Review

Inflammatory Effects of Bothrops Phospholipases A2: Mechanisms Involved in Biosynthesis of Lipid Mediators and Lipid Accumulation

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Abstract: Phospholipases A2 (PLA2s) constitute one of the major protein groups present in the venoms of viperid and crotalid snakes. Snake venom PLA2s (svPLA2s) exhibit a remarkable functional diversity, as they have been described to induce a myriad of toxic effects. Local inflammation is an important characteristic of snakebite envenomation inflicted by viperid and crotalid species and diverse svPLA2s have been studied for their proinflammatory properties. Moreover, based on their molecular, structural, and functional properties, the viperid svPLA2s are classified into the group IIA secreted PLA2s, which encompasses mammalian inflammatory sPLA2s. Thus, research on svPLA2s has attained paramount importance for better understanding the role of this class of enzymes in snake envenomation and the participation of GIIA sPLA2s in pathophysiological conditions and for the development of new therapeutic agents. In this review, we highlight studies that have identified the inflammatory activities of svPLA2s, in particular, those from Bothrops genus snakes, which are major medically important snakes in Latin America, and we describe recent advances in our collective understanding of the mechanisms underlying their inflammatory effects. We also discuss studies that dissect the action of these venom enzymes in inflammatory cells focusing on molecular mechanisms and signaling pathways involved in the biosynthesis of lipid mediators and lipid accumulation in immunocompetent cells.

Keywords: Bothrops phospholipases A2; inflammation; lipid mediators; signaling pathways

Key Contribution: This review provides an overview and recent advances in the understanding of inflammatory mechanisms triggered by svPLA2s with a focus on their actions on lipid mediator biosynthetic pathways and lipid accumulation in immunocompetent cells.

1. Introduction

Bothrops spp. snakes are responsible for the majority of snakebites in Latin America. Envenomation by these snakes induces severe pathological alterations at the site of venom injection, characterized by an intense local inflammatory reaction associated with myonecrosis, pain, and hemorrhage, potentially leading to permanent tissue damage and disability [1–6]. The local inflammatory response to bothropic envenomation involves a set of events including an increase in vascular permeability, edema formation, hyperalgesia, the activation and infiltration of immunocompetent cells, and a complex network of inflammatory mediators driving the inflammatory response [2]. These events are caused by the activation of critical host defense mechanisms of the victims by direct and indirect actions of
the toxins present in the snake venom. Proteomic studies of Bothrops snake venoms revealed that the phospholipases A$_2$ (PLA$_{2S}$) are ubiquitous components of these venoms and play an important role in the pathophysiology of envenoming by these snakes [7–11]. During envenomation, in addition to aiding prey digestion, these toxins have been described to display myotoxic, cytotoxic, hemolytic, hypotensive, anticoagulant, platelet aggregation inhibition/activation, and proinflammatory effects [12–14]. Regarding inflammation, in addition to exerting direct effects on cell membranes, these lipolytic enzymes can recruit mammalian PLA$_{2S}$ analogs of similar activity and trigger endogenous signaling systems that display and amplify the cell injury and host defense mechanisms triggered by the whole venom. This amplification is responsible for many acutely important consequences of Bothrops envenoming. Yet, as discussed below, studies with in vitro and in vivo models aiming to understand the inflammatory action of isolated venom PLA$_{2S}$ can contribute to the knowledge of the local inflammatory mechanisms induced by Bothrops snake venoms and those from the Viperidae family [3,15–17]. These studies might lead to the discovery of new therapeutic targets for a more efficient treatment of envenoming by viperid snakes, since the currently available antivenoms have low effectiveness to neutralize the local events promoted by their venoms [18]. Finally, due to the structural and functional similarities to mammalian group (G) IIA PLA$_{2}$, the Bothrops PLA$_{2S}$ can constitute useful tools for studies on the roles of human GIIA PLA$_{2}$ in inflammatory diseases. In this regard, the effectiveness of varespladib, an inhibitor of svPLA$_{2}$ [19] in attenuating inflammatory events caused by Viperidae snake venoms has been demonstrated in mice experimental model [20].

1.1. Phospholipases A$_2$

Phospholipases A$_2$ constitute a group of enzymes with diverse biological functions, ranging from homeostasis and membrane remodeling to the generation of metabolites and second messengers involved in biological processes and signal transduction. These enzymes hydrolyze the acyl ester bond at the sn-2 position of phospholipids, generating free fatty acids, such as arachidonic acid (AA) and oleic acid, and lysophospholipids, such as lyso-PAF [21,22]. Currently, PLA$_{2S}$ are classified into close families, including secretory PLA$_2$ (sPLA$_2$), cytosolic phospholipase (cPLA$_2$), and Ca$^{2+}$-independent PLA$_2$ (iPLA$_2$); platelet activating factor acetylhydrolase (PAF); and lysosomal PLA$_2$ (LPLA$_2$), PLA/acyltransferase (PLAAT), $\alpha/\beta$ hydrolase (ABHD), adipose-PLA$_2$ (AdPLA), and glycosylphosphatidylinositol (GPI)-specific PLA$_2$ [23–25]. This classification of PLA$_{2S}$ is based on the cell location, amino acid sequence, molecular weight, presence of intramolecular disulfide bridges, calcium requirement, and catalytic activity, including the hydrolysis at the sn-2 position of glycerophospholipids [22,26]. The sPLA$_2$ family is largely distributed in nature, and its components are classified into I, II, III, V, IX, X, XI, XII, XIII, and XIV groups. They are present in high concentrations in snake, bee, and wasp venoms and in several mammalian organs, cells, and pancreatic juice [21,27]. Among them, we highlight group I sPLA$_2$s, comprising the secreted enzymes found in the snake venoms of the Elapidae and Hydrophiidae families and in the pancreas secretion of mammals. Meanwhile, group II consists of sPLA$_2$s found in snake venoms of the Viperidae family and in mammalian tissues and are expressed in inflammatory processes [13,28].

For a long time, the pathophysiological activities of sPLA$_2$s were related exclusively to their enzymatic activity, capable of providing a substrate for the synthesis of second messengers and inflammatory mediators. However, alternative mechanisms of action have been associated with the ability of sPLA$_2$ to interact with receptors or even specific domains present on cell membrane surfaces, such as heparan sulfate proteoglycans, which have already been described to be important for triggering the activation of other sPLA$_2$s in the target cells [29–32]. The identification of sPLA$_{2}$ binding proteins was initially achieved by Lambeau et al. (1989) [33], who used the sPLA$_2$ from the Oxyuranus scutellatus snake venom, called OS2. Due to its prevalence in brain tissue, this protein was denominated an N (neuronal) type receptor. Then, through screening in various tissues and cells, for studies
of other OS2 binding proteins, a second type of PLA2 receptor was described, present in rabbit skeletal muscles, called an M (muscle) type receptor [34]. The presence of this latter receptor was identified in several tissues and in neutrophils, monocytes, and human alveolar macrophages [35,36], but not in murine peritoneal macrophages [37]. The M-type phospholipase A2 receptor has a high degree of homology (~30%) to mannose receptors, a member of the lectin receptor family, constitutively expressed in macrophages [38,39]. Mannose receptors are involved in phagocytosis, antigen processing [29,38,40], and the production of inflammatory cytokines by macrophages [40–42]. Additionally, this receptor has been demonstrated to play a role in cell proliferation and AA release, via MAPKs (protein kinase activated by the mitogens family), induced by the group IB sPLA2s [43–45]. Furthermore, it has been revealed that group IIA sPLA2s can bind to mannose receptors and promote the release of IL-6 by human alveolar macrophages [46].

