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INHIBITION OF ENZYMATIC ACTIVITY OF PHOSPHOLIPASES A_2 BY MINOCYCLINE AND DOXYCYCLINE

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Abstract—Extracellular phospholipases A_2 play an important role in articular and extra-articular inflammatory processes. Secretory non-pancreatic phospholipase A_2 (PLA_2) has been implicated in the pathogenesis of articular inflammation in rheumatoid arthritis, whereas pancreatic PLA_2 contributes to the tissue damage associated with acute pancreatitis. Since in experimental models lipophilic tetracyclines such as minocycline and doxycycline are antiinflammatory, we examined their effects on PLA_2 activity using two assay systems *in vitro*. We found that minocycline and to a lesser degree doxycycline were markedly inhibitory to both pancreatic and non-pancreatic PLA_2. Using [^{14}C]oleic acid labeled *Escherichia coli* membrane phospholipids as substrate, the IC_{50} values for minocycline and doxycycline were 3.6×10^{-5} M (18 μg/mL) and 0.98×10^{-4} M (47 μg/mL), respectively. In a scooting mode assay using the synthetic phospholipid 1-palmitoyl-2-(10-pyrenedecanoyl)-3-L-phosphatidylmethanol as substrate, IC_{50} values for minocycline were 5 μM (2.47 μg/mL) for non-pancreatic PLA_2 and 8 μM (3.95 μg/mL) for pancreatic PLA_2. Addition of excess calcium up to 50 mM did not reverse the inhibitory activity of tetracyclines. We conclude that lipophilic tetracyclines inhibit PLA_2, probably by interaction with the substrate, and may be a useful adjunct in the therapy of inflammatory conditions in which PLA_2 is implicated pathogenetically.

Several semi-synthetic tetracycline antibiotics, such as minocycline and doxycycline, have been found to be powerful inhibitors of the neutral matrix metalloproteinases collagenase and gelatinase [1–9]. Inhibitory activity has been demonstrated both *in vitro* and *in vivo* in tissues as diverse as inflamed gingiva, rheumatoid synovium, cornea, osteoarthritic cartilage, and cancer cells (see Ref. 10 for review). Minocycline has also been found to inhibit the synthesis of prostaglandin E_2 (PGE_2) and 6-keto $PGF_{1\alpha}$ by gingival fibroblasts [11]. It has also been reported that minocycline suppresses some clinical manifestations of adjuvant and/or collagen-induced arthritis [12–14]. However, extensive studies with minocycline and doxycycline in rats with adjuvant arthritis have failed to confirm a significant effect on joint score and paw swelling while at the same time demonstrating clearly the ability of these compounds (after *in vivo* administration) to inhibit collagenase and gelatinase [15].

Several years ago a new enzyme, low molecular weight, secretory phospholipase A_2 (PLA_2‖), was discovered [16, 17]. This PLA_2 was found to belong to the non-pancreatic group II phospholipases [18] on the basis of its novel amino acid sequence [19]. Originally, this PLA_2 was detected in rheumatoid synovial fluid (SF) [16, 17]. Serum PLA_2 activity correlates with disease activity in adult rheumatoid [20] and juvenile chronic [21] arthritis. Intra-articular injections of a native purified PLA_2 [22] or of recombinant human rh-PLA_2 [23] induce experimental synovitis closely resembling the proliferative rheumatoid process.

Since PLA_2 is proinflammatory, and present in the synovial milieu, it was of interest to investigate whether tetracycline derivatives modulate the activity of this enzyme. Our studies have shown that minocycline and, to a lesser degree, doxycycline substantially inhibit the PLA_2–substrate interaction.

METHODS

Minocycline and doxycycline were obtained from the Sigma Chemical Co., St. Louis, MO. Recombinant human group II PLA_2 (rh-PLA_2), Lot No. 2789, was a gift of Dr. J. Browning, Biogen, Cambridge, MA. Human group II extracellular PLA_2 (SF-PLA_2) was purified from rheumatoid synovial fluid as described [24]. Cell-free human synovial fluids were obtained from rheumatoid, osteoarthritic, and psoriatic inflamed joints. *Naja naja* PLA_2 was purchased from Sigma, and repurified as described [25]. *Crotalus adamanteus* PLA_2 (*Crot. adamanteus*), *Crotalus atrox* PLA_2 (*Crot. atrox*) and porcine pancreatic F[illegible] Sigma. Purified hum[illegible] of Dr. B. Sternby,

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‖ Abbreviations: PLA_2, phospholipase A_2; SF, synovial

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‖ Abbreviations: PLA_2, phospholipase A_2; SF, synovial fluid; rh, recombinant human; and TET, tetracyclines.

