

INHIBITION OF ENZYMATIC ACTIVITY OF PHOSPHOLIPASES A₂ BY MINOCYCLINE AND DOXYCYCLINE

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Abstract—Extracellular phospholipases A₂ play an important role in articular and extra-articular inflammatory processes. Secretory non-pancreatic phospholipase A₂ (PLA₂) has been implicated in the pathogenesis of articular inflammation in rheumatoid arthritis, whereas pancreatic PLA₂ 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 PLA₂ 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 PLA₂. Using [¹⁴C]oleic acid labeled *Escherichia coli* membrane phospholipids as substrate, the IC₅₀ 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, IC₅₀ values for minocycline were 5 µM (2.47 µg/mL) for non-pancreatic PLA₂ and 8 µM (3.95 µg/mL) for pancreatic PLA₂. Addition of excess calcium up to 50 mM did not reverse the inhibitory activity of tetracyclines. We conclude that lipophilic tetracyclines inhibit PLA₂, probably by interaction with the substrate, and may be a useful adjunct in the therapy of inflammatory conditions in which PLA₂ 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 E₂ (PGE₂) and 6-keto PGF_{1α} 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 A₂ (PLA₂), was discovered [16, 17]. This PLA₂ was found to belong to the non-pancreatic group II phospholipases [18] on the basis of its novel amino acid sequence [19]. Originally, this PLA₂ was detected in rheumatoid

synovial fluid (SF) [16, 17]. Serum PLA₂ activity correlates with disease activity in adult rheumatoid [20] and juvenile chronic [21] arthritis. Intra-articular injections of a native purified PLA₂ [22] or of recombinant human rh-PLA₂ [23] induce experimental synovitis closely resembling the proliferative rheumatoid process.

Since PLA₂ 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 PLA₂-substrate interaction.

METHODS

Minocycline and doxycycline were obtained from the Sigma Chemical Co., St. Louis, MO. Recombinant human group II PLA₂ (rh-PLA₂), Lot No. 2789, was a gift of Dr. J. Browning, Biogen, Cambridge, MA. Human group II extracellular PLA₂ (SF-PLA₂) 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* PLA₂ was purchased from Sigma, and repurified as described [25]. *Crotalus adamanteus* PLA₂ (*Crot. adamanteus*), *Crotalus atrox* PLA₂ (*Crot. atrox*) and porcine pancreatic F

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‡ Abbreviations: PLA₂, phospholipase A₂; SF, synovial

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‡ Abbreviations: PLA₂, phospholipase A₂; SF, synovial fluid; rh, recombinant human; and TET, tetracyclines.

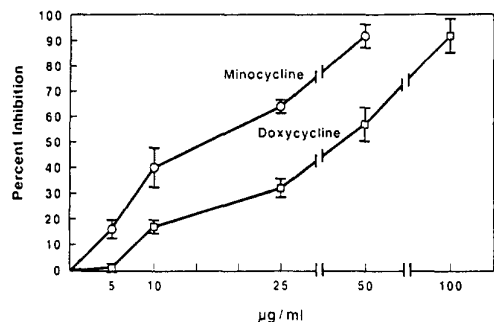


Fig. 1. Inhibition of PLA₂ purified from rheumatoid synovial fluid (SF-PLA₂) by minocycline and doxycycline. The tetracyclines were preincubated for 30 min with PLA₂. Values are means ± SD of triplicate experiments. Control value: 134 ± 7 U/mL.

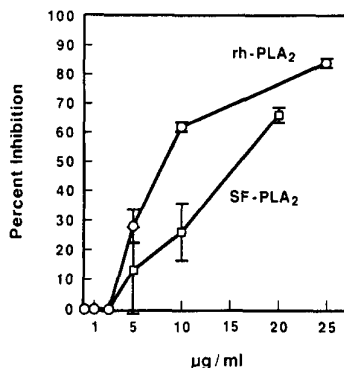


Fig. 2. Inhibition of SF-PLA₂ and of recombinant human (rh) PLA₂ by minocycline. Values are means ± SD of triplicate experiments. Respective control values were 137 ± 18 and 97 ± 2 U/mL.

osteoarthritic cartilage were prepared as described [26].

