TENIDAP (TD) was initially defined as a dual inhibitor of cyclooxygenase and lipoxygenase. This study was designed to assess its inhibitory activity against pro-inflammatory phospholipase A_2. This study shows that TD inhibits the synthesis of pro-inflammatory secretory non-pancreatic phospholipase A_2 (sPLA$_2$). Concentrations as low as 0.25μg/ml (0.725 μM) reduced the release of sPLA$_2$ by 40% from foetal rat calvarial osteoblasts stimulated with IL-1β and TNFα, whereas a concentration of 2.5 μg/ml (7.25 μM) reduced the release by over 80%. TD also markedly reduced the release of sPLA$_2$ from unstimulated cells. There was no direct inhibition of sPLA$_2$ enzymatic activity by TD *in vitro*. Northern blot analysis showed that TD did not affect the sPLA$_2$ mRNA levels; however, immunoblotting showed a dose-dependent reduction in sPLA$_2$ enzyme. These results, together with a marked reduction in sPLA$_2$ enzymatic activity, suggest that TD inhibits sPLA$_2$ synthesis at the post-transcriptional level. Therefore TD seems to inhibit the arachidonic acid cascade proximally to cyclooxygenase and lipoxygenase and its anti-inflammatory activity may be related at least in part to the inhibition of sPLA$_2$ synthesis.

Key words: Phospholipase A_2, Rat calvarial osteoblasts, Tenidap

Tenidap sodium inhibits secretory non-pancreatic phospholipase A₂ synthesis by foetal rat calvarial osteoblasts

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Introduction

Tenidap sodium ([Z]-5-chloro-2,3-dihydro-3[hydroxy-2-thienylmethylene]-2-oxo-1-H-indole-1-carboxyamide, sodium salt) (TD) is a new anti-inflammatory agent which was originally designated as a dual inhibitor of cyclooxygenase and lipoxygenase.[1,2] It soon became apparent that TD had other biological properties such as inhibition of IL-1 production in LPS-stimulated cells,[3,4] inhibition of the release and/or activity of collagenase and myeloperoxidase[5] and suppression of the expression of circulating acute phase reactants such as serum amyloid A in rheumatoid patients treated with TD[6] and of C-reactive protein in rats with adjuvant arthritis.[7] TD also inhibits bone resorption induced by cytokines IL-1 and TNF.[8] Since some of the above inhibitory activities resemble those of other agents which are known inhibitors of phospholipase A_2 (PLA$_2$), we undertook a study of the impact of TD on the synthesis of secretory non-pancreatic PLA$_2$ (sPLA$_2$) by foetal rat calvarial osteoblasts. The data suggest that TD is a potent inhibitor of sPLA$_2$ synthesis. These findings add a new aspect to the spectrum of biological activities of this compound and may lead to a better understanding of its observed anti-inflammatory effects.

Materials and Methods

Tenidap sodium, MW 343.75 was the kind gift of Pfizer Canada Inc. sPLA$_2$ activity was assessed as described in detail[9] using radiolabelled *Escherichia coli* membrane phospholipid substrate. Foetal rat calvarial cells (FRCO) were prepared as reported previously.[10] Confluent cultures of FRCO were incubated in the presence of TD in concentrations ranging from 0.25 μg/ml (0.725 μM) to 50 μg/ml (145 μM) for 24 and 48 h. Foetal calf serum was withdrawn from the medium when TD and/or IL-1 and TNF were added to the cultures. FRCO were co-stimulated with IL-1β (100 U/ml or 0.2 ng/ml) and TNFα (500 U/ml or 25 ng/ml) as described previously.[10] FRCO viability as tested by trypan blue exclusion was > 95%. The cells were counted at the end of each experiment. Each experiment was performed in triplicate and repeated at least twice. sPLA$_2$ activity was expressed as units per ml of the medium and as units per 1×10^6 cells/ml.[9]

RNA isolation and Northern blot analysis: RNA was isolated from cultured FRCO by the method of Chomczynski and Sacchi.[11] Briefly, 5×10^7 to 1×10^8 cells, were homogenized using lysing buffer containing 4 M guanidium thiocyanate. RNA was purified by

phenol:chloroform:iso-amyl alcohol extraction, followed by two iso-propanol precipitations. RNA was stored as an ethanol precipitate at −70°C until tested. Northern blot analysis of total RNA was performed on 1% agarose/formaldehyde gels and the RNA was blotted onto nitrocellulose.[12] The probe used for hybridization was the rat sPLA$_2$ cDNA.[13] The DNA fragment was labelled by random priming (Pharmacia) and prehybridization was done in a 50% formamide buffer at 42°C. The blot was washed in 1×55C 0.5% SDS at 50°C and exposed to Kodak XAR-film at −80°C with an intensifying screen.

