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Preliminary Studies on Phospholipase A_2-Induced Mouse Paw Edema as a Model to Evaluate Antiinflammatory Agents

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Phospholipase A_2 (PLA_2) is a key component of the inflammatory process because of its role in the generation of eicosanoids and platelet-activating factor (PAF). Manipulation of PLA_2 activity offers a novel therapeutic approach for the development of antiinflammatory agents; however, there is a need for a suitable in vivo model. Injection of 1 μg of snake venom PLA_2 (*A. piscivorus piscivorus*, D-49) into the mouse hind footpad produced a significant three- to four-fold rise in paw edema within 10 min, compared to the saline control. Edema formation depended on enzyme concentration and appeared specific for PLA_2 since edema was negated by enzyme pretreatment with p-bromophenacyl bromide, a nonspecific PLA_2 inhibitor. Moreover, injection of a protein such as bovine serum albumin did not result in significant edema. Coinjection of phenidone (lipoxygenase inhibitor, 50 μg), indomethacin (cyclooxygenase inhibitor, 50 μg), cyproheptadine (antihistamine/antiserotonin, 50 μg), aristolochic acid (putative PLA_2 inhibitor, 100 μg), or kadsurenone (PAF antagonist, 50μg) with PLA_2 (1 μg/paw) resulted in partial reduction (44.5, 34.2, 54.7, 64, and 50% inhibition, respectively) of edema formation. Oral administration of cyproheptadine (10 mg/kg), indomethacin (10 mg/kg), BW 755c (100 mg/kg), or dexamethasone (1 mg/kg) 1–3 h before challenge also decreased PLA_2-induced edema (63.0, 30.1, 47.8, or 62.5% inhibition, respectively). The data suggest that mouse paw edema resulting from PLA_2 injection is a multicomponent event, influenced by both autacoids and lipid mediators of inflammation.

Key words: platelet-activating factor, prostaglandins, D-49 snake venom PLA_2, inflammation, leukotrienes

Phospholipase A_2 (PLA_2) is implicated in the generation of inflammatory events in several diseases [1,2]. The synthesis of inflammatory mediators such as prostanoids (PGs) and leukotrienes (LTs) depends on the PLA_2-catalyzed hydrolysis of arachidonic acid (AA) from the second carbon position of cellular phospholipids. Furthermore, lysophospholipids generated from the deacylation of membrane phospholipids have been associated with tissue damage [3], and the ether-linked analogue of lyso-

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phosphatidylcholine is a precursor of platelet activating factor (PAF), a potent inducer of vascular-dependent inflammatory events. Indeed, antiinflammatory steroids indirectly reduce PLA_2 activity through the action of an endogenous inhibitory protein named lipocortin [4], leading to a reduction in both cyclooxygenase (CO) and 5-lipoxygenase (LO) metabolites. Since the adverse reactions of corticosteroids have not been associated with PLA_2 inhibition, nonsteroidal PLA_2 inhibitors may retain antiinflammatory activity with a more acceptable safety profile. Toward an understanding of the pathophysiology of PLA_2, we examined and pharmacologically characterized the inflammatory response initiated by the injection of snake venom PLA_2 into the mouse paw. The data suggest that this model is suitable for evaluating PLA_2 inhibitors in vivo.

MATERIALS AND METHODS

Materials

Snake venom PLA_2(D-49 PLA_2) was generously provided by Dr. Paul Sigler (University of Chicago, Chicago, IL) in crystalline form (>99% pure). The monomeric PLA_2 (D-49 isozyme) was isolated from the venom of the diamondback cottonmouth snake, *A. piscivorus piscivorus* (A.p.p.). [^{3}H]-arachidonic acid labelled *E. coli* (12.8×10^6 cpm/1704 nmole *E. coli phospholipid* phosphorus/6.4×10^{10} *E. coli*) was obtained from the laboratory of Dr. Richard Franson (Virginia Medical College, Richmond, VA). Tween-80 (polyoxyethylene sorbitan monooleate) was obtained from Fisher Scientific Co. (Fairlawn, NJ). Drugs used were cyproheptadine, indomethacin, dexamethasone, and kadsurenone (Merck Sharp and Dohme, Rahway, NJ), aristolochic acid, p-bromophenacyl bromide (BPB), mepacrine, and bovine serum albumin (BSA) (Sigma Chemical Co, MO), and phenidone and BW755C (Burroughs Wellcome, Research Triangle Park, NC).

