New Mechanisms by Which Secretory Phospholipase A₂ Stimulates Neutrophils to Provoke the Release of Cytotoxic Agents

Garret Zallen, MD; Ernest E. Moore, MD; Jeffrey L. Johnson, MD; Douglas Y. Tamura, MD; Michael Barkin; Hubertus Stockinger, PhD; Christopher C. Silliman, MD, PhD

Background: Secretory phospholipase A₂ (sPLA₂) is a potent proinflammatory enzyme that stimulates inflammation through the production of reactive lipids. However, enzymatic inhibitors have been disappointing in their effectiveness in halting hyperinflammation.

Objective: To determine whether sPLA₂ acts directly on neutrophil plasma membrane lipids or via a nonenzymatic mechanism.

Design: Isolated neutrophils (PMNs) were incubated with 3 types of sPLA₂, and elastase and superoxide release from PMNs was measured. Ethyleneglycoltetraacetic acid was used as a selective enzymatic inhibitor. The PMNs were exposed to sPLA₂ in the presence and absence of ethyleneglycoltetraacetic acid and the release of elastase was measured.

Setting: Urban trauma research laboratory.

Results: The sPLA₂ acted directly on plasma membrane lipids to stimulate the PMN to produce superoxide and release elastase. This mechanism is blocked with enzymatic inhibition of sPLA₂. The sPLA₂ also provokes elastase release from PMNs independently of its enzymatic function. This mechanism is not blocked with traditional enzymatic inhibitors.

Conclusions: These data indicate that the sPLA₂ can act directly on PMNs to stimulate the release of inflammatory mediators via enzymatic degradation of plasma membrane lipids. In addition, sPLA₂ can act as a ligand and stimulate the PMN independently of its enzymatic activity.

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CIRCULATING LEVELS of secretory phospholipase A₂ (sPLA₂) have recently been identified as a sensitive marker of injured and septic patients at risk for multiple organ failure (MOF). The magnitude of sPLA₂ elevation correlates with the development of the adult respiratory distress syndrome and with MOF. Our animal models of gut ischemia and reperfusion have demonstrated the importance of the enzymatic function of PLA₂ in producing lung and liver injury. Other animal models of sepsis have shown that enzymatic inhibitors of sPLA₂ improve survival. However, results of trials using enzymatic inhibitors of sPLA₂ in the clinical setting have been disappointing. The link between sPLA₂ and inflammation has been attributed to its calcium-dependent enzymatic function. Secretory PLA₂ catalyzes the hydrolysis of the 2-ester bond of 3-sn-phosphoglycerides. This produces reactive lipid mediators such as platelet-activating factor and arachidonic acid. Previous work has focused on sPLA₂ cleavage of plasma lipids to activate inflammatory cells. However, the exact mechanism of cellular activation remains unclear.

Our research and that of others have demonstrated the pivotal role of neutrophils (PMNs) in the pathogenesis of MOF. When stimulated, PMNs increase surface expression of adhesion molecules, firmly adhere to and diapedes through the endothelium, generate reactive oxygen species, and release proteases, which in turn leads to endothelial leak and organ damage. Understanding what stimulates PMNs to perform these processes will add further understanding to the pathophysiological mechanisms of MOF and may provide insight into new therapeutic strategies. Circulating sPLA₂ has been thought to signal PMNs via its enzymatic metabolism of phospholipids. However, because of the inability of en-
MATERIALS AND METHODS

EXPERIMENTAL DESIGN

To investigate signaling pathways in the PMN, we studied isolated cells, thus removing any plasma lipids that could act as substrate for the sPLA₂. Superoxide and elastase release were measured to provide downstream evidence of cellular activation in the PMN. We investigated 3 types of sPLA₂: Naja naja (snake venom), pancreatic, and synovial. Synovial sPLA₂ is now recognized as the type of sPLA₂ that is the most sensitive marker for subsequent MOF development. The name synovial sPLA₂ is somewhat misleading. This subclass of sPLA₂ was named synovial when it was initially isolated from arthritic joints, but subsequently it has been shown to be produced by platelets, monocytes, PMNs, and endothelial cells. Of interest, synovial sPLA₂ is the type of sPLA₂ whose levels are elevated after trauma and during sepsis. Acknowledging that sPLA₂ requires calcium for its enzymatic function, we were able to selectively inhibit enzymatic function with ethyleneglycotaeraetacetic acid (EGTA), a highly specific extracellular scavenger of free calcium.