Investigations into the biological role of mammalian group IIA PLA2, also known as inflammatory PLA2, in the development of several pathologies of inflammatory and immunological origin have been described. Several studies have revealed that group IIA sPLA2s are present in high levels in rheumatoid arthritis [47–50], acute pancreatitis [51–53], septic shock [54,55], Crohn’s disease and ulcerative colitis [56–58], respiratory distress syndrome [59–61], bronchial asthma and allergic rhinitis [59,62], atherosclerosis [63,64], autoimmune diseases [65], and cancer [66–69]. These observations imply that both local and systemic inflammation are associated with the release of sPLA2s in vivo, thus raising the unclear question of the role of these PLA2s in inflammatory reactions. Additionally, it was found that proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α), induce, in a variety of tissues, the gene transcription of sPLA2s and the subsequent increase in their secretion, thus, supporting the hypothesis of the involvement of sPLA2s in inflammation [70–73]. In addition, sPLA2s activate intracellular signaling events in cells that participate in inflammatory processes, caused by the generation of second messengers, and the phosphorylation of kinases as of MAPK [74–77]. Thus, sPLA2s represent an important target for investigations regarding the mechanisms of inflammatory events.

1.2. Inflammation—General Concepts and Signaling Pathways

Inflammation is a response of body tissues to noxious conditions for restoring homeostasis, setting the stage for the healing and reconstitution of injured tissue. The acute inflammatory response to injury involves functional alterations of microvessels that occur early after injury and develop at varying rates. The major features of these alterations include transient vasoconstriction, followed by vasodilation, then, leakage of protein-rich fluid from the microcirculation leading to edema formation, and movement of phagocytic leukocytes into the site of injury followed by local pain [78]. Immunocompetent cells, such as neutrophils and monocytes, found in blood circulation are capable of rapidly infiltrating tissues; macrophages and dendritic cells reside within tissue and play key roles in tissue surveillance and antigen presentation [79,80]. The vascular and cellular reactions are triggered and highly regulated by chemical factors, called inflammatory mediators, which include cytokines, chemokines, vasoactive amines, and eicosanoids and are produced by plasma components or are released in close proximity to the injury by endothelial cells, tissue-resident leukocytes such as mast cells, and macrophages at the early stages, followed by infiltrated leukocytes. The effects of inflammatory mediators involve the engagement of specific receptors, which then display signaling pathways responsible for the immune response [81–83]. Parallel to changes in blood flow, the margination of leukocytes begins, and leukocytes adhere to the microvascular endothelium through rolling and firm adhesion, and then moving through the vascular wall into the interstitial tissue. The various steps in the leukocyte migration are regulated by different subsets of cell-adhesion molecules expressed by both leukocyte and endothelial cells [84,85]. Thereafter, the phagocytosis of offending agents by migrated leukocytes occurs, followed by a release of lysosomal enzymes and an increase in the oxidative metabolism in leukocytes, known as respiratory burst,
resulting in the production of microbicidal agents, such as superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) [86,87]. Four major classes of receptor-mediated phagocytosis exist: receptors of complement that recognize complement-coated particles; Fcg receptors, which are constitutively active for phagocytosis of IgG-coated particles; mannose receptors that recognize mannose and fucose on the surface of pathogens; and β-glucan receptors that recognize β-glucans-bearing ligands [87]. Furthermore, receptors for the Fc portion of immunoglobulin G and mannose/fucose residues lead to the release of proinflammatory mediators and reactive oxygen [88].

Despite the stereotyped features associated with an inflammatory response, the signal pathways involved in this response are determined by the nature of the inflammatory trigger, the sensors that detect them, the inflammatory mediators released, and the tissue affected. A number of surface and cytosolic receptors expressed in innate immune cells can sense pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), or venom-associated molecular patterns (VAMPs) with high sensitivity and specificity. The recognition of these molecules is achieved by interacting with pattern recognition receptors (PRRs) [89]. These sensing receptors include Toll-like receptors (TLRs) [90,91], C-type lectin receptors (CLR), RIG-I-like receptors (RLR), and nucleotide-binding domain leucine-rich repeat (NLRs) or nucleotide-binding and oligomerisation domain (NOD)-like receptors [92,93]. Among them, TLRs (TLR1-TLR10) are highly conserved transmembrane proteins that play an important role in recognizing microbial pathogens and endogenous damage molecules, thereby triggering the generation of signals for the production of proinflammatory proteins and cytokines, via cooperation of adaptor proteins (MyD88, TIRAP, TRIF, and TRAM) [94,95]. The recognition of specific molecular patterns by NLRs induces the oligomerisation of proteins in the cytosol, generating platforms called inflammasomes [96]. The inflammasome is a high molecular weight protein complex that elicits the activation of inflammatory caspases and the processing of pro-interleukin-1β (pro-IL-1β) and pro-IL-18, generating the mature biologically active cytokines and a rapid inflammatory form of cell death termed pyroptosis [97]. Several distinct inflammasomes have been identified, including NLR and the pyrin domain containing receptor 1 (NLRP1), NLRP3, and NLR; the caspase recruitment domain containing receptor 4 (NLRC4); and the AIM2-like receptors (ALR) family [98,99]. These receptors positively regulate genes related to inflammatory mediators, including cytokines and key enzymes in the biosynthetic pathways of lipid mediators known as eicosanoids [100].

The early induction of most inflammatory transcripts depends on networks of transcription factors whose activation is coupled to pathways of signal transduction. The nuclear factor-kappa B (NF-κB) is a major and the best-studied transcription factor of inflammatory response [101,102]. The binding of activated NF-κB to the nuclear promoter region of diverse inflammatory factors leads to the transcriptional activation and expression of inflammatory mediators and enzymes. Currently, the major signaling pathways involved in inflammation are mediated by the cascade phosphorylation of protein kinases, such as the mitogen activated kinases (MAPKs) encompassing ERK1/2, SAPK, c-Jun NH2-terminal or JNK and p38MAPKs, phosphatidylinositol 3 kinase (PI3K), protein kinase C (PKC), and protein tyrosine kinase (PTK). These kinases mediate distinct intracellular signaling pathways associated with cytokines production, cytokine receptors, growth factors, mobilization of intracellular Ca$^{2+}$, and regulate a variety of functions of immunocompetent cells, including cell migration, phagocytosis, degranulation, respiratory burst, and programmed cell death [103–112].

An effective acute inflammatory response results in the removal of noxious factors followed by the resolution and repair stages [113]. The shift in inflammatory markers, including the switch from proinflammatory mediators to anti-inflammatory, resolution-inducing mediators (lipoxins, maresins, protectins, and resolvins), is vital for the change from inflammation to resolution [114]. This switch drives the transition from neutrophil to monocyte recruitment into the affected sites, resulting in the clearance of dead cells and other debris, assisted by the lymphatic system, and the initiation of tissue repair at
the damaged site [115]. However, if the acute events are not properly controlled, the inflammatory response becomes detrimental to the host. Yet, if the acute response does not succeed in neutralizing the injurious stimulus, the resolution phase might not be appropriately induced, and a chronic inflammatory state may ensue, leading to several inflammatory-mediated diseases [116,117].

2. Inflammatory Effects of sPLA₂ from Bothrops spp. Venoms

2.1. Bothrops svPLA₂s Induce Inflammatory Events and Activate Defense Functions in Leukocytes

Phospholipases A₂ of GIIA are major components of Bothrops spp. snake venoms and play important roles in the pathophysiology of envenoming by these snakes, including the inflammatory response. Although these enzymes conserve a chemistry and catalytic structure, the natural evolution of viperid venoms introduced alterations in their primary amino acid residues, generating various other biological and toxicological effects [118]. In general, the GIIA sPLA₂s found in viperid snake venoms are classified as sPLA₂s, known as ‘classic’, containing an amino acid aspartate at position 49 (Asp49) and catalyzing the hydrolysis of the ester bond at position sn-2 of glycerophospholipids in a Ca²⁺ dependent manner. Meanwhile, the other type of sPLA₂ is described as ‘variant’ and contains a lysine at the same position 49 (Lys49), with or without low catalytic activity [119]. Such a substitution affects the ability of these proteins to bind to Ca²⁺, an essential cofactor for the stabilization of tetrahedral intermediate, which occurs in the catalytic reaction performed by the Asp49-sPLA₂s [120]. Despite the lack of enzymatic activity, sPLA₂s-Lys49 homologues maintain their damaging capacity in membranes through a mechanism that is not completely understood and independent of Ca²⁺ [12,121,122].