Phospholipase A_2 Assay

PLA_2 activity was measured by the hydrolysis of [^{3}H]-AA labelled autoclaved *E. coli*. Unless otherwise stated, incubation mixtures (100 μl) routinely contained the following: 100 mM tris buffer, pH 7.5, 5 mM $CaCl_2$ and 2.5×10^8 *E. coli* substrate (equivalent to 5 nmol of phospholipid). Incubations were carried out at 37°C in a shaking water bath for 10 min since preliminary experiments showed that the rate of hydrolysis was linear for 10 min. After terminating the reaction with 2 ml of tetrahydrofuran (THF), hydrolyzed [^{3}H]-AA was separated from unhydrolyzed phospholipid by solid phase extraction with Bond Elute NH_2 columns (Analytichem International, Harbor City, CA; 100 mg/ml). Columns were conditioned with 0.5 ml THF followed by 0.5 ml THF:H_2O (20:1, v/v). Samples were loaded onto columns, and free [^{3}H]-AA was eluted with 1 ml THF:glacial acetic acid (49:1, v/v) with greater than 95–99% recovery. Radioactivity of the eluant was quantitated by liquid scintillation counting.

After correcting for nonenzymatic hydrolysis, PLA_2 activity was expressed as percentage acylhydrolysis of [^{3}H]-AA *E. coli* added.

Phospholipase A_2-Induced Mouse Paw Edema

Groups of six male CD-1 mice (Charles River, Kingston, NY), weighing 28–32 g were used in these experiments. PLA_2 at the concentrations indicated was injected

subplantar into the right paw in 50 μl of pyrogen-free saline. A.p.p. D-49 snake venom was used as the source of snake venom over other commercially available enzymes because it could be obtained in relatively pure form, free of possible proinflammatory contaminants, and is available in sufficient quantity. Right paw volumes (μl) were measured before PLA_2 injection (t = 0 reading) with a mercury plethysmograph. Treated paws were then remeasured at the times indicated (0–180 min) and paw edema was calculated by subtracting the zero time reading from the readings taken after injection. Percentage change from control was calculated to determine drug activity.

Drug Studies

Drugs were solubilized in either dimethylsulfoxide (DMSO) (not greater than 0.2% final concentration) or sterile saline alone when coinjected into the right paw with the PLA_2 enzyme in a total volume of 50 μl. For oral administration drugs were suspended in 0.5% Tween-80 in H_2O and administered in a volume of 0.5 ml 1 h before PLA_2 injection.

Statistics

Data were analyzed with one-way analysis of variance and Student Neuman Keuls multiple comparisons test($\alpha = 0.05$). Data were expressed as mean ± 1 standard deviation (S.D.) of the mean. Comparisons of drug treatment to the control were performed with the Dunnett's test ($\alpha = 0.05$).

RESULTS

Effect of Injection of D-49 Phospholipase A_2 on Mouse Paw Edema

Injection of PLA_2 in the mouse hind footpad resulted in a significant dose-dependent rise in paw edema, compared with the saline control. An injection of 3.3 μg/paw produced a maximal response (three- to four-fold above the control). Injection of higher doses (5 or 10 μg/paw) did not cause a greater increase in paw edema. Edema formation was rapid, peaking at 5 min, was slightly reduced between 10 and 20 min; and then continued to decline slowly over 60 min (Fig. 1). At 60 min the paw edema was approximately 30% the edema measured at 5 min. The dose response of edema formation (0.3 μg–3.3 μg/paw) directly correlated with the increase in hydrolysis of [^{3}H]-AA-*E. coli* associated with increasing the concentration of the D-49 PLA_2 preparations (Table I).