PLA₂ enzymatic activity. PLA₂ activity was assayed using [¹⁴C]oleic acid-labeled *Escherichia coli* membrane phospholipid as substrate [24]. Reaction mixtures contained 10 mg bovine serum albumin, 2 mM CaCl₂, 8 × 10⁸ 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].

PLA₂ scooting mode assay. A scooting mode assay [27, 28] has been developed for the PLA₂ from synovial fluid. The activities of the rh SF-PLA₂ and purified PLA₂ 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 CaCl₂, 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-PLA₂ in a concentration-dependent manner. The IC₅₀ 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 IC₅₀ of 0.98 × 10⁻⁴ M (47 µg/mL) (Fig. 1). Comparison of SF-PLA₂ and

Table 1. Inhibitors of group I and group II phospholipases A₂

| Inhibitor | IC ₅₀ (µM) | |
|------------------------|--------------------------|-------------------------------|
| | rh SF-PLA ₂ * | 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) PLA₂.

† Purified PLA₂ from porcine pancreas.

‡ PE = ethanolamine head group.

§ PC = choline head group.

recombinant human PLA₂ showed that minocycline was more inhibitory to rh-PLA₂ (Fig. 2).

Minocycline also inhibited the rh-PLA₂ and the porcine pancreatic PLA₂ when 10-Py-PM was used as the substrate in the assay. The IC₅₀ values obtained for the rh-PLA₂ and the porcine pancreatic PLA₂ 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 PLA₂ 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-PLA₂ and the porcine pancreatic PLA₂. 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

Table 2. Inhibition of group I and group II phospholipase A₂ 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) PLA₂.

‡ Values in parentheses indicate mean percent of inhibition.

§ Native purified synovial fluid PLA₂.

and group II phospholipases A₂ (Table 2) showed that minocycline was consistently more inhibitory than doxycycline. Minocycline inhibited PLA₂ 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) PLA₂ activity was strongly inhibited by minocycline (72%) but not by doxycycline (12%). Phospholipases A₂ 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 furbiprofen 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 PLA₂ from venom of *Crot. adamanteus* [36]. It was also reported that minocycline added to the cultures of gingival fibroblasts inhibits the synthesis of PGE₂ and 6-keto PGF_{1α} [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 A₂. Since in several models, low molecular weight extracellular PLA₂ 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 A₂ in both *E. coli* assay and in the scooting mode assay. Minocycline was a more potent inhibitor than doxycycline. Both TET showed

selectivity towards certain PLA₂s. Minocycline was markedly inhibitory to both group I and II PLA₂ of human origin. In the snake venom PLA₂s minocycline was slightly more inhibitory to group I PLA₂ (*Naja naja*) than to group II (*Crot. atrox* and *Crot. adamanteus*). Doxycycline substantially inhibited human group II PLA₂s, but had no inhibitory activity against pancreatic PLA₂ and snake venom PLA₂s. 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 PLA₂ inhibition.

Many group I PLA₂s 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 PLA₂ 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 adsorbed 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 PLA₂ by desorption of the enzyme from the substrate interface (nonspecific inhibitors) and compounds which inhibit PLA₂ directly by binding at the active site. In the scooting mode the PLA₂ 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 PLA₂ by affecting interface structure include lipocortin [40], alkanols [41], cationic amphiphiles [42] and aristolochic acid [43]. None of these compounds has any effect on PLA₂ activity in the scooting mode [29].

The group II rh SF-PLA₂ 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 PLA₂ 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-PLA₂ and the porcine pancreatic PLA₂. In the case of the amidophospholipid analogue possessing the ethanalamine head group (Dennis-PE), 50% inhibition of the rh SF-PLA₂ 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-PLA₂ and the porcine pancreatic PLA₂. 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-PLA₂ (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 PLA₂ converts lecithin to lysolecithin which may cause marked inflammatory reaction [44]. In acute pancreatitis the release of large amounts of pancreatic PLA₂ into the pancreatic and adjacent tissues has been well documented [45]. Furthermore, the circulating levels of PLA₂ in the serum of patients with pancreatitis may increase up to 800-fold from the normal level [46]. Since TET markedly inhibit pancreatic PLA₂-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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