It has long been demonstrated that viperid sPLA₂s are potent inductors of inflammation. Although they present differences in their catalytic activity, both viperid Asp49 and Lys49 PLA₂ homologues are capable of inducing local inflammation in diverse experimental models [123–125]. As such, this group of enzymes is considered to be a major component responsible for the severe local edema in envenomings by Bothrops spp. The inflammatory response to venom PLA₂ is characterized by edema and the marked infiltration of leukocytes into the site of toxin injection. Studies on the mechanism of local edema induced by viperid svPLA₂s (svPLA₂s) have demonstrated an early increase in vascular permeability and a local release of inflammatory mediators, which act synergistically to cause the initiation and development of the inflammatory events. Among these mediators are vasoactive amines, including histamine, serotonin, and substance P, as well as vasodilating prostaglandins. Yet, in vivo studies employing a pharmacological approach have demonstrated that antagonists of serotonin and H₁ receptors of histamine reduced the progression of edema induced by both catalytically active and inactive variants of sPLA₂ isolated from B. asper [126], B. neuwiedii [127], B. jararacussu [128,129], or B. insularis [130]. In support of these reports, the release of histamine and serotonin by mast cells was observed following stimulation with bothropic sPLA₂s from B. jararacussu [128,131]. Consistent with this evidence, the contribution of mast cells to edema formation induced by viperid PLA₂ was further observed in vitro experimental models demonstrating the ability of sPLA₂s isolated from B. pirajai, B. jararacussu, and B. atrox snake venoms to degranulate mast cells [125,128,131,132]. It is well known that upon activation, mast cells secrete and synthesize an array of inflammatory mediators, which trigger the earliest events of inflammation [133,134]. Moreover, the contribution of the catalytic activity for the edematogenic effect of the enzymatic active Asp49 from bothropic PLA₂ was suggested by studies, revealing that the chemical modification of this sPLA₂ by p-bromophenacyl bromide inhibited edema formation induced by these viperid PLA₂ [126–128]. In addition, the role of lipid mediators, such as PAF and eicosanoids, for hyperalgesia induced by catalytic active venom PLA₂ was highlighted by studies using a pharmacological approach [135]. These authors suggested that the enzymatic hydrolysis of membrane phospholipids played a role in these events by directly releasing the precursors of lipid mediators, such as lyso-PAF and AA.
As mentioned previously, leukocytes are central components of inflammation. An important cellular component exists in the inflammatory response to *Bothrops* sPLA2s. As such, the stimulatory effect of piratoxin-I, bothropstoxin-I, and -II from *B. pirajai* and *B. jararacussu*, respectively, on neutrophil chemotaxis was demonstrated in an in vitro experimental model [136]. This effect was revealed to involve the interaction of these sPLA2s with surface heparan binding sites of neutrophils, followed by the release of chemotactic mediator leukotriene B4 (LTB4) and PAF, and is independent of enzyme activity. Furthermore, the ability of these venom PLAs to recruit an endogenous PLA2 when the activation of GTP-binding protein and PKC was added to the mechanisms by which they cause neutrophil migration [137]. Moreover, studies conducted using in vivo experimental models have demonstrated the ability of *Bothrops* sPLA2s to induce a marked influx of polymorphonuclear and mononuclear cells into the site of their injection, as demonstrated for both catalytic active and non-catalytic venom PLAs, such as MT-III and MT-II from *B. asper* snake venom [123,138]. A similar effect was reported by other authors, investigating various sPLA2s isolated from different *Bothrops* spp. snake venoms, such as bothropstoxin (BthTX-I) and BthTX-II; *B. jararacussu* [131], BnSP-7, a catalytically inactive PLA2 from *B. pauloensis* [139], BatroxPLA2 from *B. atrox* [140] and BJ-PLA2-I from *B. jararaca* [141] in in vivo experimental models. The sPLA2-induced leukocyte migration was linked to the upregulation of adhesion molecules, such as l-selectin, LFA-1, and CD18, which in turn was associated with the release of inflammatory cytokines IL-1β, IL-6, and TNF-α with chemotactic activity by resident leukocytes, primarily macrophages [123]. Cytokines, chemokines, and leukotriene B4 are among the major mediators regulating the expression of adhesion molecules and chemotaxis of leukocytes [142–144]. Consistent with this information, increased serum levels of IL-6, IL-1, and TNF-α induced by Bbl-TX from *B. bilineata* snake venom were observed in a mouse experimental model [145]. In addition, there are reports that two Lys49 PLAs isolated from *B. mattragrossensis* (BmaTX-I and BmaTX-II) venom were able to induce the release of IL-1β by murine neutrophils in culture [146] and that BatroxPLA2, an acidic sPLA2 from *B. atrox* venom, induced the release of IL-6, PGE2, and LTB4 from murine macrophages in culture [140]. In this context, the involvement of inflammasomes in the production of IL-1β induced by *Bothrops* sPLA2s was recently investigated. The participation of NLRP3 inflammasome via the activation of caspase 1 in the production of IL-1β induced by BthTX-I, a Lys49-PLA2 from *B. jararacussu* venom, injected into mouse gastrocnemius muscle was reported [147]. In addition, the participation of inflammasomes in BthTX-I-induced production of IL-1β was demonstrated in peritoneal macrophages. This effect was demonstrated to be dependent on caspase 1/11, ASC, and NLRP3 and was associated with the activation of ATP and activation of P2X7 receptors [148]. Despite the importance of cytokines, chemokines, and eicosanoids in orchestrating the events of inflammation and the potent proinflammatory effects triggered by viperid sPLA2s, including those from *Bothrops* genus, a complete picture of the inflammatory mediators released by immunocompetent cells upon stimulus by *Bothrops* sPLA2s has yet to be further investigated. Moreover, the mechanisms involved in the production and release of these mediators and the possible crosstalk between them remain to be better clarified. Regarding the mechanisms involved in the biosynthesis of lipid mediators induced by *Bothrops* sPLA2s, the progress made is presented in this review as a separate item (Section 2.2).

It is well recognized that the activation of innate effector functions, such as phagocytosis, and the production of microbicidal substances in leukocytes are critical for host defense and tissue repair. Regarding phagocytosis, studies have demonstrated the activity of *Bothrops* sPLA2s to induce phagocytosis following the activation of distinct receptors in immune-competent cells. In this sense, it was demonstrated that MT-II and MT-III, isolated from *B. asper* snake venom, can directly stimulate phagocytosis by macrophages in culture. MT-II significantly increased phagocytosis mediated by all classes of receptors, whereas MT-III increased phagocytosis via only mannose and beta-glucan receptors. This suggests that although the catalytic activity of *Bothrops* sPLA2s is not an essential requirement for enhancing macrophage phagocytosis, it may drive the class of phago-
cytosis receptors involved in this process. Molecular regions distinct from the catalytic network are likely involved in this effect [138]. In addition, the signaling pathways mediating zymosan phagocytosis, induced by both MT-II and MT-III, were investigated, with a focus on lipid second messengers. This study demonstrated that whereas the effect of MT-III, catalytically active, was dependent on the activation of endogenous iPLA₂, the effect of MT-II was dependent on both endogenous iPLA₂ and cPLA₂. Likewise, COX-2 and 5-LO-derived metabolites in addition to PAF were involved in the signaling events required for phagocytosis induced by both venom sPLA₂ [138]. In line with these data, BaltTX-I, devoid of catalytic activity and isolated from B. alternatus snake venom, was reported to activate the phagocytosis of serum-opsonized zymosan by murine macrophages, indicating the involvement of complement receptors. In addition, the participation of PKC was demonstrated. Nonetheless, BaltTX-II, a catalytically active sPLA₂ isolated from the same venom did not stimulate phagocytosis in macrophages, lending support to previous findings that the catalytic activity of Bothrops sPLA₂ is not essential for the stimulation of phagocytosis via complement receptor [149]. In addition, the sPLA₂ isolated from Panamanian B. asper snake venom, pMTX-III (catalytically active Asp49) and pMTX-II and -IV, two enzymatically inactive Lys49 isoforms, were described to induce phagocytosis via mannose receptor and superoxide production in macrophages [150]. The mechanisms underlying the differences between the catalytic and non-catalytic active Bothrops PLA₂, regarding the activation of phagocytosis in macrophages and the participation of distinct receptors in their effects, require further clarification.