Injection of BSA at a concentration of 10–100 μg/paw (18 ± 6 μl; 10 min, n = 12) did not produce edema above that observed in the saline control (44 ± 4 μl; 10 min, n = 6). Three concentrations of D-49 PLA_2 (e.g., those producing a maximal response [1.0 μg], 60% of maximum [0.3 μg], and 30% of maximum [0.1 μg]) were exposed to p-BPB (100 μM, 8 h in Ca^{++}-free medium), an alkylating agent that inactivates PLA_2. The same solutions were injected into the mouse hind footpad.

The edema formed by coinjection of PLA_2 and pBPB is shown in Figure 2 and is compared with edema formed by PLA_2 dissolved in saline or PLA_2 in the vehicle (0.2% DMSO in saline) control. Again, increasing concentrations of PLA_2 initiate edema formation from 20% to 400% greater than that observed with the saline injection alone. Neither pBPB itself nor saline containing vehicle (0.2% DMSO) altered PLA_2-induced edema formation when compared to saline vehicle control alone. The

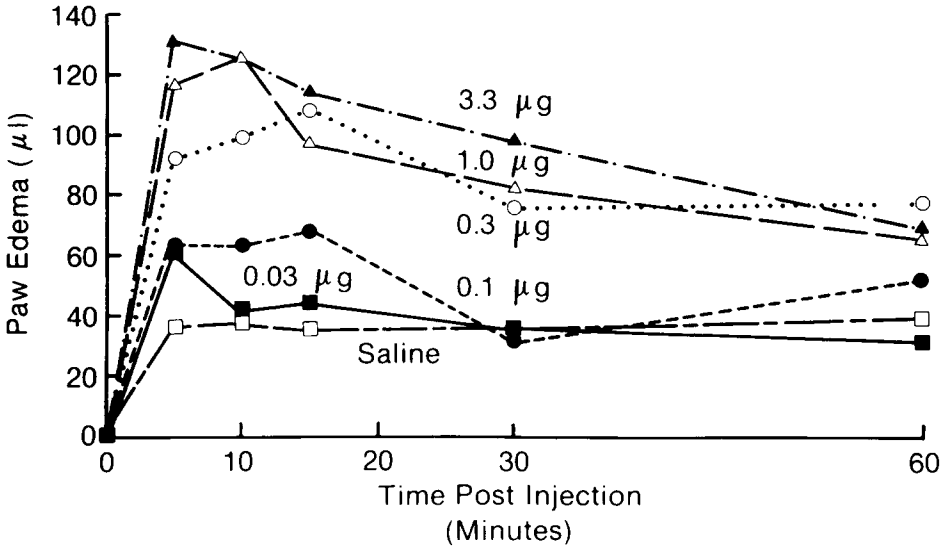

Fig. 1. Effect of different concentrations of D-49 snake venom PLA_2 on mouse paw edema; 3.3 μg to 0.03 μg of PLA_2 were injected into the mouse paw and edema measured over 1 h. Points represent mean edema from six mice.

presence of pBPB during injection of D-49 PLA_2 resulted in a total inhibition of edema at all levels of PLA_2 injected. When the PLA_2-pBPB solutions were tested for acylhydrolysis of [^{3}H]-AA *E. coli* containing 0.1, 0.3, or 1.0 μg PLA_2, activity was reduced by 96%, 85%, or 69%, respectively.

Effect of Pharmacological Agents

To characterize D-49 PLA_2-induced paw edema, various antiinflammatory drugs were prepared with the PLA_2 enzyme (0.3 μg/paw) and then coinjected as a single solution into the hind footpad. Paw edema was measured at 10 min and compared with edema resulting from injection of PLA_2 alone. Figure 3 shows that aristolochic acid, indomethacin, phenidone, cyproheptadine, and kadsurenone (50 μg/paw) significantly reduced edema by 64%, 35%, 45%, 49%, and 50%, respectively, ($\alpha = 0.05$) when coinjected individually with PLA_2. Mepacrine, a nonspecific PLA_2 inhibitor, did not affect PLA_2-induced paw edema at 100 μg/paw. When tested for [^{3}H]-AA *E. coli* acylhydrolytic capability, none of the drugs themselves inhibited PLA_2-induced acylhydrolytic activity except for phenidone ($P < .001$) (Table II).

