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Inhibition of phospholipase A_2 purified from human herniated disc

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Abstract—The effect on human herniated intervertebral disc phospholipase A_2 (HD-PLA_2) of a number of retinoids, antirheumatic drugs and reported PLA_2 inhibitors was evaluated using autoclaved [1-^{14}C]-oleate-labeled *Escherichia coli* membranes as the substrate. Dexamethasone, non-steroidal antiinflammatory drugs, aristolochic acid and retinol were inactive, whereas a marked inhibition was found for manoalide, retinal, nordihydroguaiaretic acid and *p*-bromophenacyl bromide after preincubation with the enzyme (IC_{50} values 0.25, 4, 5 and 5 μM, respectively). The results are parallel to those obtained with the PLA_2 purified from human synovial fluid.

The deacylation of phosphoglycerides at position 2 leading to fatty acids and lysophospholipids is catalysed by phospholipase A_2 (PLA_2*; phosphatidate-2-acylhydrolase, EC 3.1.1.4) [1]. This enzyme may have a crucial role in the process of inflammation through regulation of free arachidonate and eicosanoid production [2]. Moreover, the produced lysophospholipids are cytotoxic and C_{16}-C_{18} 1-alkyl lysophosphatidylcholines are precursors of platelet-activating factor, a potent biochemical mediator [3]. The implication of PLA_2 enzymes in inflammatory conditions has been demonstrated in several animal models and in humans [4]. PLA_2 activity is markedly elevated in the serum and synovial fluid of patients with rheumatoid arthritis [5] and it has been detected in disc samples removed from patients with lumbar disc diseases [6]. Thus, selective inhibition of PLA_2 is considered as an interesting target for the pharmacological control of inflammation [7].

There are no selective PLA_2 inhibitors currently used in the treatment of human arthritic diseases. Antiinflammatory steroids are not able to inhibit PLA_2 *in vitro*, as is the case for most of the non-steroidal antiinflammatory drugs (NSAIDs) [8]. On the other hand, retinoids are able to inhibit human synovial fluid PLA_2 (HSF-PLA_2) *in vitro* [9 10], as well as to block arachidonic acid release from rat peritoneal macrophages stimulated with calcium ionophore A23187 [11]. These properties could be responsible, at least in part, for the antiinflammatory activity shown by some retinoids in animal models of arthritis [12, 13]. The same correlation may be valid for some PLA_2 inhibitors of marine origin, such as manoalide and luffariellolide, which display antiinflammatory activity in the phorbol 12-myristate 3-acetate-induced mouse ear edema test [14].

Recently we have observed similarities between PLA_2 enzymes isolated from human synovial fluid and herniated discs as regards in their substrate preference [15]. In this paper we compare the inhibitory activity on purified HD-PLA_2 and HSF-PLA_2 of some antiinflammatory drugs, retinoids and other reported PLA_2 inhibitors.

Materials and Methods

Chemicals. Retinal, retinol, retinoic acid, *p*-bromophenacyl bromide (p-BPB), dexamethasone, ibuprofen, naproxen, nordihydroguaiaretic acid (NDGA) and piroxicam were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) Oleoyloxyethylphosphorylcholine (OPC) was from Biomol (Plymouth Meeting, PA, U.S.A.) and manoalide was from Calbiochem (San Diego, CA, U.S.A.). All other chemicals and reagents used in this study were analytical grade.