Concomitantly with phagocytosis, there is an increase in the oxidative metabolism, also referred to as respiratory burst, in leukocytes. In this context, the literature reveals that viperid sPLA₂s can trigger the respiratory burst in immunocompetent cells. In the first study describing the ability of Bothrops sPLA₂ to induce the release of microbicidal agents, the authors demonstrated that MT-II and MT-III, isolated from B. asper snake venom, induced the release of H₂O₂ by macrophages, with MT-III being the more potent stimulator [151]. In agreement with this evidence, it has been demonstrated that BaltTX-I and BaltTX-II from B. alternatus snake venom induced superoxide production by macrophages in culture in a process mediated by PKC [149]. In addition, other authors have revealed that the three sPLA₂s from B. atrox venom, namely BaTX-I, a Lys49 variant devoid of catalytic activity; BaTX-II, a catalytically active Asp49; and BaPLA₂, an acidic Asp49 sPLA₂ induced the release of the superoxide anion by the J774A.1 lineage macrophages in culture [152]. BaTX-I was the only sPLA₂ able to stimulate complement receptor-mediated phagocytosis, but all studied sPLA₂s could increase the macrophage lysosomal volume [152]. These data demonstrate the ability of Bothrops PLA₂s to trigger the respiratory burst, which is an essential process for the elimination of harmful agents. Although the structural determinants of such an effect were not investigated, it is likely that neither the enzymatic activity nor the basic or acidic characteristic of PLA₂ is essential for the activation of the respiratory burst.

An additional defensive strategy important for host defense is the neutrophil extracellular trap, or ‘NET’. The formation of NET (NEToxis) occurs through the release of nuclear DNA, forming a sticky ‘net’ of extracellular fibers that can halt the dissemination of pathogens and toxins [153,154]. Despite its importance in the inflammatory response, little attention has been paid to the involvement of this defense mechanism in the effects of viperid sPLA₂s. Yet, a report indicates that BaTX-II, an Asp49 PLA₂ isolated from B. atrox snake venom, can activate human neutrophils in culture to produce hydrogen peroxide via the PI3K signaling pathway. Furthermore, this sPLA₂ stimulated neutrophils to secrete MPO, NETs, and inflammatory mediators, including IL-1β, IL-8, and LTB₄ [155]. Therefore, the activation of neutrophilic functions, including toxin trapping and inactivation, is likewise involved in the inflammatory response to Bothrops sPLA₂s. Further studies are necessary to amplify the knowledge regarding the participation of NETs in inflammation induced by Bothrops spp. sPLA₂s. Interestingly, in contrast to the reported ability of Bothrops sPLA₂s to activate distinct inflammatory functions in leukocytes, a report revealed that CB (Crototoxin B), a catalytically active sPLA₂ isolated from Crotalus durissus terrificus,
which is a subunit of crotoxin complex [156,157], could, per se, display inhibitory effects in macrophage functions, including spreading and phagocytosis [158]. Such an inhibitory effect suggests an anti-inflammatory activity for this particular viperid sPLA2 [159]. In agreement with this idea, CB was reported to reduce the release of inflammatory cytokines, including IL-6 and TNF-α, and increase the release of PGE2 and lipoxin A4, both immunomodulatory lipid mediators, in dendritic cells [160]. A summary of the inflammatory activities of svPLA2s is illustrated in Figure 1. In Table 1, the svPLA2s-induced inflammatory responses are summarized according to the amino acid residue at position 49 and basic and acidic characteristics.

![Figure 1. Scheme of inflammatory activities of svPLA2s. The svPLA2s induce inflammatory events, characterized by activation of innate immune cells and endothelial cells and release of several inflammatory mediators that interfere in the vascular dynamic. svPLA2s induce mast cells degranulation and activation of resident macrophages with release of inflammatory mediators such as prostaglandins (PGs), histamine, serotonin, and substance P, which lead to vasodilation, increase of vascular permeability, culminating in edema formation and pain. In addition, svPLA2s activate phagocytosis by macrophages and increase the local production of oxygen reactive species (ROS). Furthermore, svPLA2s, along with vascular alterations and produced inflammatory mediators, increase the expression of adhesion molecules such as LFA, CD-18 and L-selectin. These adhesion molecules, in turn, promote chemotaxis and leukocyte migration. The svPLA2s induce production of myeloperoxidase (MPO) and release of NETs by neutrophils. Both neutrophils and macrophages release proinflammatory mediators such as platelet-activating factor (PAF), IL-8, LTB4, IL-1β, IL-6, and TNF-α. These last three mediators are involved in the upregulation of COX-2 isoform, and release of PGs, thus amplifying the inflammatory response induced by svPLA2s.](image-url)
| PL\(A_2\) | Origin | Basic or Acid | Type of PL\(A_2\) Variant | Inflammatory Activity/Experimental Model | Refs. |
|---|---|---|---|---|---|
| Piratoxin-I | \(B.\ pirajai\) | Basic | Lys49 | Increase in vascular permeability (in vivo) Mast cell degranulation, neutrophil chemotaxis (in vitro) | [125,136] |
| P-1 | \(B. neuwiedii\) | Acidic | nd | Edema (in vivo) | [126] |
| P-2 | \(B. neuwiedii\) | Acidic | nd | Edema (in vivo) | [126] |
| SIISPIIA | \(B. jararacussu\) | Acidic | Asp49 | Edema (in vivo) | [129] |
| SIISPIIB | \(B. jararacussu\) | Acidic | Asp49 | Edema (in vivo) | [129] |
| SIISPIIIA | \(B. jararacussu\) | Acidic | Asp49 | Edema (in vivo) | [129] |
| SIISPIIIIB | \(B. jararacussu\) | Acidic | Asp49 | Edema (in vivo) | [129] |
| BintTX-I | \(B. insularis\) | Acidic | Asp49 | Edema (in vivo) | [130] |
| Bothropstoxin-I (BthTX-I) | \(B. jararacussu\) | Basic | Lys49 | Edema, leukocyte migration, mast cell degranulation (in vivo) Neutrophil chemotaxis, activation of inflammasome (in vitro) | [128,131,136,147,148] |
| Bothropstoxin-II (BthTX-II) | \(B. jararacussu\) | Basic | Asp49 | Edema, leukocyte migration, mast cell degranulation (in vivo) Neutrophil chemotaxis (in vitro) | [128,131,136] |
| Myotoxin-II (MT-II) | \(B. asper\) | Basic | Lys49 | Increase in vascular permeability, leukocyte migration, release of mediators, hyperalgesia, eicosanoid production, COX-2 expression (in vivo) Phagocytosis, \(H_2O_2\) production COX-2 expression, lipid droplet formation (in vitro) | [123,126,135,138,161–167] |
| Myotoxin-III (MT-III) | \(B. asper\) | Basic | Asp49 | Increase in vascular permeability, leukocyte migration, release of mediators, hyperalgesia, eicosanoid production; COX-2 expression (in vivo) Phagocytosis, \(H_2O_2\) production, COX-2 expression, lipid droplet formation, preadipocyte activation (in vitro) | [123,126,135,138,162–166,168–170] |
| BrSP-7 | \(B. pauloensis\) | Basic | Lys49 | Edema (in vivo) | [139] |
| BatroxPLA\(_2\) | \(B. atrox\) | Acidic | Asp49 | Leukocyte chemotaxis, mediators release (in vivo) Mast cell degranulation (in vitro) | [140] |
| BJ-PLA\(_2\)-I | \(B. jararaca\) | Acidic | Asp49 | Leukocyte migration, mediators release (in vivo) | [141] |
| Bbil-TX | \(B. bilineata\) | Basic | nd | Neutrophil migration, mediators release (in vivo) | [145] |
| BmaTX-I | \(B. mattogrossensis\) | Basic | Lys49 | Mediator release (in vitro) | [146] |
| BmaTX-II | \(B. mattogrossensis\) | Basic | Lys49 | Mediator release (in vitro) | [146] |
| BaltTX-I | \(B. alternatus\) | Basic | Lys49 | Phagocytosis, superoxide production (in vitro) | [149] |
| BaltTX-II | \(B. alternatus\) | Basic | Asp49 | Superoxide production (in vitro) | [149] |
| pMTX-II | \(B. asper\) | Basic | Lys49 | Phagocytosis, superoxide production (in vitro) | [150] |
| pMTX-III | \(B. asper\) | Basic | Asp49 | Phagocytosis, superoxide production (in vitro) | [150] |
| pMTX-IV | \(B. asper\) | Basic | Lys49 | Phagocytosis, superoxide production (in vitro) | [150] |
| BaTX-I | \(B. atrox\) | Basic | Lys49 | Superoxide production, lipid droplet formation (in vitro) | [155] |
Table 1. Cont.