The effect of antiinflammatory drugs administered orally 1 h before PLA_2 (0.3 μg/paw) challenge was also examined (Fig. 4). Indomethacin (10 mg/kg), cyproheptadine (10 mg/kg), or BW 755c, a dual 5-LO/CO inhibitor (100 mg/kg), reduced paw edema by 31%, 63%, or 47%, respectively (Fig. 4). Oral administration of aristolochic acid (100 mg/kg) and phenidone (100 mg/kg) failed to significantly alter paw edema. Dexamethasone (1 mg/kg), a steroid that is reported to inhibit PLA_2 activity through the induction of a PLA_2-inhibitory protein, lipocortin, was dosed p.o. 3 h before PLA_2 challenge. This resulted in a 63% reduction in paw edema relative to the Tween-80 control which was also administered 3 h before challenge (Fig. 4).

TABLE I. Comparison of [^{3}H]-AA-*E. coli* Acylhydrolytic Activity With Mouse Paw Edema Inducing Capability of D-49 PLA_2

D-49 PLA_2/50 μl	Acylhydrolysis[a] (% of [^{3}H]-AA *E. coli*)	Paw edema[b] (μl)
Saline	0.05 ± 0.0	37.0 ± 25
0.03 μg	2.8 ± 0.1	41.8 ± 12
0.1 μg	25.3 ± 0.4	63.0 ± 11
0.3 μg	51.2 ± 1.6	99.5 ± 11
1.0 μg	64.3 ± 2.2	126.8 ± 14
3.3 μg	76.4 ± 1.7	125.0 ± 21

[a]Reaction time was 10 min at 37°C (n = 2) (see Materials and Methods).
[b]Paw edema measured 10 min post PLA_2 injection (n = 6 mice).

