 min, 1 h, 2 h, and 4 h of injection. The release of PGE_2, LTB_4, and TXB_2 induced by total RVV and purified PLA_2 reached the highest at 2 h, and dropped between 2 and 4 h. However, the purified PLA_2 fraction could potentially induce all mediators more than total RVV (Fig. 3A, B, and C).

4. Discussion

Snakebite is not only a condition mediated directly by venom, but also by the amplification of signals dysregulating inflammation, coagulation, neurotransmission, and cell survival (Bickler, 2020). Herein, the evaluation of total RVV or purified PLA_2 to activate leukocyte cell on local inflammatory response was performed at mouse peritoneal cavity. The leukocytes cells are the cells of the immune system which involved the body protection against infectious diseases and foreign invaders. Our results might help for a better understanding of envenomation pathology and patient assistance after RVV envenomation. It is possible that the protein cocktail in RVV are balancing proteins could reduce the effect of each other (Slagboom et al., 2020; Bickler, 2020). The purified PLA_2, which was separated from those other proteins cocktail showed more predominant inflammatory response at site then total RVV. Therefore, PLA_2 inhibitors can relief the local pathological symptoms. For example, PLA_2 inhibitor cream showed 69.9% reduction of

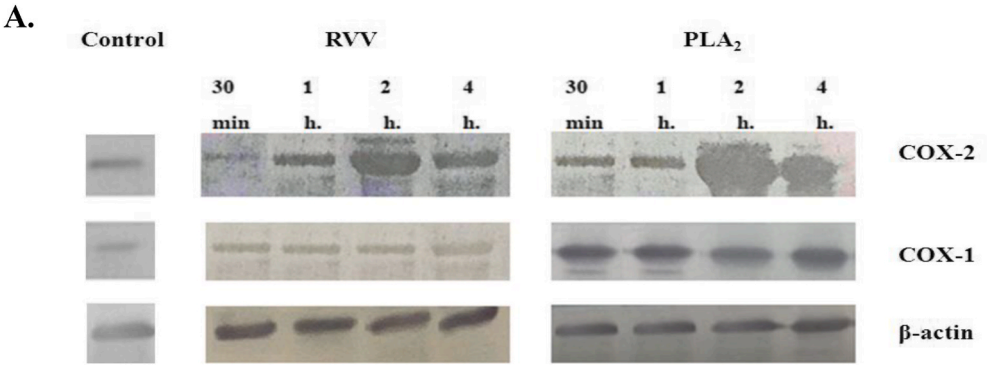

Fig. 2. The COX-1 and COX-2 protein expression in peritoneal leukocytes were induced by 0.5 μg total RVV/mouse and 0.15 μg purified PLA_2/mouse, compared with a control group. The leukocytes cells were harvested at 30 min, 1 h, 2 h, and 4 h after injection of venom or PLA_2. β-actin was used as a loading control in the western blotting analysis.