Enzyme sources and assay of enzyme activity. Human intervertebral disc material was obtained from patients who underwent laminectomy and discectomy and were supplied by Hospital Germans Trias i Pujol, Badalona (Barcelona). Human synovial fluid was obtained from arthritic patients at the Hospital Mutua de Terrassa (Barcelona). PLA_2 was purified from both sources as described previously [15]. During purification PLA_2 activity was determined by a spectrophotometric method [16] using the NEFA-C Test from Boehringer Ingelheim. Protein was determined by the Pierce Micro BCA Protein Assay method [17] using bovine serum albumin as standard. Assays for PLA_2 activity inhibition were performed using [1-^{14}C]oleate-labeled autoclaved *Escherichia coli* as substrate [18]. Inhibitory potencies of compounds listed in Table 1 were measured in two different conditions: (i) by adding the compound directly to the reaction mixture and (ii) after preincubation with the enzyme for 30 min at 25° before addition of the substrate. All compounds were dissolved in dimethyl sulfoxide (less than 5% final concentration of the solvent). The reaction mixture was incubated at 37° in a shaking water bath for 5 min and the hydrolysis was stopped by addition of 3 mL of $CHCl_3$:CH_3OH (1:2, v/v). Lipids were extracted by the method of Bligh and Dyer [19] and separated by TLC. Radioactivity was quantitated by liquid scintillation.

Results and Discussion

Antiinflammatory activity of corticosteroids is associated with a reduction in the biosynthesis of arachidonic acid metabolites. However, it has been described that this action does not involve a direct inhibition of proinflammatory HSF-PLA_2 [8]. This enzyme is also insensitive *in vitro* to NSAIDs acting through inhibition of cyclooxygenase or 5-lipoxygenase [8]. Accordingly, we found that dexamethasone and several NSAIDs (naproxen, ibuprofen, ketoprofen and piroxicam) are completely inactive as HSF-PLA_2 inhibitors, after preincubation for 30 min with the enzyme. These compounds also lack appreciable inhibitory activity upon HD-PLA_2 in concentrations up to 100 μM (Table 1). Thus, our results with two human pro-inflammatory PLA_2 from different sources confirm that classical antiinflammatory drugs are not inhibitors of these enzymes. Both HSF-PLA_2 and HD-PLA_2 activities were not affected by the dual cyclooxygenase/5-lipoxygenase inhibitor NDGA when directly added to the reaction mixture. However, after preincubation of these enzymes for 30 min with NDGA, a strong inhibition of hydrolytic activity was observed (Table 1). The IC_{50} values measured against HSF and HD enzymes were comparable, being 10 and 5 μM, respectively. The effect of this phenolic compound on HSF-PLA_2 activity had been previously

* Abbreviations: p-BPB, *p*-bromophenacyl bromide; NDGA, nordihydroguaiaretic acid; HD, herniated disc; HSF, human synovial fluid; PLA_2, phospholipase A_2; OPC, oleoyloxyethylphosphorylcholine; NSAID, non-steroidal antiinflammatory drug.

Table 1. IC_{50} values (μM) for the inhibition of HD-PLA_2 and HSF-PLA_2 activities using [1-^{14}C]oleate-labeled *E. coli* (10 nmol of phospholipid, equivalent to 10,000 cpm)

Compound	HD-PLA_2		HSF-PLA_2	
	No preincubation	Preincubation	No preincubation	Preincubation
Cyclooxygenase inhibitors				
Naproxen	—	>100	—	>100
Ibuprofen	—	>100	—	>100
Ketoprofen	—	>100	—	>100
Piroxicam	—	>100	—	>100
Dual cyclooxygenase/5-lipoxygenase inhibitors				
NDGA	>100	5	>100	10
Retinoids				
Retinol	>100	>100	>100	45
Retinoic acid	20	26	25	20
Retinal	30	4	25	3
Others				
Dexamethasone	—	>100	—	>100
Aristolochic acid	>100	>100	>100	>100
OPC	28	8	25	40
Manoalide	>2	0.25	>2	0.23
p-BPB	>100	5	>100	>100 (40)*

Reaction was performed in 0.5 mL of a mixture containing 50 mM Hepes buffer (pH 7.5) 5 mM $CaCl_2$, 150 mM NaCl; all compounds were added in dimethyl sulfoxide solution (<5% of the final volume).

Results are expressed as IC_{50} values (μM) and are the mean of at least two determinations. Preincubation of enzyme–compound was carried out at room temperature for 30 min and hydrolysis was started by addition of labeled *E. coli*.

* After preincubation for 60 min in the absence of Ca^{2+} the reaction was started by simultaneous addition of 5 nmol of substrate and 5 mM $CaCl_2$.

