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Selective Inhibition of Cyclooxygenase-2 (COX-2) by 1α,25-Dihydroxy-16-ene-23-yne-Vitamin D_3, a Less Calcemic Vitamin D Analog

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Abstract Inducible cyclooxygenase-2 (COX-2) has been implicated to play a role in inflammation and carcinogenesis and selective COX-2 inhibitors have been considered as anti-inflammatory and cancer chemopreventive agents. 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), the active hormonal form of vitamin D_3 also has been considered to be a cancer chemopreventive agent in addition to its important role in maintaining calcium homeostasis. Based on these observations, we studied the direct effect of 1α,25$(OH)_2D_3$ and one of its less calcemic synthetic analogs, 1α,25$(OH)_2$-16-ene-23-yne-D_3 on the activity of both COX-1 and COX-2 in an in vitro enzyme assay. Preliminary data indicated that both 1α,25$(OH)_2D_3$ and 1α,25$(OH)_2$-16-ene-23-yne-D_3 inhibited selectively the activity of COX-2 with no effect on the activity of COX-1. Out of the two compounds, 1α,25$(OH)_2$-16-ene-23-yne-D_3 was found to be more effective with an IC_{50} of 5.8 nM. Therefore, the rest of the experiments were performed using 1α,25$(OH)_2$-16-ene-23-yne-D_3 only. 1α,25$(OH)_2$-16-ene-23-yne-D_3 inhibited the proliferation of lipopolysaccharide (LPS) stimulated mouse macrophage cells (RAW 264.7) with a reduction in the expression of COX-2 along with other inflammatory mediators like inducible nitric oxide synthase (iNOS) and interleukin-2 (IL-2). Furthermore, 1α,25$(OH)_2$-16-ene-23-yne-D_3 also inhibited carrageenan induced inflammation in an air pouch of a rat and effectively reduced the expression of COX-2, iNOS, and IL-2 in the tissues of the same air pouch. In both cases, 1α,25$(OH)_2$-16-ene-23-yne-D_3 did not show any effect on the expression of COX-1. In summary, our results indicate that 1α,25$(OH)_2$-16-ene-23-yne-D_3, a less calcemic vitamin D analog, exhibits potent anti-inflammatory effects and is a selective COX-2 inhibitor. J. Cell. Biochem. 104: 1832–1842, 2008.

Key words: COX-2; vitamin D analogs; inflammation

Cyclooxygenase (COX) also referred to as prostaglandin H_2 synthase catalyzes the rate limiting conversion of arachidonic acid to prostaglandin H_2 (PGH_2) [Smith et al., 1996; Smith and Langenbach, 2001; Ratliff, 2005]. These prostaglandins by interacting with specific family of G-protein coupled cell membrane receptors produce many cellular responses and pathophysiological processes such as inflammation, gastrointestinal cytoprotection, cancer, and atherosclerosis. There are two known isofoms of this enzyme, COX-1, and COX-2. COX-1 is constitutively expressed and COX-2 is inducible and belongs to "immediate early gene" family. The COX-1 is mainly responsible for the synthesis of prostaglandins which exert cytoprotective effect in the gastrointestinal tract and control renal function, where as, the COX-2 is induced selectively

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by pro-inflammatory cytokines and growth factors (EGF, PDGF) and facilitates the release of prostaglandins involved in the inflammatory process [Vane et al., 1998; Smith et al., 2000]. In the early 1970s it was found that non-steroidal anti-inflammatory drugs (NSAIDs) prevent the production of PGs by inhibiting the COX enzyme. Inhibition of this enzyme led to decreased production of pro-inflammatory prostaglandins in inflamed tissues. But use of NSAIDs is known to cause complications like development of gastric ulcers and renal failure because of the inhibition of essential COX-1 [Mantry et al., 2003]. This led researchers to develop selective COX-2 inhibitors, so that the important physiological roles of COX-1 in stomach, liver, and kidney can be maintained.