Western blot analysis: SDS–polyacrylamide gel electrophoresis was done on 12% polyacrylamide gels using the method of Laemmli.[14] Proteins were then electrophoretically transferred to nitrocellulose (Hybond™–ECL, Amersham Canada, Ltd)[15] for immunoblot analysis. The primary antibody was a rat sPLA$_2$ monoclonal antibody (mAb 2E7 plus 2B9).[16] The blot was developed using enhanced chemiluminescence (ECL) as described by the manufacturer (ECL™ Western blotting protocols, Amersham International plc). The differences between sPLA$_2$ activity in control cultures supernatants and TD treated cells were assessed using Student's test.

Results

sPLA$_2$ activity in culture supernatants of unstimulated FRCO was 96 ± 21 U/10^6 cells whereas cells incubated in the presence of TD 5 μg/ml (14.5 μM) for 48 h released 19 ± 13 U/10^6 cells ($p <$ 0.01). Final concentration of cells was 0.72 ± 0.02 × 10^6/ml in controls and 0.67 ± 0.03 × 10^6/ml in TD treated cultures. FRCO stimulated with IL-1β and TNFα released 8944 ± 798 (SD) U/10^6 cells of sPLA$_2$ compared with 110 ± 16 U/10^6 cells in unstimulated controls. TD in concentrations of 0.25 μg/ml–0.5 μg/ml (0.725 μM to 1.450 μM) reduced the release of sPLA$_2$ by 40%, whereas a concentration of 1.25 μg/ml (3.625 μM) suppressed sPLA$_2$ activity by 68%. Concentrations of 2.5 μg/ml (7.25 μM) reduced sPLA$_2$ activity by over 80% (Fig. 1). TD in concentrations over 1.25 μg/ml was slightly inhibitory to cell proliferation, reducing the final number of cells from 0.92 ± 0.02 × 10^6/ml in controls to 0.78 ± 0.02 × 10^6/ml; concentrations over 2.5 μg/ml reduced final cell counts to 0.63 ± 0.03×10^6/ml.

Northern blot analysis of FRCO total RNA showed that TD at 1.25 μg/ml did not affect the level of sPLA$_2$ mRNA in unstimulated or IL-1/TNF stimulated cells (Fig. 2). In contrast, immunoblot analysis revealed reduction in the quantity of sPLA$_2$ in FRCO treated with 0.25 μg/ml (0.725 μM) TD (Fig. 3). At the TD concentration of 3.75 μg/ml (10.875 μM) the sPLA$_2$ protein became undetectable. These results suggest
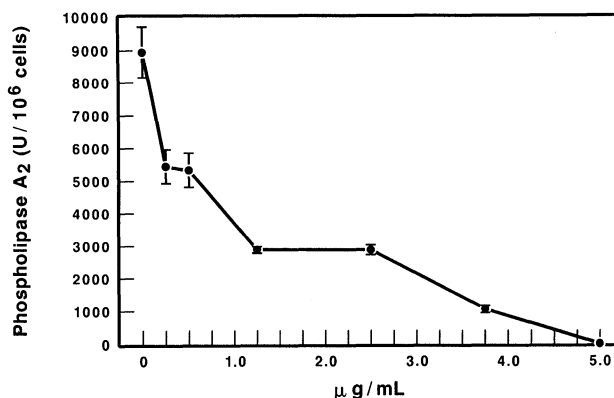
FIG. 1. Inhibition by Tenidap of the activity of phospholipase A$_2$ (sPLA$_2$) in the supernatant of FRCO stimulated by IL-1/TNF. One μg = 2.7 μM.

that TD may have a post-transcriptional effect on sPLA$_2$ synthesis by FRCO.