| PLA2       | Origin | Basic or Acid | Type of PLA2 Variant | Inflammatory Activity/Experimental Model | Refs. |
|------------|--------|---------------|----------------------|------------------------------------------|-------|
| BaTX-II    | B. atrox | Basic         | Asp49                | Superoxide and H2O2 production, MPO release, NET formation, lipid droplet formation (in vitro) | [155] |
| BaPLA2     | B. atrox | Acidic        | Asp49                | Superoxide production, lipid droplet formation (in vitro) | [140,155] |
| BaPLA2-I   | B. atrox | Basic         | nd                   | Mast cell degranulation, edema (in vivo)  | [132] |
| BaPLA2-III | B. atrox | Neutral       | nd                   | Degranulation, edema (in vivo)           | [132] |

nd, not described.

2.2. Influence of Bothrops svPLA2s on Pathways of Arachidonic Acid Metabolism

It is well established that sPLA2s play key modulatory roles in numerous cellular processes in physiological and pathological conditions by regulating the release of AA from membrane phospholipids [27,171]. It has long been recognized that the AA-derived lipid mediators are potent mediators of inflammation [83]. The AA is rapidly metabolized by several enzyme complexes, including cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP450). These enzymatic pathways promote the synthesis of oxygenated and bioactive products, generically called eicosanoids, which include prostaglandins (PG), leukotrienes (LT), hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), epoxides (EETs), and lipoxins (LX) [172–178]. A summary of the cascades involved in biosynthesis of eicosanoids is shown in Figure 2.

It is important to emphasize that COX-1 is a constitutive isoform present in most tissues and is responsible for generating PGs for diverse physiological functions [179–182]. In contrast, COX-2 is upregulated by inflammatory cytokines and growth factors [183,184] and is constitutively expressed in some tissues [185,186].

Regarding the production of inflammatory eicosanoids by Bothrops svPLA2s, studies have demonstrated that the intraperitoneal injection of MT-III [187] and MT-II [162] in mice induced an early and transient release of PGD2, followed by a rapid and sustained release of PGE2. Likewise, in mice injected with BatroxPLA2 [140], from B. atrox snake venom, and BJ-PLA2-I from B. jararaca [141], an early release of PGE2 was observed. The in vivo experimental models of previous studies have revealed that B. asper sPLA2s induce the release of other eicosanoids, such as thromboxane A2 (TXA2) and LTB4 [123]. Moreover, an Asp49 svPLA2 from B. atrox venom [140] stimulated the production of LTB4, lipoxin, and PGE2.

PGE2 and PGD2 are important modulators of vasodilation, and PGE2 can potentiate an increase in vascular permeability, promoted by mediators of this phenomenon, with a consequent formation of edema [188,189]. Studies using pharmacological treatment with non-steroidal anti-inflammatory compounds were crucial in demonstrating the participation of these COXs-derived lipid mediators on edema [126,190] and hyperalgesia [135], induced by B. asper sPLA2s. In addition, studies demonstrating that MT-III and MT-II upregulated COX-2 protein expression in peritoneal leukocytes without altering the constitutive expression of COX-1 evidenced the ability of these venom PLA2s to influence downstream cyclooxygenase isozymes and suggested this as a mechanism by which these bothropic sPLA2s induced the production of prostaglandins [162,163]. Moreover, these findings suggested that the catalytic activity of these bothropic PLA2s did not contribute to the induction of PG biosynthesis, since MT-II, devoid of catalytic activity, caused the same effect.

In this regard, studies have demonstrated that the IkB phosphorylation inhibitor TPCK effectively prevented both MT-II- or MT-III-induced COX-2 expression, suggesting that the activation of NF-κB was critical for the induction of COX-2 expression by these bothropic svPLA2s. The involvement of NF-κB as the mechanism underlying this venom svPLA2s-induced upregulation of COX-2 expression was further confirmed by results that revealed
the inhibition of the NF-κB nuclear translocation site, markedly reduced svPLA$_{2s}$-induced COX-2 expression and, as a consequence, reduced PGE$_2$ production by macrophages in culture [162,164].

Figure 2. Scheme of arachidonic acid metabolism by several enzymatic pathways leading to production of bioactive lipid mediators. Abbreviations: (PLA$_2$) phospholipase A$_2$, (Lyso-PAF) lysophospholipid-platelet-activating factor, (COX-1) cyclooxygenase-1, (COX-2) cyclooxygenase-2, (5-LO) 5-lipoxygenase, (12-LO) 12-lipoxygenase, cytochrome P450, (PGD$_2$) prostaglandin D$_2$, (PGJ$_2$) prostaglandin J$_2$, (PGF$_2$) prostaglandin F$_2$, (PGH$_2$) prostaglandin H$_2$, (TXA$_2$) thromboxane A$_2$, (PGE$_2$) prostaglandin E$_2$, (PGD$_2$) prostaglandin D$_2$, (PGJ$_2$) prostaglandin J$_2$, (PGF$_2\alpha$) prostaglandin F$_2$ alpha, (PGJ$_2$) prostacyclin, (15-HPETE) 15-hydroperoxyeicosatetraenoic, (15-HETE) 15-hydroxyeicosatetraenoic acid, (12-HPETE) 12-hydroperoxyeicosatetraenoic acid, (12-HETE) 12-hydroxyeicosatetraenoic acid, (5-HPETE) 5-hydroperoxyeicosatetraenoic acid, (5-HETE) 5-hydroxyeicosatetraenoic acid, (LTA$_4$) leukotriene A$_4$, (LTB$_4$) leukotriene B$_4$, (LTC$_4$) leukotriene C$_4$, (LTD$_4$) leukotriene D$_4$, (LTE$_4$) leukotriene E$_4$, (LXA$_4$) lipoxin A$_4$, (LXB$_4$), lipoxin B$_4$, (19-HETE) 19-hydroxyeicosatetraenoic acid, (EETs) epoxyeicosatrienoic acids.