The active hormonal form of vitamin D$_3$, 1α,25(OH)$_2$D$_3$ is mainly involved in maintaining calcium homeostasis through its effect on intestinal calcium absorption and bone calcium mobilization [Dusso et al., 2005]. At the same time, 1α,25(OH)$_2$D$_3$ also has been shown to possess many non-calcemic actions which include anti-proliferative and pro-differentiation effects in both normal and many malignant cells including colon, breast and prostate cancer cells [Abe et al., 1981; Holick, 1995; Bouillon et al., 1995a; Campbell et al., 1997, 1999; Moreno et al., 2005]. For a comprehensive review on the subject of the role of 1α,25(OH)$_2$D$_3$ as a cancer chemopreventive agent, the reader may refer to a recent review [Deeb et al., 2007]. Like 1α,25(OH)$_2$D$_3$, selective COX-2 inhibitors are also being considered as cancer chemopreventive agents. Thus, a possible association between 1α,25(OH)$_2$D$_3$ and COX-2 has been entertained. For example, 1α,25(OH)$_2$D$_3$ has been shown to modulate arachidonic acid release, as well as PGE$_1$ and E$_2$ levels [Boyan et al., 2002]. The retinoid X receptor (RXR), the nuclear receptor for 9-cis retinoic acid (9-cis RA) which forms a heterodimer with the Vitamin D receptor also influences COX-2 expression [Thompson et al., 2001]. Only recently, a definite association between 1α,25(OH)$_2$D$_3$ and COX-2 has been established. It has been shown that 1α,25 (OH)$_2$D$_3$ may exhibit anti-proliferative and chemopreventive activities in prostate cancer cells by inhibiting the expression of COX-2, induction of 15-prostaglandin dehydrogenase and decreasing the expression of EP (PGE$_2$ receptor) and FP (PGF$_{2\alpha}$ receptor) [Krishnan et al., 2007; Swami et al., 2007].

In spite of the extensive literature indicating 1α,25(OH)$_2$D$_3$ as a possible cancer chemopreventive agent, the use of 1α,25(OH)$_2$D$_3$ as a drug has not been successful because of its potent calcemic effects. As a result, many less calcemic vitamin D analogs were synthesized during the past two decades [Bouillon et al., 1995b]. For a comprehensive review on the development of less calcemic vitamin D analogs and their potential use in the treatment of hyper-proliferative conditions such as psoriasis and different types of cancer, the reader is referred to a recent review [Masuda and Jones, 2006]. One of the synthetic vitamin D analogs, 1α,25(OH)$_2$-16-ene-23-yne-D$_3$, has been investigated extensively for its various non-calcemic actions which are discussed in detail later in this paper. The chemical structure of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ differs from that of 1α,25(OH)$_2$D$_3$ by the presence of a double bond in the D-ring at C-16 and a triple bond on the side chain at C-23 (Fig. 1). In the present study, we demonstrated that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ selectively inhibits COX-2 and exhibits anti-inflammatory effects in mouse macrophage cells (RAW 264.7) stimulated with LPS and in the tissues of a rat air pouch, in which inflammation was induced by carrageenan.

MATERIALS AND METHODS

Chemicals

1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-16-ene-23-yne-D$_3$, were synthesized at Hoffmann La-Roche Co., Nutley, NJ. Arachidonic acid, Hematin, Tetramethylene para-phenylenediamine (TMPD), Protease inhibitor cocktail, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] and L-NAME [N (G)-nitro-L-arginine methyl

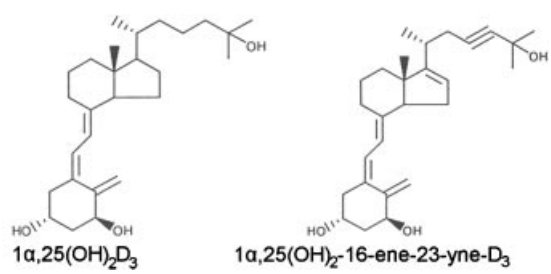

Fig. 1. Chemical structures of 1α,25(OH)2D3 and 1α,25(OH)2-16-ene-23-yne-D3.

ester] were purchased from Sigma Chemicals Co. (St. Louis, MO). Affinity purified goat polyclonal antibodies for COX-1 and COX-2 were purchased from Santa Cruz, CA. Penicillin/streptomycin, fetal bovine serum, Dulbecco's Modified Eagle's Medium (DMEM) were obtained from GIBCO, Life Technologies, Inc., MD.