EXPERIMENTAL PROCEDURES

PMN Isolation

The PMNs were isolated from heparinized blood of healthy volunteers by means of dextran sedimentation followed by centrifugation through a ficoll-hypaque density gradient as previously described. The resulting PMNs were washed once and resuspended in Kreb Ringer phosphate with dextrose at pH 7.35 to a final concentration of 2.5 × 10⁵ cells per millilter. The final cell population was more than 98% PMNs by differential staining and more than 98% viable by trypan blue exclusion.

Superoxide Anion Assay

Superoxide anion generation by PMNs was measured by superoxide dismutase–inhibitable cytochrome c reduction in 96-well microplates. Isolated PMNs (3.75 × 10⁵) were incubated with sPLA₂ for 5 minutes. All wells contained cytochrome c (80 µmol/L) and blank wells contained superoxide dismutase (15 µg/mL) to achieve a total reaction volume of 150 µL. All priming assays were completed at 37°C in duplicate with a separate superoxide dismutase blank. The respiratory burst was initiated with the addition of 1-µmol/L N-formyl-methionyl-leucyl-phenylalanine (fMLP) to experimental wells. The maximal rate of superoxide anion production was determined by the slope of the absorbance curve over 5 points by means of Softmax (Molecular Devices Corp, Menlo Park, Calif) software. The data are recorded as the maximal rate of superoxide anion production (nanomoles of O₂⁻/3.75 × 10⁵ cells per minute).

Elastase Assay

Elastase release by PMNs was measured by N-methoxy succinyl-alanine-alanine-pro-val p-nitroanilide ketone inhibitible by N-methoxy succinyl-alanine-alanine-pro-val p- nitroanilide cleavage in 96-well microplates. Isolated PMNs (6.25 × 10⁵) were incubated with sPLA₂ for 5 minutes, and EGTA was added 2 minutes before the addition of the sPLA₂. The incubated cells were pelleted by centrifugation at 400 g for 5 minutes at 25°C. The supernatants (50 µL per well) were incubated in a 96-well plate at 37°C for 1 hour. Elastase release was measured as a percentage of total elastase content as determined by Triton X-100 (Sigma Chemical Co, St Louis, Mo) lysis.

Materials

Naja naja and pancreatic sPLA₂ were obtained commercially (Sigma Chemical Co). Human synovial sPLA₂ was obtained as a gift (H.S., Department of Biochemicals, Boehringer Mannheim GmbH, Penzberg, Germany). All other chemical reagents were purchased (Sigma Chemical Co) unless specified. Ficoll-Hypaque was purchased (Pharmacia Biotech, Uppsala, Sweden).

RESULTS

DIRECT STIMULATION OF PMNs BY sPLA₂

To determine whether sPLA₂ is able to stimulate PMNs in the absence of plasma lipids acting as substrate, isolated PMNs were incubated with the 3 types of sPLA₂ (Figure 1). All 3 types of sPLA₂ provoked significant release of elastase compared with paired control PMNs (unstimulated). Synovial sPLA₂ was used at a lower concentration because we only had a more dilute supply of synovial sPLA₂. Pancreatic sPLA₂ had a response at 100 units that was equivalent to that of platelet-activating factor (200 nmol/L)–primed and fMLP (1 µmol/L)–activated PMN elastase release. Superoxide release was performed with both Naja naja and pancreatic sPLA₂. Because of reagent constraints, synovial sPLA₂ was not tested. Neither pancreatic nor Naja naja sPLA₂ directly stimulated PMNs for superoxide release (data not shown). However, Naja naja was able to prime PMNs for fMLP–activated superoxide release (Figure 2).