There was no direct inhibition by TD of the enzymatic activity of recombinant human sPLA$_2$ or of the extracellular PLA$_2$ of rat osteoblasts. Enzymatic activity of recombinant human sPLA$_2$ was assessed after 60 min incubation with TD in concentrations between 1 μg/ml and 50 μg/ml (2.9 μM–145 μM). sPLA$_2$ activity varied from 185 to 198 U/ml as compared with the control of 195 U/ml. The activity of
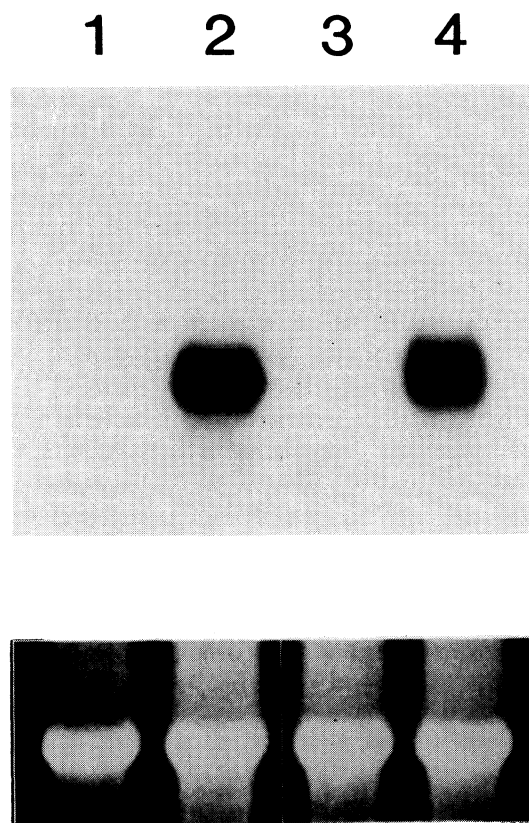

FIG. 2. Effect of Tenidap on expression of phospholipase A$_2$ (sPLA$_2$) mRNA levels in FRCO. Northern blot hybridization using the rat sPLA$_2$ cDNA probe was run by using isolated total cellular RNA obtained from FRCO (10 μg/lane). Lane 1, Control cells. Lane 2, IL-1/TNF treated cells. Lane 3, Tenidap (1.25 μg/ml) treated cells. Lane 4, IL-1/TNF/Tenidap treated cells. The lower panel shows ethidium bromide stain of 18S RNA to show equal loading of RNA per lane of gel.
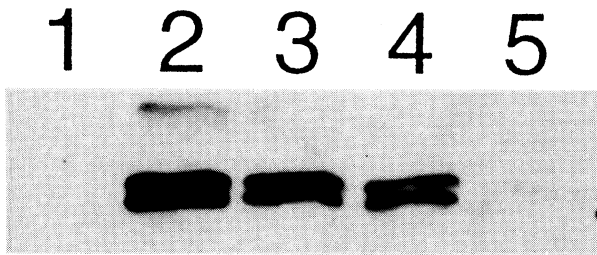
FIG. 3. Immunoblotting assay. Supernatants of FRCO cultures were used for Western blotting and phospholipase A$_2$ staining. Lane 1, Control. Lane 2, FRCO stimulated with IL-1β and TNFα. Lane 3, FRCO stimulated with IL-1β and TNFα, coincubated with Tenidap 0.25 μg/ml (1 g/ml = 2.7 μM). Lane 4, FRCO stimulated with IL-1β and TNFα, coincubated with Tenidap 1.25 μg/ml. Lane 5, FRCO stimulated with IL-1β and TNFα, co-incubated with Tenidap 3.75 μg/ml. Double lower bands are caused by some protein oxidation. Upper band in Lane 2—dimer of PLA$_2$. (See References 16 and 40).

FRCO extracellular sPLA$_2$ incubated with TD (50 μg/ml) was 63 U/ml compared with 62 U/ml in the control.