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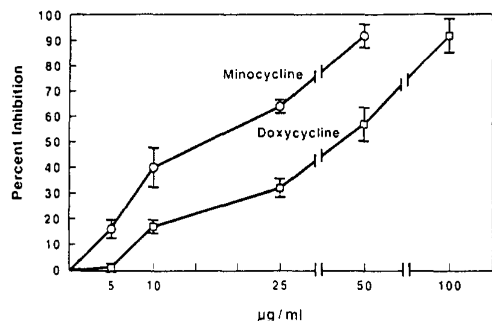

Fig. 1. Inhibition of PLA_2 purified from rheumatoid synovial fluid (SF-PLA_2) by minocycline and doxycycline. The tetracyclines were preincubated for 30 min with PLA_2. Values are means ± SD of triplicate experiments. Control value: 134 ± 7 U/mL.

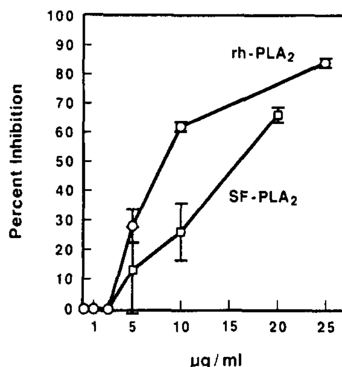

Fig. 2. Inhibition of SF-PLA_2 and of recombinant human (rh) PLA_2 by minocycline. Values are means ± SD of triplicate experiments. Respective control values were 137 ± 18 and 97 ± 2 U/mL.

osteoarthritic cartilage were prepared as described [26].

PLA_2 enzymatic activity. PLA_2 activity was assayed using [^{14}C]oleic acid-labeled *Escherichia coli* membrane phospholipid as substrate [24]. Reaction mixtures contained 10 mg bovine serum albumin, 2 mM $CaCl_2$, 8×10^8 radiolabeled *E. coli* and 0.1 M Tris–HCl buffer, pH 7.5, in a volume of 1.5 mL. Reaction mixtures were incubated at 37° for 30 min and the reaction was terminated by filtration through a 0.45 µm Millipore filter. Assays were performed in duplicate in substrate excess. Enzyme activities were corrected for non-enzymatic hydrolysis [24].

PLA_2 scooting mode assay. A scooting mode assay [27, 28] has been developed for the PLA_2 from synovial fluid. The activities of the rh SF-PLA_2 and purified PLA_2 from porcine pancreas were measured using the synthetic phospholipid 1-palmitoyl-2-(10-pyrenedecanoyl)-3-L-phosphatidylmethanol (10-Py-PM). Vesicles containing different mole fractions of minocycline were prepared by mixing chloroform solutions of the phospholipid and the inhibitor, followed by removal of the solvent under a stream of nitrogen. The phospholipid–inhibitor mixtures were then resuspended in 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) (pH 9.0) and sonicated in a bath sonicator. Reactions contained 10 µM 10-Py-PM, 2.5 mM $CaCl_2$, 100 mM KCl, and 50 mM Tris–HCl (pH 9.0). Reactions were incubated at 37° for 4 min and quenched, and the products were analyzed by HPLC (Street *et al.*, unpublished observations).

RESULTS

Using *E. coli* membrane phospholipids, minocycline in concentrations of 1×10^{-5} M to 1×10^{-4} M (5–50 µg/mL) was found to inhibit substrate hydrolysis by SF-PLA_2 in a concentration-dependent manner. The IC_{50} was 3.6×10^{-5} M (18 µg/mL). Doxycycline tested in concentrations from 0.1×10^{-4} M to 2.0×10^{-4} M (5–100 µg/mL) was also inhibitory with an IC_{50} of 0.98×10^{-4} M (47 µg/mL) (Fig. 1). Comparison of SF-PLA_2 and recombinant human PLA_2 showed that minocycline was more inhibitory to rh-PLA_2 (Fig. 2).

Table 1. Inhibitors of group I and group II phospholipases A_2

Inhibitor	IC_{50} (µM) rh SF-PLA_2*	IC_{50} (µM) Pancreatic PLA_2†
Gelb inhibitor	65	2
Dennis inhibitor (PE)‡	0.048	0.005
Dennis inhibitor (PC)§	0.69	0.14
Minocycline	5	8

* Recombinant human (synovial type) PLA_2.
† Purified PLA_2 from porcine pancreas.
‡ PE = ethanolamine head group.
§ PC = choline head group.

Minocycline also inhibited the rh-PLA_2 and the porcine pancreatic PLA_2 when 10-Py-PM was used as the substrate in the assay. The IC_{50} values obtained for the rh-PLA_2 and the porcine pancreatic PLA_2 were 5 µM (2.47 µg/mL) and 8 µM (3.95 µg/mL), respectively. For comparison a number of substrate analogue-inhibitors were also tested for their ability to inhibit PLA_2 in the 10-Py-PM assay (Table 1). The phosphonate substrate analogue, described by Jain *et al.* [29] and called Gelb inhibitor, and the amidophospholipids, described by Yu *et al.* [30] and called Dennis inhibitors, were found to be effective inhibitors of both the rh-PLA_2 and the porcine pancreatic PLA_2. The impact of calcium concentration on the inhibitory activity of minocycline and doxycycline was tested at concentrations from 7 to 50 mM. Addition of excess calcium did not reverse the inhibitory activity of the tetracyclines (data not shown).