ENZYMATIC INDEPENDENCE OF sPLA₂

Elastase assays were performed in the presence or absence of EGTA (25 mmol/L). The EGTA at 25 mmol/L was sufficient to chelate enough calcium to inhibit the enzymatic function of all 3 of the sPLA₂s (data not shown). The addition of EGTA alone did not cause any increased release of elastase, nor did it cause cell membrane damage (PMNs 98% viable after EGTA treatment by trypan blue staining). Pancreatic sPLA₂ was able to cause elastase release independently of its enzymatic function. Synovial sPLA₂ was able to stimulate elastase re-
Multiple organ failure remains the leading cause of death late after injury in the intensive care unit. The pathophysiologic process is characterized by malignant systemic inflammation, but the fundamental mechanism remains unclear. The PMN is a pivotal effector in MOF, and our Trauma Research Program has focused on the PMN as a surrogate for systemic inflammation. Understanding the mediators that prime PMNs to adhere to the endothelium and have maximal potential for cytotoxic effects and the mediators that activate the primed PMN to cause organ injury may lead to therapy that will ameliorate this self-destructive process. In this context, sPLA2 has emerged as a potential key proinflammatory agent in the pathogenesis of MOF.

Phospholipase A2 is a large family of enzymes that have a broad range of physiological roles, including digestion, fertilization, neurotoxic effects, bactericidal activity, and cell signaling. The PLA2s are classified into 2 main categories: cytosolic and secretory. Cytosolic PLA2s are intracellular signaling proteins that are activated by multiple receptor-mediated pathways. Cytosolic PLA2s are typically 85 kd in size and are activated by phosphorylation. The other group, secretory PLA2s, are 14 kd in size and are secreted by multiple cell lines. When high circulating levels were identified in pancreatitis and sepsis and these levels correlated with mortality, sPLA2 became recognized as a proinflammatory mediator. For many years, the proinflammatory role of sPLA2 was thought to be mediated by its enzymatic effects on circulating lipids. Efforts at inhibiting this enzymatic function had success in animal models, but have not proved useful in the clinical setting.

The 3 subclasses of sPLA2 used in this study were chosen because of their unique characteristics. Naja naja (snake venom) has strong enzymatic activity, while pancreatic sPLA2 has been implicated in proinflammatory states such as sepsis and pancreatitis. The third type, synovial sPLA2, was originally isolated from inflamed synovial fluid, but it is now recognized as the key circulating proinflammatory sPLA2 in trauma, sepsis, and pancreatitis. Receptor-mediated signaling of PMNs is crucial to their function and plays a major role in the development of hyperinflammatory states, ultimately culminating in MOF. Understanding how proinflammatory agents such as sPLA2 signal the PMN may identify targets to modify these signaling cascades in severely injured patients.

We have identified 2 novel mechanisms of sPLA2 action on PMNs. First, we have demonstrated, by studying isolated PMNs, that sPLA2 can act on the membrane lipids of healthy and intact PMNs directly. The enzymatic activity on plasma lipids causes both the release of elastase and primes for fMLP-activated superoxide release. The ability of pancreatic sPLA2 to attack intact plasma membranes has not been previously demonstrated, but it may be anticipated because of its digestive function. However, the nonenzymatic effects of sPLA2 appear to ameliorate any enzymatic signaling, as inhibition of its enzymatic activity does not decrease its signaling potential for elastase release. Conversely, synovial sPLA2, which has purely an inflammatory role, is able to attack intact cell membranes to produce reactive lipid species that can in turn signal PMNs to degranulate. This mechanism implies that future therapeutic enzymatic inhibitors of sPLA2 not only will need...
to inhibit the free enzyme, but also may have to intercalate into lipid bilayers to inhibit the breakdown of plasma membrane lipids. Since this mechanism was sufficient to activate the PMNs, we did not evaluate the ability of sPLA₂ to act as a priming agent. However, future exploration with different doses may demonstrate that sPLA₂ can also act as a priming agent.

We have also demonstrated that pancreatic sPLA₂ and synovial sPLA₂, which have been found previously to bind to sPLA₂ receptors,²⁶⁻³² are able to stimulate elastase release independently of their enzymatic function. These findings provide indirect evidence that there are sPLA₂ membrane receptors on PMNs that activate the cell to release inflammatory mediators. While there are no readily available antibodies to these receptors, the fact that both pancreatic and synovial sPLA₂ can act as a ligand defines an entirely new role for these inflammatory proteins. Traditional enzymatic inhibitors should not block the ligand effects unless they also interfere with the binding of these proteins to their receptors. This mechanism has not been tested on enzymatic inhibitors and is an area for potential improvement in their development. Because there are currently no antibodies specific for the sPLA₂ receptor, we are attempting other techniques, such as cell signaling pathways and exploration of PMN membranes.