Discussion

Early studies have identified TD as dual inhibitor of cyclooxygenase and lipoxygenase.[1,2] TD inhibited PGE$_2$ and LTB$_4$ synthesis by ionophore-stimulated human PMNs.[2] In rat basophilic leukaemia cells, TD inhibited 5-HETE and LTB$_4$ synthesis as well as PGD$_2$.[17] However, compared with cyclooxygenase, the inhibition of lipoxygenase required a 14-fold higher concentration of TD. Similar observation was made when plasma-free leukocyte suspensions were tested in the rat.[18] The IC$_{50}$ of TD for cyclooxygenase and lipoxygenase was 0.05 μM and 10 μM respectively. When whole blood was used, TD did not suppress 5-lipoxygenase at all. It was therefore concluded that, *in vivo*, TD is essentially a selective inhibitor of cyclooxygenase.[18]

Subsequent studies of TD have detected its impact on cytokine production. Endotoxin-induced IL-1 synthesis in murine peritoneal macrophages was markedly inhibited by TD (IC$_{50}$ 1 μg/ml). Subcellular studies identified the block of pro-IL-1α in the cells.[4] Higher concentrations of TD (10 μg/ml) also inhibited IL-1 activity in LPS-stimulated human monocytes[19] and production of IL-6, and to the lesser degree of TNF and IL-1 in LPS stimulated human peripheral blood mononuclear cells.[20] TD inhibited the release of activated collagenase and of myeloperoxidase from neutrophils.[5] It also inhibited the expression of IL-1 receptor mRNA and IL-1 receptor levels and reduced collagenase and stromelysin activity in cultured normal and osteoarthritis chondrocytes.[21] The observation that rats with adjuvant arthritis treated with TD show marked reduction in the paw swelling and circulating CRP[7] and that human patients with rheumatoid arthritis show reduction in circulating SAA and CRP[6,20] may be

related to the above suppressive activity. Yet, in cytokine-stimulated Hep-3B hepatoma cells *in vitro*, TD was unable to block SAA synthesis. TD used together with chemically modified tetracycline, synergistically inhibited the tissue activity of collagenase and gelatinase in adjuvant arthritis and substantially reduced radiological severity of joint damage.[22] It also inhibited cytokine-induced bone resorption of ^{45}Ca-labelled mouse calvaria.[8] Some clinical improvement was noted in a few short-term open trials of rheumatoid arthritis and osteoarthritis.[23–25]

Since TD may be potentially useful in the therapy of inflammatory conditions, its impact on the inflammatory cascade is of significant interest. Secretory non-pancreatic phospholipase A$_2$ has been identified as one of the pivotal pathogenetic agents in both local[26–28] and systemic[29–31] inflammatory conditions. Marked elevation of circulating sPLA$_2$ was found in adult and juvenile rheumatoid arthritis, correlating well with the disease activity.[32,33] sPLA$_2$ also correlated significantly with the complications and outcome of septic shock[31] and multi-organ failure.[34] sPLA$_2$ injected into joints of experimental animals induced dose-dependent synovitis.[26,35] The synthesis of sPLA$_2$ by osteoblasts,[10] chondrocytes[36] and smooth muscle cells[37] is induced and enhanced by cytokines, especially IL-1 and TNF. It has recently been reported that some agents that inhibit collagenase[38] also inhibit sPLA$_2$ interaction with the substrate.[39] We hypothesized that TD might also inhibit sPLA$_2$ synthesis. Indeed, this study has shown that concentrations as low as 0.25 μg/ml (0.725 μM) markedly inhibited the synthesis of sPLA$_2$ by foetal rat calvarial osteoblasts. We have shown that the synthesis of sPLA$_2$ was most probably blocked at the post-transcriptional level. It has recently been reported that TD generally inhibits protein synthesis in cells.[41,42] Thus, inhibition of sPLA$_2$ synthesis by TD seems to be an important part of this general effect on cell metabolism. TD had no direct effect on PLA$_2$ enzymatic activity.

The fact that TD inhibits the synthesis of sPLA$_2$ adds to the repertoire of its biological activities and shows that this agent may inhibit the arachidonic acid cascade, at a level proximal, to that of cyclooxygenase and lipoxygenase. Marked inhibition of sPLA$_2$ synthesis by TD may be partially responsible for its anti-inflammatory activity.

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