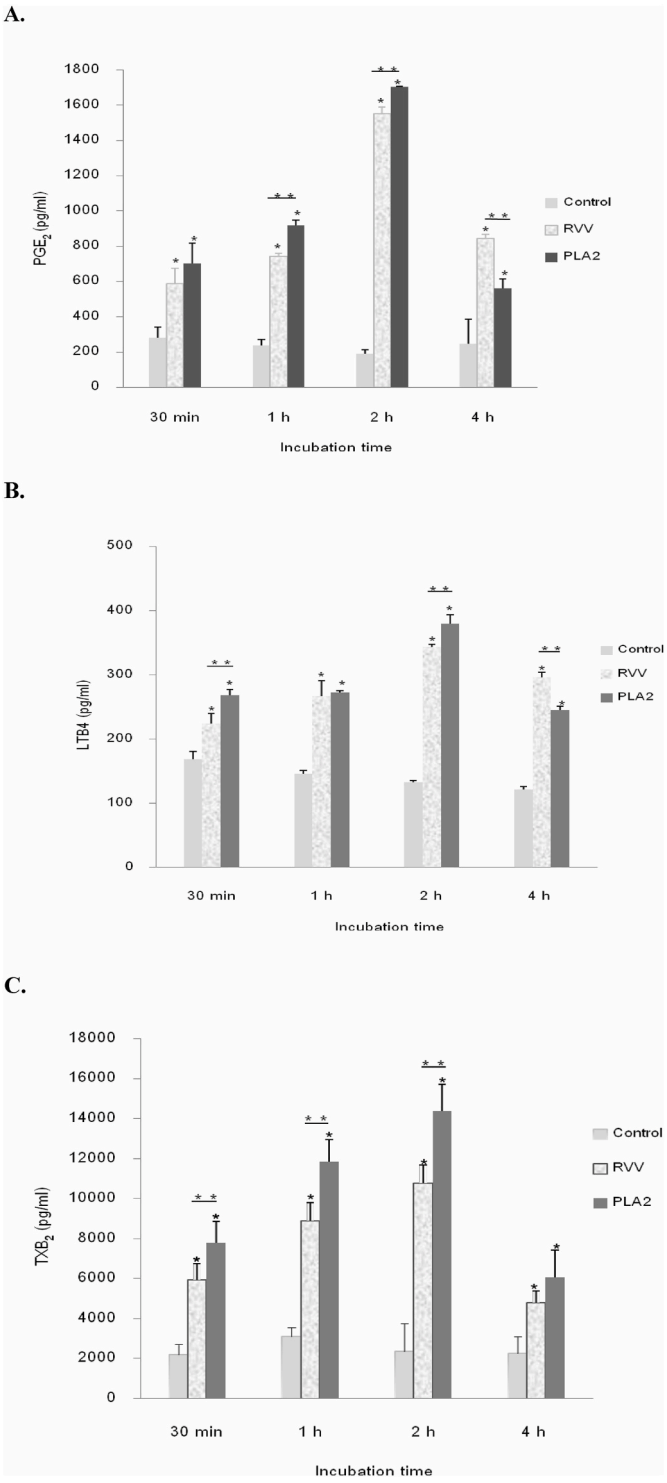

Fig. 3. PGE_2 (A), LTB_4 (B), and TXB_2 (C) concentrations in mouse peritoneal fluid after injection with 0.5 μg total RVV/mouse and 0.15 μg purified PLA_2/mouse for 30 min, 1 h, 2 h, and 4 h, measured by a specific ELISA kit. Mice received saline were used as a control group. Each point represented the mean ± SD of 5 mice. $^*p < 0.05$ when compared with the control group, meanwhile, $^{**}p < 0.05$ compared between total RVV and purified PLA_2.

dermatitis which indicated the inhibitor would contribute to treat the skin inflammation (Ingber et al., 2007). Many reports revealed that PLA_2 inhibitors from various natural sources could inhibit PLA_2 activities in vitro and decreased edema induced by snake venom (Samy et al., 2012).

COXs pathway could be activated by snake venoms with the hydrolyzing of the membrane phospholipids of mice peritoneal cavity cells. This is followed by the release of the amounts of arachidonic acids (AA) and led to initial inflammatory process in immune response (Moreira et al., 2011). The amount of leukocyte cells was significant increased in early 30 min which indicated that the macrophages and neutrophils may be the initial immune cells against the venoms, similarly to *Bothrop* sp venom (Wanderley et al., 2014). Both total RVV and purified PLA_2 could induce the release of lipid mediators; PGE_2 and TXB_2 which are the products of COXs pathway. The TXB_2 was the major inflammatory metabolite produced against the venoms. In evidence, comparing with the control group of each experiment, the increase TXB_2 at 30 min was higher than PGE_2 and LTB_4 at 2 h which was the highest concentration of each group (Fig. 3). TXB_2 is a stable degradation product of TXA_2 which is metabolized and release after inflammatory stimuli, including ischemia-reperfusion injury, hepatic inflammatory processes and acute hepatoxicity. In this regard, it could mean that this venom might cause of hepatotoxicity. Inhibition of TXB_2 or TXA_2 activity can also reduce the inflammatory response against RVV. In addition, the venoms might activate the influx of leukocytes and the inflammatory process was initiated at the local site via COX-2 activity (Fig. 2). The highest expression of COX-2 was at 2 h, then decreased at 4 h. The results of purified PLA_2 showed that PLA_2 in RVV could induce COX-2 protein expression leading to inflammatory response. It indicated that the local inflammatory response induced by RVV venom might be relieved by COX-2 inhibitors such as NSAIDs, especially whose selective to COX-2 (Chicoine, 2010). Whereas, COX-1 protein expression was increased at 30 min, then constantly expressed until 4 h, which might be the body response of animals. In contrast, total RVV could not increase COX-1 expression after venom injection.

Our results indicate that PLA_2 seems to be an important component of RVV, which a significant role on the pathological effects. PLA_2 would activate local inflammatory response of COX-2 pathway via TXB_2 as the major mediator. COX-2 and TXB_2 inhibitors for the local pathological and treatments should be considered in further study. Understanding of the mechanism and underlying signaling pathway of PLA_2 might help to treat snakebite envenoming.

Author contributions

Suchitra Khunsap: Conceptualization, Formal analysis, Writing - Review & Editing and Visualization, Kanyanat Promruangreang: Formal analysis, Validation and Resources, Sunutcha Suntrarachun: Writing - Review & Editing, Jureeporn Noiphrom: Methodology, Orawan Khow: Methodology.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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