Preincubation of 40 µM minocycline (20 µg/mL) and 104 µM doxycycline (50 µg/mL) with group I

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Table 2. Inhibition of group I and group II phospholipase A_2 activity by minocycline and doxycycline

Phospholipase A_2	Minocycline, 40 μM (20 μg/mL)	Doxycycline, 104 μM (50 μg/mL)
	Activity (U/mL)	
Control	91 ± 2*	134 ± 1
rh-PLA_2†	14 ± 1 (85)‡	30 ± 3 (78)
Control	137 ± 18	68 ± 8
SF-PLA_2§	47 ± 4 (66)	36 ± 3 (53)
Control	106 ± 2	106 ± 2
Human pancreatic	5 ± 3 (95)	95 ± 9 (10)
Control	223 ± 4	223 ± 4
Porcine pancreatic	100 ± 3 (55)	194 ± 13 (13)

* Mean ± SD of 3 experiments in duplicate.
† Recombinant human (synovial type) PLA_2.
‡ Values in parentheses indicate mean percent of inhibition.
§ Native purified synovial fluid PLA_2.

and group II phospholipases A_2 (Table 2) showed that minocycline was consistently more inhibitory than doxycycline. Minocycline inhibited PLA_2 in rheumatoid, osteoarthritic and psoriatic synovial fluids (62–68% inhibition) and in the homogenate of superficial and deep human osteoarthritic articular cartilage (62–71% inhibition). The respective inhibition by doxycycline was 26–57 and 33–38%. *Naja naja* (group I) PLA_2 activity was strongly inhibited by minocycline (72%) but not by doxycycline (12%). Phospholipases A_2 from the venoms of *Crot. atrox* and *Crot. adamanteus* (Group II enzymes) were inhibited by minocycline (22 and 43%, respectively) but not by doxycycline. Preincubation of minocycline (40 μM, 20 μg/mL) and doxycycline (104 μM, 50 μg/mL) for 30 min with the substrate showed inhibition essentially identical to that obtained when the tetracyclines were preincubated with the enzymes (data not shown).

DISCUSSION

Minocycline and doxycycline are highly lipid soluble tetracyclines (TET) that easily penetrate body tissues and fluids including synovial fluid [31]. Both these TET, as well as others which have been modified so as to remove their antibiotic activity, were found to exert marked inhibitory activity on matrix metalloproteinases, such as collagenases, gelatinase and macrophage elastase (see Ref. 10 for review).

Synovial fluids and tissues from patients with rheumatoid arthritis treated with minocycline have markedly lower collagenase activity as compared to pretreatment values [5]. Doxycycline inhibits digestion of exogenous type XI collagen by homogenates of human articular cartilage [8].

These studies [1–10] prompted trials to attenuate experimental arthritis by TET. It was suggested that minocycline suppressed collagen II and adjuvant arthritis in the rat [12–14]. However, in another study, oral TET given as a single agent to rats had no substantial antiinflammatory activity as assessed by joint swelling and paw diameter but it inhibited collagenase activity. A combination of the non-steroidal agent flurbiprofen and TET potently inhibits inflammation and normalized radiographic joint damage [15]. Thus, simultaneous administration of cyclooxygenase inhibitors and lipid-soluble TET seems to have a more pronounced beneficial effect in experimental arthritis than when each agent is given separately.

In several small series of patients with rheumatoid or reactive arthritis, minocycline and doxycycline were shown to have some beneficial effect on arthritis [32–34]. Tetracycline hydrochloride given to patients with rheumatoid arthritis showed no significant benefit [35]. However, these were all open trials and were statistically inconclusive.

The impact of tetracyclines on the arachidonic acid cascade has not been studied extensively. Preliminary study has shown that tetracycline and minocycline inhibit the activity of partially purified PLA_2 from venom of *Crot. adamanteus* [36]. It was also reported that minocycline added to the cultures of gingival fibroblasts inhibits the synthesis of PGE_2 and 6-keto $PGF_{1\alpha}$ [11].