Studies employing mouse resident peritoneal macrophages or neutrophils in culture revealed that viperids sPLA$_{2s}$s induced a marked release of PGE$_2$ in cell supernatants, accompanied by the release of AA [162,165,166,191]. These data support the results in vivo and serve as evidence that immune innate leukocytes, such as resident macrophages and neutrophils, are important sources of PGs under in vivo stimuli by sPLA$_2$ from Bothrops spp. snake venoms. Interestingly, studies have demonstrated that the incubation of resident peritoneal macrophages with MT-II or MT-III significantly increased the concentration of AA [162,164]. Although the release of AA induced by the Asp49 sPLA$_2$ was approximately 20 times greater than that induced by MT-II, it demonstrated that the catalytic activity of viperid sPLA$_{2s}$ was not an essential requirement for inducing COX-2 expression and PGE$_2$ production. According to Kini and Evans (1995) [192], in mechanisms independent
on catalytic activity, as in the case of MT-II, the interaction of sPLA2s to acceptor regions can cause the biological effect or interfere with the interaction of target proteins with their physiological ligands. Furthermore, some effects may result from combinations of both enzymatic and non-enzymatic mechanisms [192], leading to the activation of several signaling pathways; this should be considered when interpreting the effects of group IIA Lys49 svPLA2s. In this context, since crosstalk among sPLA2, cPLA2, and iPLA2s has been demonstrated to occur in several physiological and inflammatory conditions [193–196], the contribution of prey/victim cPLA2 and/or iPLA2 to the increased production of PGs and upregulation of COX-2 protein expression induced by svPLA2 variants was evaluated in diverse in vitro experimental models [162,165,187]. Thus, the pharmacological treatment of cells with the cPLA2 inhibitor but not the iPLA2 inhibitor decreased the release of AA and the production of PGE2 and PGD2 induced by svPLA2. In contrast, these pretreatments did not modify the MT-III-induced COX-2 expression but reduced the COX-2 expression induced by MT-II. These results demonstrate that cPLA2 is required for distinct actions of MT-II in the PG biosynthetic pathway in macrophages [162,187]. This is consistent with the reported functional cooperation between intracellular PLA2s and GIIA sPLA2 for PG biosynthetic responses in several other cell systems [171,197–199]. The role of cPLA2 as a key enzyme in supplying AA for COX-2-dependent PGs production is well established [171,200,201]. Taken together, the available data demonstrate that the Asp49 svPLA2s are functionally coupled with cPLA2, since prior activation of cPLA2 is required for MT-III to act with downstream enzymes for PG biosynthesis in macrophages and neutrophils [165,187]. Interestingly, the association of Lys49 sPLA2 with cPLA2 in addition to being important for the supply of AA for the production of PG, appears to modulate the transcription and protein expression of COX-2 inflammatory isoform. The mechanisms involved in the coupling between the venom GIIA sPLA2s and mammalian cPLA2 have yet to be investigated. One possibility is that GIIA svPLA2s activate cPLA2 by distinct signaling cascades that mimic the transducing mechanism conveyed by physiological activators of cPLA2, such as MAPKs, since this enzyme family is likewise important for the activation of NF-κB [202]. In line with this concept, the Asp49 svPLA2, MT-III, in addition to Lys49 PLA2, MT-II, from B. asper snake venom, were revealed to stimulate the phosphorylation of protein kinases, including the MAPKs, such as p38MAPK and ERK1/2 [167,169,171]. Moreover, other protein kinases, including PI3K, PKC, and PTK, were reported to be phosphorylated in macrophages stimulated by both MT-III and MT-II [162,164]. In this regard, studies have demonstrated that MT-III (Asp49 sPLA2) stimulates PKC and p38MAPK pathways to positively modulate PGE2 production and COX-2 expression via NF-κB, while MT-II (Lys49 PLA2) displays similar effects by activating PKC, ERK1/2, and PTK in murine peritoneal macrophages [167,169,171]. Since PTK is involved in the activation of MAPKs, which, in turn, are essential for cPLA2 activation, this signaling protein might be involved in the activation of cPLA2 by MT-II [202–204]. Furthermore, another pathway implicated in the release of PGE2 and the expression of COX-2 induced by MT-III was demonstrated to be independent of NF-κB activation. This pathway involved the activation of ERK1/2 by the 12-HETE pathway, the main product of 12-LO [166]. The involvement of another transcription factor in these MT-III-induced effects was suggested by the authors. Together, these findings reveal the variety and complexity of the mechanisms involved in the effects of svPLA2 leading to the generation of lipid mediators. The signaling molecules and pathways acting in an innate immune cell (macrophage) upon stimulus either by Asp49 or Lys49 svPLA2 are summarized in Figure 3.

Although the participation of the M-type PLA2 receptor or another type of interaction of Bothrops sPLA2 with membrane sites for the stimulation of signal transduction pathways has not yet been demonstrated, a study revealed for the first time the involvement of the TLRs in the inflammatory response induced by MT-III (Asp49-PLA2) from B. asper in macrophages [205]. The involvement of TLR2 and MyD88 adapter molecules was demonstrated to be critical in producing PGs, COX-2 protein expression, and cytokines IL-1β and IL-10 induced by this svPLA2. An indirect mechanism for the activation of
TLRs through the release of DAMPs was suggested by the authors, since the analysis of the fatty acids released by the hydrolysis of membrane phospholipids by MT-III revealed high levels of oleic and palmitic acids. In this context, it is known that arachidonic, oleic, and palmitic acids produced by membrane cleavage by sPLA2s are important bioactive mediators involved in the induction and release of COX-2 and PGE2, respectively, through the activation of intracellular signaling mechanisms in several cell types [206–209].

Figure 3. Schematic representation of signaling pathways stimulated by Asp49 and Lys49 PLA2s from B. asper snake venom to produce prostaglandins in macrophages. Asp49 PLA2 induces AA and fatty acids release from macrophage membrane. Free fatty acids can act as DAMPs and activate TLR2 or other TLRs (still unknown), via activation of adapter protein MyD88, leading to COX-2 protein expression and release of PGs, Asp49PLA2 upregulates the 12-LO pathway, culminating the release of 12-HETE. 12-HETE, in turn, activates the ERK1/2 pathway, leading to COX-2 protein expression and PG release, independent on NF-κB translocation. Asp49PLA2 also activates the signaling protein PI3K leading to COX-2 expression and production of PGs independent on NF-κB activation. Asp49 PLA2 also activates PKC and p38 MAPK pathways promoting COX-2 expression and production of PGs via NF-κB activation. In addition, Asp49PLA2 provides AA for activation of COX-1 activity which is followed by production of proinflammatory PGs. Asp49 and Lys49 PLA2s both produce PGs by pathways independent on iPLA2. Although both sPLA2 from bothropic venom produce PGs via crosstalk with cPLA2, only Lys49PLA2 induces COX-2 expression dependent on this cytosolic PLA2. Lys49PLA2 activates signaling pathways mediated by p38 MAPK, PTK, PKC, and ERK1/2. All these kinase pathways, except for p38 MAPK, are involved in NF-κB activation and COX-2 protein expression and PG production. Full arrows indicate actions already studied and demonstrated. Dotted arrows indicate hypothesized or unknown effects.

2.3. Bothrops svPLA2s Trigger Lipid Accumulation in Immunocompetent Cells

As mentioned, the high enzymatic activity exhibited by ophidian sPLA2 provides a microenvironment rich in free fatty acids that exert stimulating effects in immunocompetent cells, leading to the biosynthesis of lipid mediators. Moreover, excess free fatty acids exert cytotoxic effects that trigger the activation of cellular mechanisms capable of converting
free fatty acids into metabolites of lower toxicity, known as neutral lipids (triacylglycerol and cholesterol—energetic body reserve) [210]. In recent decades, numerous studies have demonstrated the activation of intracellular metabolic pathways responsible for the metabolism of free fatty acids in neutral lipids and the consequent formation of dynamic organelles called lipid droplets (LDs) [211,212]. These organelles are composed of a hydrophobic neutral lipid core surrounded by a phospholipid monolayer membrane, which contains numerous proteins related to cellular activation in addition to structural proteins, such as perilipin 2 (PLIN2), which plays an important role in LD assembly and the formation of foam macrophage [213], marker cells in metabolic diseases, such as atherosclerosis and obesity [214]. LDs are commonly present in adipocytes due to the already-established role of these cells in the supply of energy in mammalian organisms [215]. In addition to the relevance regarding lipid homeostasis, the direct relationship between increased LD formation and inflammatory processes was evidenced by numerous studies [216–218]. In this sense, mammalian group IIA sPLA₂s have been identified as potential plasma biomarkers for diseases related to lipid imbalance, such as atherosclerotic cardiovascular disease and obesity [219]. Alongside a marked inflammatory reaction, these metabolic diseases are characterized by lipid accumulation in immunocompetent cells [220,221]. In line with the ability of svPLA₂S to elicit an inflammatory response characterized by a high level of inflammatory mediators and free fatty acids, it was demonstrated that MT-III (Asp49 PLA₂), isolated from B. asper venom, induced LD formation enriched by PLIN2 protein in mice peritoneal macrophages [170]. This effect was likewise observed in rat vascular smooth muscle cells isolated from the thoracic aorta stimulated by MT-III [166]. Moreover, the ability of MT-II, a Lys49 PLA₂ homologue devoid of catalytic activity from B. asper venom, to directly activate macrophages to form LDs was reported [167]. This effect was reproduced by a synthetic peptide corresponding to the C-terminal sequence 115–129 of MT-II, evidencing the critical role of C-terminus for the MT-II-induced effect [167]. Similarly, BaTX-I, a catalytically inactive Lys49 variant; BaTX-II, a catalytically active Asp49; and BaPLA₂, an acidic catalytically active Asp49 PLA₂, isolated from B. atrox snake venom, increased the number of LDs on murine macrophages cell line J774A.1 [152]. The formation of LDs upon stimulus by MT-III was likewise demonstrated in human monocytes of peripheral blood [222].