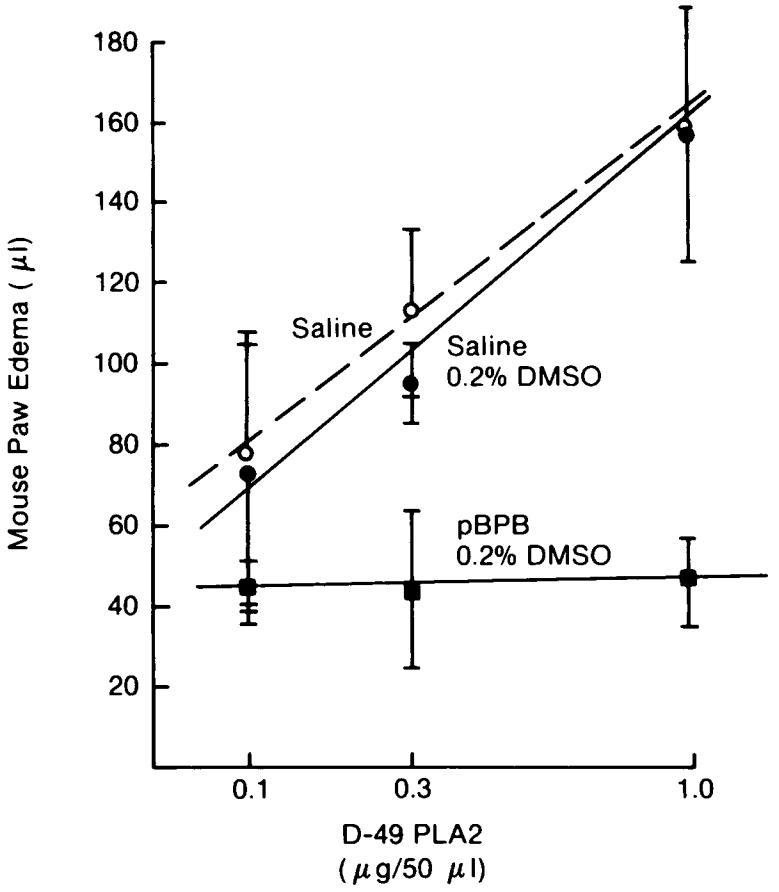

Fig. 2. Effect of pBPB on PLA_2-induced mouse paw edema. D-49 PLA_2 was injected in saline or saline containing 0.2% DMSO or was coinjected with 100 μM pBPB. Data represent mean ± SD (n = 6) of paw edema volume measured at 10 min. Edema values obtained in 10 min for the controls include saline (38 ± 13; n = 6) 0.2% DMSO in saline (61 ± 5; n = 6) and pBPB in 0.2% DMSO (48 ± 10; n = 6).

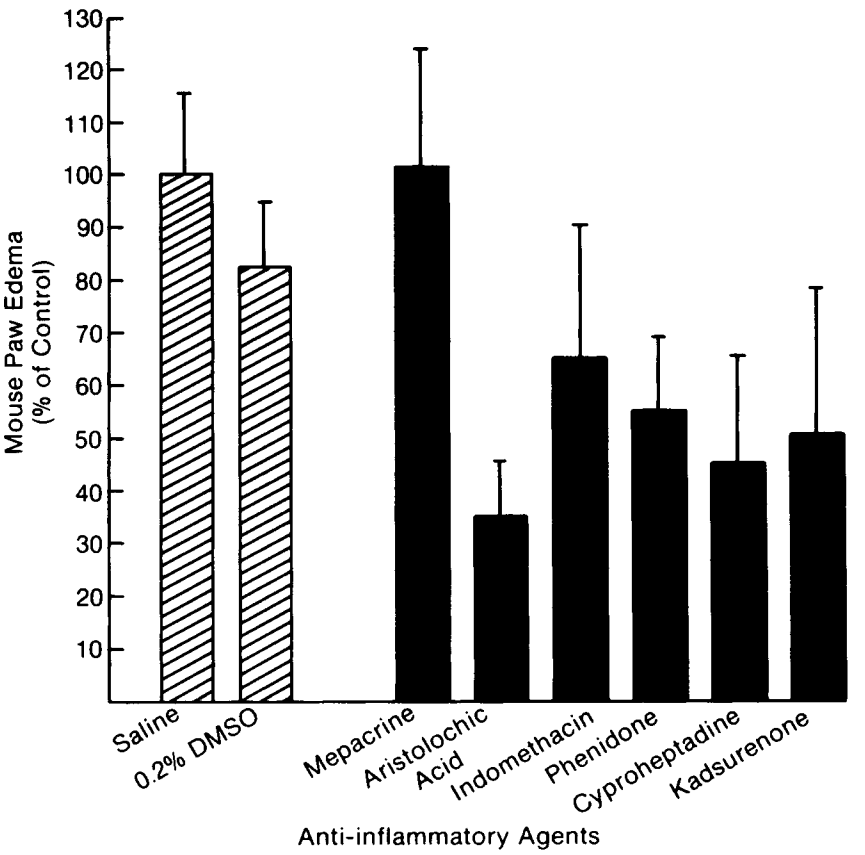

Fig. 3. Effect of drug coinjection on D-49 PLA_2-induced mouse paw edema. The following drugs were injected simultaneously with 0.3 μg D-49 PLA_2: mepacrine (100 μg), aristolochic acid (50 μg), indomethacin (50 μg), phenidone (50 μg), cyproheptadine (50 μg), and kadsurenone (50 μg). Paw edema was measured at 10 min. Bars represent mean ± SD, n = 6.