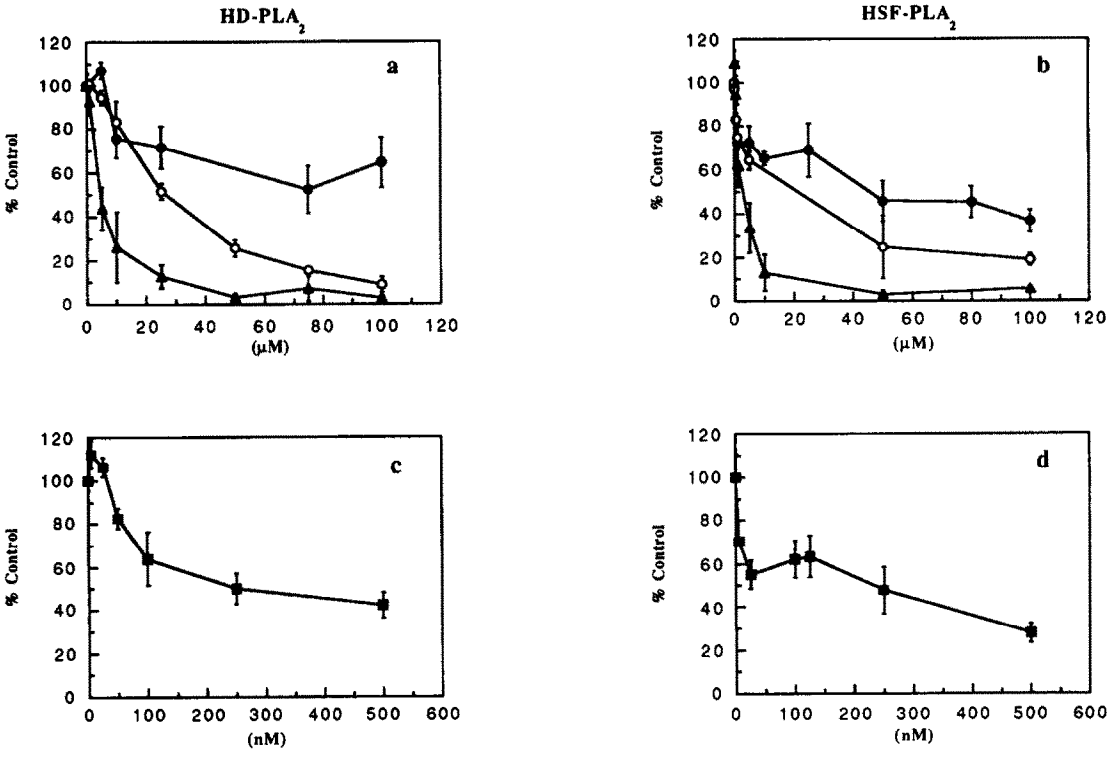

Fig. 1. Inhibition of HD-PLA_2 (plots a and c) and HSF-PLA_2 (plots b and d) by retinol (●), retinal (▲), retinoic acid (○) and manoalide (■). All compounds were preincubated with the enzyme for 30 min at 25° before addition of the substrate. Enzyme activity is expressed as per cent of the hydrolysis measured in the controls; each point represents the mean ± SD of three experiments.

reported [8] and may be related to its antioxidant properties. Indeed, other phenolic derivatives such as quercetin or rutin inhibit HSF-PLA_2 *in vitro*, probably acting through interaction with the substrate [9].

Aristolochic acid has been described as a weak inhibitor of several PLA_2, including the human synovial enzyme [8, 20, 21]. However, in our experiments this compound showed no significant inhibitory activity against both HSF-PLA_2 and HD-PLA_2 ($IC_{50} > 100\ \mu M$, Table 1), even after preincubation with the enzymes. On the other hand, the reported substrate analog PLA_2 inhibitor OPC [22] was able to inhibit both PLA_2 with similar potencies (IC_{50} 25 and 28 μM, respectively). Preincubation with the enzymes for 30 min enhanced the inhibitory activity against HD-PLA_2 (IC_{50} 8 μM), although this effect was not observed with HSF-PLA_2.