Considering the above information, LD formation induced by svPLA₂S in phagocytes proved to be inherent to the action of sPLA₂S regardless of the catalytic activity. The capability of Bothrops svPLA₂S to induce the formation of LDs is related to the activation of PRRs of an innate immune response, kinase proteins, and intracellular PLA₂ signaling pathways involved in cellular metabolism, proliferation, and differentiation [170,205]. Hence, by using gene knockout mice cells and a pharmacological approach, the participation of TLR2, MYD 88 adaptor protein, and CD36 in LD formation in macrophages in culture has been reported [170,205]. In addition, the upregulation of CD36 receptors was observed in these cells. Considering the participation of the MYD 88 adaptor molecule, the involvement of other TLRs has yet to be investigated. In line with the reported Asp49 svPLA₂ action on PRRs, the upregulation of both SRA-1, from the scavenger receptor family, and LOX-1, an LDL receptor, was demonstrated in mouse aortic smooth muscle cells (VSMCs) [168]. These findings demonstrate that the inflammatory response elicited by Asp49 svPLA₂ likewise involved the upregulation of PRRs associated with lipid uptake in immunocompetent cells. This fact indicates that svPLA₂S can be useful tools in studies aiming to understand the diseases associated with lipid imbalance.

In addition to providing the synthesis of mediators, TLR2 activation elicited by MT-III action was related to cytoskeleton activation [205], a critical step in the transport of structural proteins into LDs, such as PLIN2. Cytoskeletal activation involves the activation of kinase proteins [223]. Consistent with this information, the participation of kinase proteins in LD formation induced by both Asp49 and Lys49 svPLA₂S from B. asper venom has been demonstrated through a pharmacological approach and the detection of phosphorylated kinase proteins. Yet, MT-II- and MT-III-induced LD biogenesis is dependent on the ac-
tivation of PKC, PI3K, p38MAPK, and ERK1/2 signaling pathways in mice peritoneal macrophages [224]. It is well known that PKC regulates a variety of processes associated with lipid droplet biology, such as adipocyte differentiation [225], magnolol-induced lipolysis [226], cholesterol-induced targeting of caveolin to lipid droplets [227], and the expression of the PAT family [228]. Hence, the activation of the PKC signaling pathway in macrophages stimulated by the Bothrops PLA2s MT-II and MT-III in peritoneal macrophages may be implicated in an increase in PLIN2 protein expression, since LD formation induced by both svPLA2s has always been accompanied by an increase in PLIN2 protein expression in macrophages.

PI3K/AKT is a classical pathway involved in insulin resistance, cell growth, and lipid metabolism associated with the inhibition of cholesterol efflux leading to LD formation [229]. In this sense, the participation of the PI3K signaling pathway in MT-III-induced LDs in macrophages and vascular smooth muscle cells stimulated with MT-III has been demonstrated [167,224]. In the case of vascular smooth muscle cells, the activation of PI3K was related to the uptake of fatty acids to LDs by macropinocytosis [167]. Furthermore, the findings that MT-III increases phagocytic activity and upregulates macrophage markers in VSMCs reinforce the importance of this class of enzymes as inducers of factors implicated in the formation of foamy cells in both mononuclear phagocytic cells and VSMCs, which are key elements in the development of metabolic diseases.

The MAPK signaling pathway has been revealed to mediate the activation of intracellular PLA2s in physiologic and inflammatory contexts [230,231]. It has been demonstrated that the ERK1/2 signaling pathway is implicated in LD formation via the activation of phospholipase D (PLD) and phosphorylation of dynein [232,233]. Consistent with this information, the critical role of ERK1/2 for LD formation induced by MT-II and MT-III in macrophages and VSMCs was reported [167,224]. Regarding p38 MAPK, the literature evidenced its importance in the development of atherosclerosis [234] and the apoptosis of foam macrophages. Macrophage death is a feature of atherosclerotic plaque linked to necrosis and plaque destabilization [235]. Interestingly, although MT-III has been demonstrated to activate apoptotic pathways, including the p38 MAPK signaling pathway, DGAT, ACAT, cPLA2, and LD formation in macrophages, no change in cell viability was observed. Further studies may clarify this lack of apoptotic effect.

It is known that the MAP kinases signaling pathway is implicated in intracellular PLA2 activation, including the Ca\(^{2+}\)-dependent cytosolic group IVA PLA2 (cPLA2\(\alpha\)) and the Ca\(^{2+}\)-independent group VIA PLA2 (iPLA2) involved in both physiological and pathophysiological conditions [236,237]. The biogenesis of LDs in CHO-K1 cells submitted to an enriched environment of fatty acids demonstrated ERK, p38, and JNK signaling pathway activation, with JNK cascade being responsible for cPLA2\(\alpha\) phosphorylation in this event. Of note, cPLA2 was likewise implicated in LD biogenesis stimulated by MT-III associated with the activation of ERK and the p38 MAPK signaling pathway [224]. Considering the ability of cPLA2 to mobilize cell membrane fatty acids [238], the aforementioned activation should amplify the action of MT-III and provide a greater substrate for the metabolization and formation of LDs. Moreover, the biogenesis of LDs induced by the svPLA2s MT-III and MT-II is dependent on the activation of iPLA2 signaling pathways [167,224]. This signaling pathway was associated with the processing of fatty acids into triacylglycerol, a relevant component in the constitution of LDs [214]. Hence, the crosstalk already evidenced between intracellular PLA2 and svPLA2 to elicit inflammatory conditions [169] might contribute to elucidating mechanisms related to the formation of LDs.

The peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the family of nuclear receptors that regulate glucose homeostasis, inflammation, and lipid metabolism. Three proteins, encoded by distinct genes, have been identified: PPAR-\(\alpha\), PPAR-\(\beta/\delta\), and PPAR-\(\gamma\), which control gene expression by binding to PPREs in the promoters [239]. The activation of PPARs is a tightly regulated process implicated in the control of lipid homeostasis, which involves the biogenesis of LDs and protein expression involved in lipid uptake, including PRRs and structural protein PLIN2, and
enzymes implicated in neutral lipid synthesis (triacylglycerol and cholesterol) [240]. PPARs have been demonstrated to increase in foam macrophages [241]. In this sense, it was revealed that MT-III induced the upregulation of the transcription factors PPAR-γ and PPAR-β/δ, in addition to the translocation of these factors to the nucleus of mouse peritoneal macrophages. The pharmacological blockage of the PPAR-β/δ transcription factor abolished the increase in PLIN2 and CD36 protein expression induced by MT-III. Moreover, the PPAR-γ blockage caused a reduction in LD formation and abolished CD36 receptor protein expression induced by MT-III. Since an increased expression of CD36 and PLIN2 is related to macrophage differentiation into foam cells [242,243], these findings suggest that MT-III induces foam cell formation by this route. In addition, MT-III caused an increase in the levels of triacylglycerol and cholesterol due to the uptake of free fatty acids. These effects were mediated by DGAT and ACAT enzymes, which are involved in the synthesis of triacylglycerol and cholesterol, respectively [244]. In agreement with this study, a significant increase in triacylglycerol and cholesterol levels was observed in human monocytes under MT-III stimulation [222]. This effect was dependent on fatty acid reacylation. Moreover, the fatty acid composition of triacylglycerol and cholesterol induced by MT-III was compatible with fatty acids released by the enzymatic action of this svPLA2 on cell membranes. According to the above information, the mechanisms triggered by MT-III, in both mice peritoneal macrophages and human monocytes, align with macrophages differentiation into foamy cells, a cell type characteristic of inflammatory metabolic diseases, such as atherosclerosis [245]. Similarly, MT-III could stimulate LD formation in VSCMs. This lipid accumulation was likewise mediated by the activation of transcription factors PPAR-γ and PPAR-β/δ and DGAT and ACAT enzymes. Moreover, it is noteworthy that VSCMs under stimulation of MT-III exhibited an increase in the protein levels of PRRs, SRA-1 (scavenger receptor type 1), and LOX-1 (lectin-like oxidized low-density lipoprotein receptor-1). Interestingly, the blockage of these receptors did not alter the formation of LDs induced by MT-III, but the upregulation of LOX-1 was associated with an increased uptake of acetylated-low density lipoprotein (acLDL) in VSMCs stimulated by this svPLA2. This higher uptake of acLDL by VSMCs identifies new pathways involved in the accumulation of lipids triggered by a sPLA2 that is not directly linked to the reacylation of free fatty acids. In addition, lipid accumulation induced by MT-III in VSCMs was related to the expression of ATP-binding cassette transporters ABCA1 and ABCG1, responsible for the efflux of cholesterol of macrophage-derived foam cells [242]. Although the signaling pathway by which MT-III induces an increased expression of the factors implicated in lipid homeostasis has not been fully elucidated, these studies have broadened the knowledge about the actions of svPLA2s on the formation of LDs and the synthesis of lipid mediators and provided new insights into the actions of group IIA sPLA2s in diseases related to lipid imbalance. The pathways and factors involved in lipid accumulation in an innate immune cell (macrophage) upon stimulus by svPLA2s are summarized in Figure 4.

Another aspect related to the metabolism of free fatty acids is the biosynthesis of lipid mediators [246–248]. On the one hand, it is known that the synthesis of eicosanoids is closely related to the triggering of the inflammatory process induced by svPLA2s, regardless of the catalytic activity on the cell membranes [13]. In this sense, the immunofluorescence approach has demonstrated that LDs stimulated by svPLA2s synthesize PGE2 [167,249]. On the other hand, some eicosanoids are implicated in the resolution of inflammatory processes, such as PGJ2. This mediator was co-located in LDs in macrophage peritoneal mice stimulated by MT-II [161], indicating, for the first time, that the LDs not only are related to the production of inflammatory mediators but also might play a role in regulating this process.

Adipose tissue is the principal organ responsible for balancing energy metabolism in the mammalian body. An imbalance in adipose tissue functions is linked to the triggering of the inflammatory process observed in metabolic diseases, including obesity [250,251]. Recently, it has been demonstrated that MT-III activated proinflammatory mechanisms in 3T3-L1 preadipocytes, including the biosynthesis of PGE2 and PGJ2, lipid mediators impli-
In macrophages, svPLA2 acts on membrane phospholipids generating free fatty acids, which are ligands of and may activate TLR2 and the Myd88 adaptor molecules participate in the recruitment of the PLIN2 protein via cytoskeleton activation stimulated by svPLA2. Furthermore, MT-III upregulated the gene expression of the adipokines leptin and adiponectin in preadipocytes [169]. These mediators have been described as regulating appetite and satiety, glucose and lipid metabolism, inflammation, and immune functions [252-255]. Although the mechanisms related to the release of adipokines and the ability of MT-III to induce lipid accumulation in adipocytes have not yet been investigated, these data offer new directions for investigating the actions triggered by svPLA2s and mammalian GIIA sPLA2s.

![Schematic representation of the mechanisms and factors involved in lipid accumulation induced by svPLA2s, in macrophages. svPLA2 acts on membrane phospholipids generating free fatty acids, which are ligands of and may activate the TLR2, CD36, and cytoplasmic transcription receptors and factors PPARs. svPLA2 induces the activation of transcription factors PPAR-γ and PPAR-δ/β and increases protein expression of PPARs and CD36. PPAR-γ, PPAR-β/δ, TLR2/MyD88, and CD36 receptors, as well as DGAT and ACAT enzymes are involved in the lipid droplets formation stimulated by MT-III. PPAR-β/δ, but not PPAR-γ, is implicated in upregulation of PLIN2 protein expression, induced by MT-III. Moreover, TLR2 and the Myd88 adaptor molecules participate in the recruitment of the PLIN2 protein via cytoskeleton activation stimulated by MT-III. In addition, LD formation induced by svPLA2 is dependent on activation of PKC, PI3K, p38MAP, ERK, cPLA2, and iPLA2 signaling pathways.](image-url)

**Figure 4.**

**3. Conclusions**

The existing literature demonstrates that the svPLA2s trigger a cascade of inflammatory events including edema formation, leukocyte recruitment into tissues, release of a complex network of inflammatory mediators, and increased oxidative stress in experimental animal models that mimic the inflammatory responses elicited by viperid snake
venoms, especially those from the Bothrops genus, in the victims. The catalytic activity of the svPLA₂s is not strictly required by these proteins for the triggering of all the inflammatory responses, since the catalytically inactive Lys₄₉ PLA₂ variants can display inflammatory events that are qualitatively similar to those of Asp₄₉ PLA₂s. In addition to cell migration, the svPLA₂s can activate distinct functions of immunocompetent cells that include phagocytosis, the respiratory burst, NET formation, production of cytokines, chemokines and multiple reactive cleavage products such as lysophospholipids, polyunsaturated fatty acids and eicosanoids, as well as formation of LDs. The highly complex network of mediators, particularly lipid mediators, modulates a variety of inflammatory events triggered by this class of snake venom toxins. The effects triggered by svPLA₂s in inflammatory cells that lead to generation of lipid mediators has been associated with the activation of distinct signaling pathways of inflammatory kinase proteins by mechanisms dependent and independent of NF-κB. Moreover, the inflammatory response elicited by svPLA₂s in leukocytes also involves upregulation of PRRs of innate immune response, the crosstalk between the svPLA₂ and intracellular PLA₂s, and upregulation of factors implicated in lipid homeostasis. Although much has been learned regarding the inflammatory actions of svPLA₂s, many knowledge gaps still exist and need to be addressed. There is still considerable work to be done before we fully understand the complex interactions that occur among svPLA₂s and immunocompetent cells and tissues that lead to inflammation. The cell acceptors and/or receptors involved in the actions of svPLA₂s in these cells and the signaling pathways elicited and how they interact with each other remain to be clarified. In addition, the actual types and subtypes of receptors activated by the principal mediators produced by svPLA₂s and the mechanisms involved in coupling between the svPLA₂ and endogenous PLA₂s have yet to be investigated. Recently, the stimulatory activity of a svPLA₂ on adipose tissue cells leading to increased biosynthesis of PGE₂ and other inflammatory mediators including adipokines was demonstrated. This information offers new directions for investigating the actions triggered by svPLA₂s and mammalian GIIA sPLA₂ and gives insights into the potential role of the adipocytes as target cells for viperid snake venoms. Finally, a deeper and comprehensive understanding of the mechanisms underlying the inflammatory actions of svPLA₂s will give new insights into (i) the actions of group IIA sPLA₂s in diseases related to lipid imbalance and inflammation and (ii) a better understanding of the pathophysiology of Bothrops envenomation. Within this frame, the acquired knowledge might pave the way for the development of novel therapeutic approaches aimed at counteracting the prominent inflammation caused by Bothrops snakebite envenoming.

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