In Vitro Enzyme Assay to Measure the Activity of Both COX-1 and COX 2

COX-1 enzyme was isolated from the microsomal fraction of ram seminal vesicles containing COX-1 activity and purified as per the methods described earlier [Reddy et al., 2000]. Recombinant human COX-2 enzyme was expressed in Sf-9 (*Spodoptera frugiperda*) insect cells as described earlier [Reddy et al., 2000]. The activity levels of COX-1 and COX-2 were measured by chromogenic assay [Reddy et al., 2000] based on the oxidation of TMPD during the reduction of PGG_2 to PGH_2. The assay mixture contained Tris–HCl buffer (pH 8.0, 100 mM) hematin (15 μM), EDTA (3 μM), enzyme (COX-1 or COX-2, 100 μg) in a final volume of 1 ml. The mixture was preincubated at 25°C for 15 min and then the reaction was initiated by the addition of arachidonic acid (100 μM) and TMPD (120 μM). The enzyme activity was measured by estimating the velocity of TMPD oxidation during the first 25 s of the reaction. TMPD oxidation was monitored by following the increase in absorbance at 603 nm. Each test compound, dissolved in ethanol, was then added to the assay mixture at different concentrations to calculate its IC_{50} value. A low rate of non-enzymatic oxidation, which was observed in the absence of either COX-1 or COX-2 was subtracted from the experimental value while calculating the percent inhibition.

Cell Lines and Animals

The mouse macrophage cell line (RAW 264.7) obtained from National Centre for Cell Sciences (NCCS), Pune was grown in DMEM containing 10% fetal bovine serum with 1% penicillin/streptomycin solution at 37°C in a humidified atmosphere of 5% CO_2. Wistar male rats (150–200 g) were obtained and housed in pathogen-free conditions with free access to food and water at the institutional animal care facility. All the experiments were carried out in accordance with the guidelines of Institutional animal welfare regulations.

Assay to Measure Cell Growth

The in vitro effect of 1α,25$(OH)_2$-16-ene-23-yne-D_3 on the growth of mouse macrophage (RAW 264.7) cells was determined by MTT assay. Cells (5×10^3) were treated in triplicates with and without LPS (1 μg/ml), L-NAME (1 mM), and 1α,25$(OH)_2$-16-ene-23-yne-D_3 (0, 5, 10, 15, and 20 nM) for 24 h. In order to prevent the interference of nitric oxide (NO) in LPS stimulated RAW 264.7 cells, L-NAME, inhibitor of NO synthase (NOS) activity was employed through out the study. At the end of 24 h incubation, 20 μl of MTT (5 mg/ml) was added to each well and the incubation was continued for 4 h at 37°C. Purple blue formazan crystals formed were dissolved in DMSO and the absorbance was read at 570 nm using microtiter plate reader, Quant Bio-Tek Instruments, VT. Absorbance is directly related to viable cell number. The percent inhibition was calculated using control as 100%.

Air Pouch Model

An air pouch model to study inflammation was created as described before [Martin et al., 1994] with minor modifications. Briefly, an air cavity was produced in Wistar rats by subcutaneous injection of 20 ml of sterile air into the intracapsular area on the dorsal side of the rat. An additional 10 ml of air was injected into the cavity every 3 days to keep the space open. Seven days following the initial air injection, an inflammatory reaction was generated in the air pouch by injecting 2 ml of 1.5% (w/v) solution of carrageenan dissolved in saline directly into the pouch. Treatment group received a single dose of 1α,25$(OH)_2$-16-ene-23-yne-D_3 (10 μg/Kg body weight) along with carrageenan. Control group received only carrageenan without 1α,25$(OH)_2$-16-ene-23-yne-D_3. At different time intervals (0, 4, 8, 12, and 24 h), rats from both groups were anesthetized and sacrificed by cervical dislocation. The pouch lining was separated from the muscle and stored in liquid nitrogen for further studies. The degree of inflammation was assessed by measuring both the volume of the exudate in the pouch and the number of cells present in the exudate. To measure the total number of cells in the exudate, the pouch was first gavaged using 20 ml of saline and the fluid was collected for centrifugation at 3,000 rpm for 10 min. The pellet was then suspended in 1 ml of saline and the cells were finally counted using heamocytometer.

