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 A₂ (purified PLA₂) 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 PLA₂/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 PLA₂. Both treatments also induced the expression of COX-2 which was increased at 2 h and then decreased at 4 h, whereas only purified PLA₂ 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 PLA₂ caused the release of the eicosanoids; PGE₂, TXB₂, and LTB₄, which reached the peak after 2 h. The findings of this study indicate that purified PLA₂ has the potential effects to induce the local inflammation relating the amounts of leukocytes, 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 hematoxic 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.

PLA₂ is the major component of RVV and cause various medical problems such as hematotoxic 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; LTB₄, prostaglandins; PGE₂ and thromboxane; TXB₂ (a stable degradation product of TXA₂)), proinflammatory cytokines, and acute-phase proteins. A few minutes after cell injury, inflammatory cells release chemical mediators which intermediate inflammatory process protecting the...
Many reports reveal that the lipid mediators induced by animal and negative control. Each group consisted of 4 subgroups, 5 mice per 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 cyclo-

2.4. Induction of inflammatory reaction

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 PLA2 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).

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).

2.7. 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 PLA2 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 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
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 PLA2 then constantly expressed until 4 h (Fig. 2).

3.3. Total RVV and purified PLA2 induce the release of PGE2, LTB4, and TXB2 from leukocytes

The ability of 0.5 μg total RVV/mouse and 0.15 μg purified PLA2/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 PGE2, LTB4, and TXB2 induced by total RVV and purified PLA2 reached the highest at 2 h, and dropped between 2 and 4 h. However, the purified PLA2 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 PLA2 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 PLA2, which was separated from those other proteins cocktail showed more predominant inflammatory response at site then total RVV. Therefore, PLA2 inhibitors can relieve the local pathological symptoms. For example, PLA2 inhibitor cream showed 69.9% reduction of
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 LT$_B$ 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 hepatotoxicity. 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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