Understanding the mechanism by which sPLA₂ is able to signal PMNs for cytotoxic effects provides exciting new avenues for modulating this response. The link between sPLA₂ and adult respiratory distress syndrome and MOF was initially thought to result from the enzymatic function of sPLA₂. It is now clear that sPLA₂ is able to stimulate PMNs independently of this enzymatic action. Modulating this new mechanism may uncouple sPLA₂ from MOF.


Reprints: Ernest E. Moore, MD, Department of Surgery, Denver Health Medical Center, 777 Bannock St, Denver, CO 80204 (e-mail: emoore@dhha.org).

REFERENCES


DISCUSSION

Cora K. Ogle, PhD, Cincinnati, Ohio: This presentation along with the previous one from your group really helps elucidate how neutrophils can contribute to multisystem organ failure. I find it fascinating (as an old enzymologist) that an enzyme would have a separate receptor-mediated activity, and I wonder if you could elucidate more on the structure function of what you think the receptor is for the phospholipase.
And even though you can block enzymatic activity, could the active sites still be binding to a phospholipid causing single transduction? I know you’ve used some inhibitors of single transduction. Have you used other inhibitors of other pathways to evaluate receptor function?

The ultimate activity of all phospholipases, as you indicated, is the production of eicosanoids. I would be just a little bit curious if you would have wanted to determine if you had any increased eicosanoid production by either some autocrine stimulation of cytoplasmic phospholipases to increase eicosanoid production. And in that line, did you add any of the inhibitors of eicosanoid production to see if you could get opposite or different end results?

Dr Zallen: Thank you, Dr Ogle. I will try to answer these in the order they were given. The enzyme receptor activity or protein having both an enzyme and a receptor activity is somewhat of a novel concept; however, this type of action is really not that novel. There are many proteins, such as G proteins, that have an enzymatic effect but also have protein-binding sites on them that are important for their location within the cell, and there are numerous types of proteins that have both an enzymatic and a protein-binding activity. The receptor for sPLA₂ has been characterized to be similar to that of a mannose receptor, which neutrophils are also known to possess, and it is possible that through changes in these mannose receptors that they have been able to bind the sPLA₂. In several studies out of France, the enzymatic site on the sPLA₂ has been found to be independent of the binding to these sPLA₂ receptors; therefore, it seems that they are indeed separate events.

The question of blocking other pathways, I have, as I alluded to, looked for ERK activation. I have also tried blocking ERK 1/2 pathways and have been unable to attenuate the elastase release; therefore, the ERK pathway is probably not involved. I have also used web inhibitors that inhibit platelet-activating factor, as there is a small possibility that a small amount of platelet-activating factor can be released by these enzymes, and once again, the PAF inhibitors did not show any activity in terms of decreasing the receptor-mediated elastase release.

Eicosanoid production is an interesting topic, as the sPLA₂ receptor has been implicated in terms of cross-talk between secretory phospholipase A₂ and cytosolic phospholipase A₂. As I’m sure you all are aware, cytosolic PLA₂ resides within the cytoplasm but produces eicosanoids that can act to activate neutrophils, and it is felt that through these receptor-mediated pathways such as p38, sPLA₂ is able to activate cytosolic PLA₂ to release eicosanoids and other lipid mediators. We have not, however, looked for the production of these eicosanoids or other lipid mediators that might have been produced by the cytosolic PLA₂, but that would be an interesting study to do later on.

ARCHIVES OF INTERNAL MEDICINE

Treatment of Helicobacter pylori Infection
Julio A. Salcedo, MD; Firas Al-Kawas, MD

Since acceptance of the association between Helicobacter pylori and peptic ulcer disease, eradication of H pylori has become the standard of care in the treatment of peptic ulcer disease. Unfortunately, eradication therapy is no easy task, especially when one is faced with a myriad of drug combinations with varying degrees of efficacy and tolerability. The following is a review of the literature regarding the drugs and drug combinations used to eradicate H pylori and their effectiveness both as single agents and in combination. (1998;158;842-851)

Reprints: Firas Al-Kawas, MD, Department of Gastroenterology, Georgetown University Medical Center, 3800 Reservoir Rd NW, Room 2122, 2nd Floor/Main Building, Washington, DC 20007.