Western Blot Analysis

Western blot analysis was performed for both in vitro and in vivo samples. For in vitro studies, RAW 264.7 cells were cultured with LPS, L-NAME and with or without 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ for 24 h. Cells were collected at 0, 4, 8, 12, and 24 h time points and cell lysates were prepared by incubating in lysis buffer (10 mM EDTA, 5 mM EGTA, 1 mM PMSF and 10 μl leupeptin) for 30 min and supernatants were collected by centrifuging at 15,000 rpm for 20 min. For in vivo studies, the tissues from the air pouches were collected at 0, 4, 8, 12, and 24 h following treatment with carrageenan alone or carrageenan + 1α,25(OH)$_2$-16-ene-23-yne-D$_3$. The tissues from each air pouch were homogenized in 100 mM Tris–HCl (pH 8.0), 0.3 M mannitol, 1 mM EGTA, 1 mM EDTA, 4 mM K_2HPO_4, 1 mM DTT, 1 mM Sodium orthovandidate, 0.1% SDS, 2 mM PMSF and 40 μl/ml of complete protease inhibitor solution (Sigma Chemicals Co.). Supernatants were then collected by centrifuging the homogenized tissues at 10,000 rpm for 30 min at 4°C. Protein concentrations in the samples were determined by standard Lowry method [Lowry et al., 1951]. Equal amounts of cell lysates from in vitro studies and the tissue lysates from the in vivo studies were electrophoresed on 10% SDS–PAGE and transblotted onto a nitrocellulose membrane. Immunoblotting was performed with COX-1, COX-2, iNOS, and IL-2 antibodies according to manufacturer's procedures. Immunoblotting with GAPDH antibodies acted as loading control. The experiments were carried out in triplicates and Scion image analysis of band intensities was carried out.

Statistical Analysis

Data are presented as mean ± SEM and the P values were determined using the unpaired Student's t-test.

RESULTS

Effect of 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on the Activity of COX-1 and COX-2

To determine the direct effect of 1α,25(OH)$_2$D$_3$, and its synthetic analog 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on the activity of both COX-1 and COX-2, we performed spectrophotometric assay (TMPD assay) as described in Materials and Methods Section. Both 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ inhibited the activity of COX-2 selectively with no effect on the activity of COX-1 up to 1 μM concentration. 1α,25(OH)$_2$D$_3$ inhibited only 20% of the activity of COX-2 at 1 μM concentration. On the contrary, its analog 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ inhibited 50% of the activity of COX-2 at only 5.8 nM concentration. These effects were produced independent of the period of preincubation of the enzyme with the compounds. The efficacy of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ was also compared with known inhibitors of COX-1 and COX-2 (Table I). Since 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ has exhibited better inhibition of COX-2 than 1α,25(OH)$_2$D$_3$, further studies were performed only with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$.

Effect of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on Growth of Mouse Macrophage Cells (RAW 264.7)

We tested the effect of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on the growth of RAW 264.7 cells treated with LPS by incubating with different concentrations of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ (0–20 nM) and the viability of cells was determined by MTT assay after 24 h as described in Materials and Methods Section. A typical growth curve of cells treated with different concentrations of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ is shown in Figure 2. The IC$_{50}$ value for 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ was calculated to be 10 nM. Microscopic analysis of the cells treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ revealed pronounced morphological changes when compared to control cells treated with LPS alone. The cells showed loss of cytoplasm integrity and formation of vacuoles inside the cell (Fig. 3).

Effect of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on COX-1, COX-2, iNOS, and IL-2 Expression in RAW 264.7 Cells Treated With LPS

The effect of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on the expression of COX-1, COX-2, iNOS, and IL-2 was studied in RAW 264.7 cells treated with

TABLE I. The IC$_{50}$ of 1α25(OH)$_2$-16-ene-23-yne-D$_3$, in Relation to Known Inhibitors of COX-1 and COX-2

Compound	COX-1 (IC$_{50}$)	COX-2 (IC$_{50}$)
1α25(OH)$_2$-16-ene-23-yne-D$_3$	No effect[a]	5.8 nM
Celecoxib	25.0 μM	40 nM
Indomethacin	200 nM	100 μM

[a]Upto 1 μM concentration.