The above observation combined with the fact that TET are inhibitory to several metalloproteinases prompted us to investigate the impact of TET on a panel of structurally distinct phospholipases A_2. Since in several models, low molecular weight extracellular PLA_2 was found to have pro-inflammatory activity [22, 23, 37, 38], inhibition of this enzyme by TET would serve as an additional rationale to use TET in arthritis alone or in combination with antiinflammatory agents. Our study has shown that minocycline and doxycycline markedly inhibit proinflammatory synovial-type phospholipase A_2 in both *E. coli* assay and in the scooting mode assay. Minocycline was a more potent inhibitor than doxycycline. Both TET showed

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selectivity towards certain PLA_2s. Minocycline was markedly inhibitory to both group I and II PLA_2 of human origin. In the snake venom PLA_2s minocycline was slightly more inhibitory to group I PLA_2 (*Naja naja*) than to group II (*Crot. atrox* and *Crot. adamenteus*). Doxycycline substantially inhibited human group II PLA_2s, but had no inhibitory activity against pancreatic PLA_2 and snake venom PLA_2s. It is not clear why minocycline was found to be more inhibitory than doxycycline whereas the opposite is true in the studies of collagenase inhibition [5, 10]. Perhaps some differences in lipophilic properties may account for the above phenomenon. An increase of calcium concentration in the reaction mixtures did not reverse the inhibitory activity of TET, thereby suggesting that chelation of calcium by TET is not the mechanism of PLA_2 inhibition.

Many group I PLA_2s have a high affinity for vesicles containing anionic phospholipids, and hydrolyse these substrates without leaving the lipid-water interface [27]. Under these conditions (scooting mode hydrolysis), it is possible to differentiate between inhibitors which bind at the catalytic site of the enzyme and those compounds which exert their inhibitory effect by decreasing the affinity of the enzyme for the lipid–water interface [28].

Kinetic studies with PLA_2 are complicated by the fact that binding of the enzyme to the lipid–water interface must precede catalytic turnover. Compounds which affect the organization of the substrate interface, and consequently the amount of absorbed enzyme, can alter apparent turnover rates by up to several orders of magnitude. By monitoring the kinetics of interfacial catalysis in the scooting mode [29] it is possible to differentiate between compounds which inhibit PLA_2 by desorption of the enzyme from the substrate interface (nonspecific inhibitors) and compounds which inhibit PLA_2 directly by binding at the active site. In the scooting mode the PLA_2 has a very high affinity for the substrate interface and remains tightly bound to the substrate vesicle during and after hydrolysis of all of the available substrate [39]. The effect of nonspecific inhibitors is minimized and it is possible to detect desorption of the enzyme from the interface. Examples of compounds which inhibit the action of PLA_2 by affecting interface structure include lipocortin [40], alkanols [41], cationic amphiphiles [42] and aristolochic acid [43]. None of these compounds has any effect on PLA_2 activity in the scooting mode [29].

The group II rh SF-PLA_2 has a high affinity for the anionic interface of 10-Py-PM vesicles and remains tightly bound to the vesicle surface for many catalytic cycles (Street *et al.*, unpublished observations). Therefore, it was of interest to examine minocycline and a number of known substrate analogue inhibitors of PLA_2 in the 10-Py-PM assay system. The results presented in Table 1 show that the phosphonate inhibitor (Gelb inhibitor) and particularly the amidophospholipids described by Yu *et al.* [30] were potent inhibitors of both the rh SF-PLA_2 and the porcine pancreatic PLA_2. In the case of the amidophospholipid analogue possessing the ethanolamine head group (Dennis-PE), 50% inhibition of the rh SF-PLA_2 was observed in vesicles containing only a 0.048 μM concentration of the inhibitor.

Minocycline codispersed with 10-Py-PM was inhibitory to both the rh SF-PLA_2 and the porcine pancreatic PLA_2. However, inhibition of these enzymes was observed only when minocycline was present in the assay at concentrations approaching that of the substrate. Minocycline is one of the most lipophilic tetracyclines and will partition essentially completely into the lipid phase. Thus, at the concentration of minocycline required for 50% inhibition of the rh SF-PLA_2 (5 μM) the inhibitor constituted 30 mol% of the substrate vesicle. At this very high interfacial concentration, minocycline may cause structural changes to the substrate vesicle and it is likely that most of the inhibition is due to these effects and dilution of the substrate within the vesicle. However, as this study only involved the measurement of initial rates, the possibility that minocycline does bind at the active site cannot be excluded completely.

Irrespective of the mechanism of the inhibitory activity of TET, they may potentially be useful as adjuncts in the treatment of inflammatory arthritides. TET may also be used in acute pancreatitis. Pancreatic PLA_2 converts lecithin to lysolecithin which may cause marked inflammatory reaction [44]. In acute pancreatitis the release of large amounts of pancreatic PLA_2 into the pancreatic and adjacent tissues has been well documented [45]. Furthermore, the circulating levels of PLA_2 in the serum of patients with pancreatitis may increase up to 800-fold from the normal level [46]. Since TET markedly inhibit pancreatic PLA_2–substrate interaction, their use in conditions in which this enzyme is excessively activated and released from the pancreas may be clinically beneficial.

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