It is known that natural retinoids and some synthetic analogs related structurally show antiinflammatory activity in several animal models, but their mechanism of action has not yet been defined [10]. The fact that retinoids are able to inhibit *in vitro* several PLA_2 enzymes, including the proinflammatory HSF-PLA_2, has been suggested as a possible mode of action for this class of compounds [9, 10]. In a first set of experiments, we have evaluated the inhibition of HSF-PLA_2 and HD-PLA_2 enzymes immediately after the incorporation of retinol, retinoic acid and retinal to the reaction mixtures (Table 1). Neither HSF-PLA_2 nor HD-PLA_2 were sensitive to the presence of retinol ($IC_{50} > 100\ \mu M$), whereas both enzymes were inhibited in a concentration-dependent fashion by retinoic acid and retinal (IC_{50} values between 20 and 30 μM). Our results are in good agreement with those described by Fawzy *et al.* [9] and by Hope *et al.* [10] for HSF-PLA_2. Preincubation with the enzyme did not modify significantly the activity of retinol or retinoic acid, whereas the inhibitory potency of retinal was greatly enhanced, giving IC_{50} values of 3 and 4 μM for HSF- and HD-PLA_2, respectively (Table 1 and Fig. 1a and b). Inhibition of HSF-PLA_2 by retinal has been demonstrated to be independent of the substrate concentration, thus suggesting a direct enzyme–inhibitor interaction [9]. This fact and the observed increase in the PLA_2 inhibitory potency of retinal after preincubation may indicate that a covalent bond is developing between the aldehyde function of retinal and a nucleophilic amino acid residue on the enzyme.

Manoalide, a marine natural product from the sponge *Luffariella variabilis*, contains aldehyde groups and irreversibly inactivates PLA_2 enzymes by alkylation of lysine residues [23, 24]. This compound has been described as a very potent irreversible inhibitor of HSF-PLA_2 [25]. In our experiments, manoalide showed a strong inhibitory effect after 30 min of preincubation with HSF-PLA_2 or HD-PLA_2. Inhibition of both phospholipases was concentration-dependent and the curves are very similar (Fig. 1d and c, IC_{50} values 0.23 and 0.25 μM, respectively), suggesting a close relationship between the mechanism of inactivation of these enzymes by manoalide. When tested without preincubation, this compound showed a markedly reduced potency ($IC_{50} > 2\ \mu M$).

Finally, we tested another irreversible inhibitor of phospholipases, namely p-BPB. This compound covalently inactivates several PLA_2 enzymes, including the HSF-PLA_2 [8], by alkylation of the histidine 48 imidazole ring in the catalytic site [26]. This irreversible inactivation of HSF-PLA_2 is prevented by the presence of Ca^{2+} ions [27]. In agreement with these previously reported data, p-BPB inhibited HSF-PLA_2 only when preincubated with the enzyme for 60 min in the absence of Ca^{2+} (IC_{50} 40 μM, Table 1). However, in the case of HD-PLA_2, a strong inhibitory activity (IC_{50} 5 μM) appeared after 30 min of incubation in the usual conditions, i.e. in the presence of 5 mM Ca^{2+}. This result suggests that p-BPB is not competing with Ca^{2+} at the active site of the HD-PLA_2, in contrast with its behaviour against the synovial enzyme.

In conclusion, our experiments with the proinflammatory phospholipase purified from disc herniations point out that: (a) classical NSAIDs, dexamethasone and aristolochic acid are unable to inhibit this enzyme *in vitro*; (b) among the retinoids tested, retinal was the most potent inhibitor when preincubated with the enzyme; (c) this requirement for preincubation was observed also with other compounds, such as NDGA, manoalide, OPC and p-BPB; (d) the inhibitory potencies obtained with the tested compounds parallel those found against the HSF-PLA_2; (e) the two enzymes differed in the competition between Ca^{2+} and p-BPB at the active site.

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