DISCUSSION

The results of this investigation demonstrated that D-49 snake venom PLA_2 induces an inflammatory edema that is both dose- and time-dependent. Interestingly, the shape of the edema time curve; represented by a rapid rise within 10–20 min and then a slow decline back to baseline between 60 and 180 min (data not shown), is similar to edema induced by injection of exogenous AA or PAF into the mouse paw [5]. This similarity suggests that these two components play a contributive role in PLA_2-induced edema. In contrast, injection of carrageenan produces an edema that slowly increases, reaching its maximum by 3 h, indicating that the inflammatory edema is caused by other initiating pathways [5]. This response to D-49 PLA_2 appears to be a specific result of enzyme acylhydrolytic activity since injection of equal concentrations of protein such as BSA did not induce edema. Furthermore, addition of the alkylating agent pBPB resulted in the loss of the edema-producing capability, which directly correlated to the inhibition of enzyme activity.

Mepacrine, a putative PLA_2 inhibitor, did not alter PLA_2-induced paw edema

TABLE II. Effect of Antiinflammatory Drugs on D-49 PLA_2 Acylhydrolysis of ^{3}H-AA *E. coli* in 10 Min†

Drug	Concentration (μg/50 μl)	Acylhydrolysis (% [^{3}H]-AA *E. coli*)	
		No enzyme	0.3 μg PLA_2/50 μl
Saline	—	0.3 ± 0.1	55.0 ± 2.8
0.2% DMSO	—	0.3 ± 0.1	50.3 ± 1.8
Mepacrine	100	0.1 ± 0.02	56.6 ± 0.9
Aristolochic acid	50	0.1 ± 0.1	52.7 ± 0.1
Indomethacin	50	0.0 ± 0.00	53.0 ± 0.6
Phenidone	50	0.2 ± 0.10	50.7 ± 0.6
Cyproheptadine	50	0.2 ± 0.10	58.5 ± 0.4
Kadsurenone	50	0.1 ± 0.10	65.0 ± 0.2
pBPB	50	0.1 ± 0.1	8.3 ± 0.5*

†Data are expressed as mean ± SD of two determinations. (*) Signifies significantly different from the 0.2% DMSO vehicle control as determined by ANOVA and Dunnett's test ($\alpha = 0.05$).

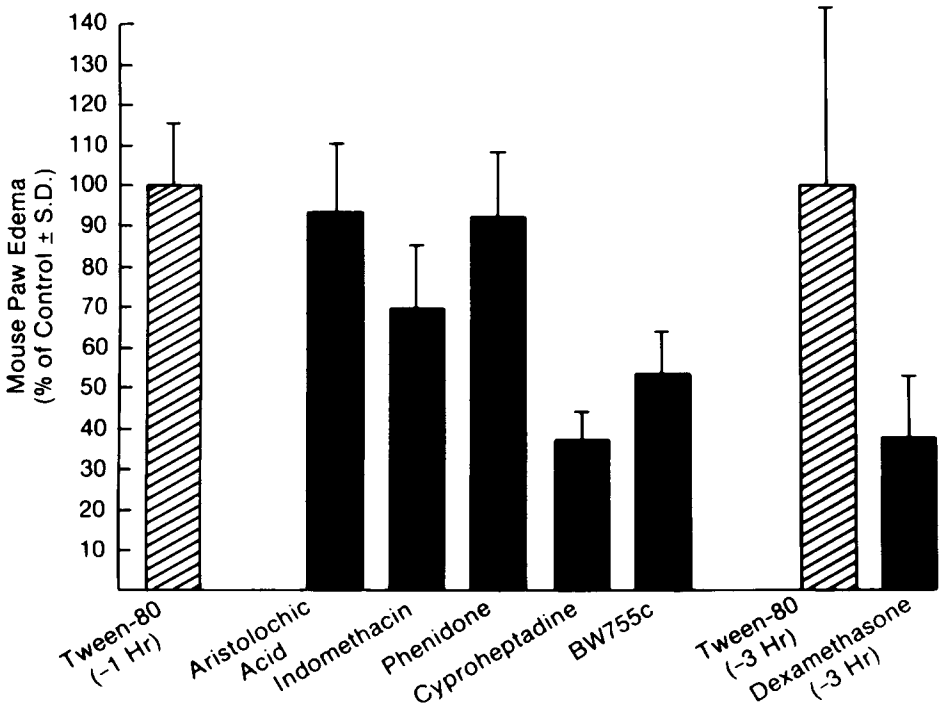

Fig. 4. Oral activity of antiinflammatory drugs in D-49 PLA_2-induced mouse paw edema. Drugs were solubilized or finely suspended in Tween-80 and administered 1 h before challenge with 0.3 μg D-49 PLA_2. Drug doses include aristolochic acid (100 mg/kg), indomethacin (10 mg/kg), phenidone (100 mg/kg), cyproheptadine (10 mg/kg), and BW 755c (100 mg/kg). Dexamethasone (1 mg/kg) was administered 3 h before PLA_2 challenge. Bars represent mean ± SD, n = 6.

(100 μg/paw), and in our hands, mepacrine did not affect D-49 [^{3}H]-AA *E. coli* hydrolytic capability up to 100 μM. Aristolochic acid, a reported snake venom PLA_2 inhibitor [6] had no direct effect on enzyme hydrolysis in vitro but reduced paw edema by 64% when coinjected with D-49 PLA_2. This finding suggests that the local antiinflammatory activity of aristolochic acid may not be occurring through PLA_2 inhibition. Aristolochic acid lost this activity when administered.

Dexamethasone, a potent corticosteroid, owes its antiinflammatory activity to the induction of an endogenous PLA_2-inhibitor protein, lipocortin [7]. Since generation of lipocortin requires protein synthesis, dexamethasone was administered orally (1 mg/kg) at 3 h before PLA_2 challenge. Dexamethasone reduced paw edema by 63%, suggesting that exogenous PLA_2 may be inhibited by lipocortin.

PLA_2 hydrolytic action also gives rise to the proinflammatory mediator, PAF [3]. Kadsurenone, a PAF antagonist that does not directly inhibit D-49 PLA_2 in vitro, reduced paw edema by 50%, suggesting that PAF may contribute to the PLA_2-induced paw edema. Moreover, our previous data [5] showed that PAF induced a similar inflammatory response in the mouse paw.

PLA_2 challenge is reported to initiate the release of PGs, hydroxyeicosatetraenoic acids (HETEs), and LTs [8]. Direct injection of these mediators in the skin is known to directly elicit an inflammatory response as well as augment inflammatory activities of other mediators [9]. Indomethacin, a CO inhibitor, reduced paw edema by 35% when coinjected with PLA_2. Similarly, a 50% reduction of paw edema was noted after oral administration of indomethacin (10 mg/kg). The known inhibition of PGs by indomethacin therefore implicates their involvement in the PLA_2-induced paw edema.

Phenidone and BW 755c, were effective in reducing PLA_2-induced paw inflammation. Phenidone is a more specific inhibitor of the LO pathway, and it reduced paw edema by 45% when coinjected with D-49 PLA_2. However, phenidone had no effect when administered orally at 100 mg/kg. BW 755c, a mixed LO/CO inhibitor, was active orally at 100 mg/kg, reducing paw edema by 47%. LTs are reported to augment vascular permeability, which may account for their implied role in mediating PLA_2-induced paw edema [10].

The involvement of histamine in PLA_2-induced edema is suggested by the ability of cyproheptadine, an antihistamine/antiserotonin compound, to reduce PLA_2-induced paw edema. This involvement is consistent with the observations that histamine release by basophils or mast cells is initiated by PLA_2 [11], and 5-LO products are thought to be obligatory for histamine release from human basophils [12].

In conclusion, the current data suggest that the synthesis of several inflammatory mediators is initiated by PLA_2 injection into the mouse paw. Further, no single pharmacological agent could abrograte the PLA_2 response totally. Studies to further resolve these complex mechanisms are ongoing. Nonetheless, we have shown that snake venom PLA_2 is proinflammatory and that it supports a role for PLA_2 in inflammation and tissue injury. With this model, a means by which novel inhibitors of PLA_2 could be identified and characterized for their in vivo antiinflammatory potential is available.

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