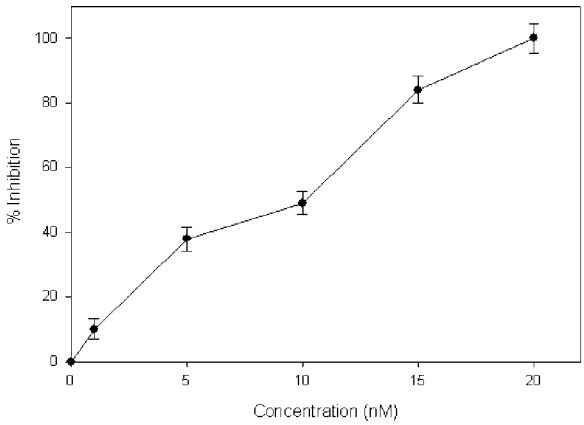

Fig. 2. Effect of 1α,25(OH)2-16-ene-23-yne-D3 on the percent inhibition in the growth of mouse macrophage cells (RAW 264.7). The cells were incubated with different concentrations of 1α,25(OH)2-16-ene-23-yne-D3 for 24 h and the survival of the cells was determined by MTT assay. Data represent the mean ± SEM values from three separate experiments.

LPS (1 μg/ml) and L-NAME (1 mM). Western blot analysis was performed using whole cell lysates, immunoblots and Scion image analysis of band intensities are shown in Figure 4. The expression of COX-1 was noted at 0 h because of its constitutive expression and there was no significant change in its intensity over a period of 24 h both in the control and the cells treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$. On the contrary, the expression of COX-2, unlike COX-1, was detected only after 4 h of treatment with LPS, and its intensity increased progressively up to 24 h. However, this gradual increase in the intensity of the expression of COX-2 over a period of 24 h was inhibited in cells treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$.

Furthermore, it can be seen in Figure 4 that the expression of both iNOS and IL-2, like COX-2, was also induced in RAW 264.7 cells only after 4 h of treatment with LPS and the intensity of their expression increased gradually to reach maximum at 24 h. However, such gradual increase in the intensity of the expression of iNOS and IL-2 was not observed in cells treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$.

Effect of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on Carrageenan Induced Inflammation in an Air Pouch of a Rat

The air pouches of the rats treated with saline only did not show any signs of inflammation (Fig. 5A,D). However, the classical signs of acute inflammation, namely redness and swelling, were observed in the air pouches of the rats treated with carrageenan (control group). The tissues of the air pouches were heavily infiltrated with blood cells at various sites, especially near the cavities as it is easy for the cells to migrate into the open areas (Fig. 5B,E) and the inflammatory reaction reached its peak at 24 h following treatment with carrageenan. These observations clearly demonstrate that carrageenan induced a marked inflammatory reaction in air pouches. On the contrary, a reduced inflammatory reaction was noted in the air pouches of the rats treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ (experimental group) (Fig. 5C,F).

Inflammatory exudate was noted in the air pouches of the control rats within 4 h after carrageenan treatment and its volume gradually increased to reach maximum at 24 h. The volume of the exudates collected in the air

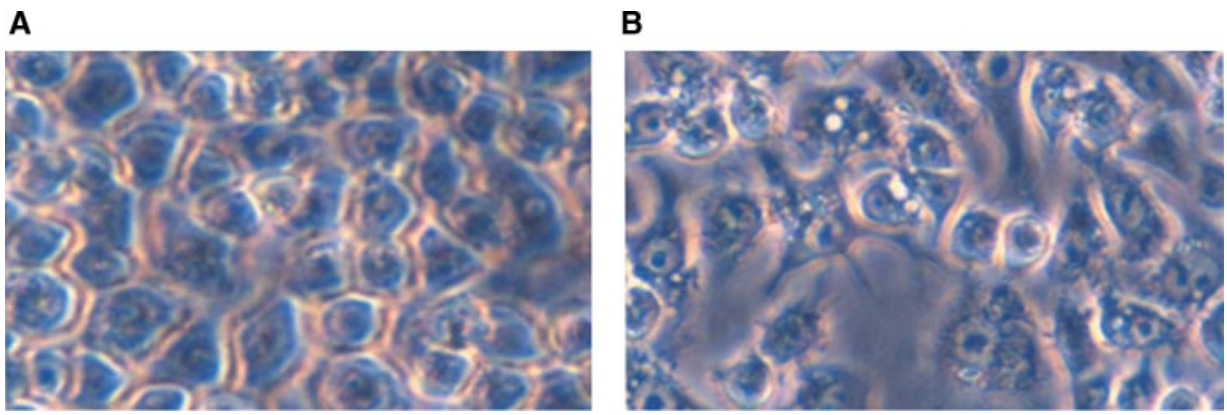

Fig. 3. Phase contrast photomicrographs (400×) of control (**A**) and 1α,25(OH)2-16-ene-23-yne-D3 (10 nM for 24 h) treated (**B**) mouse macrophage cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

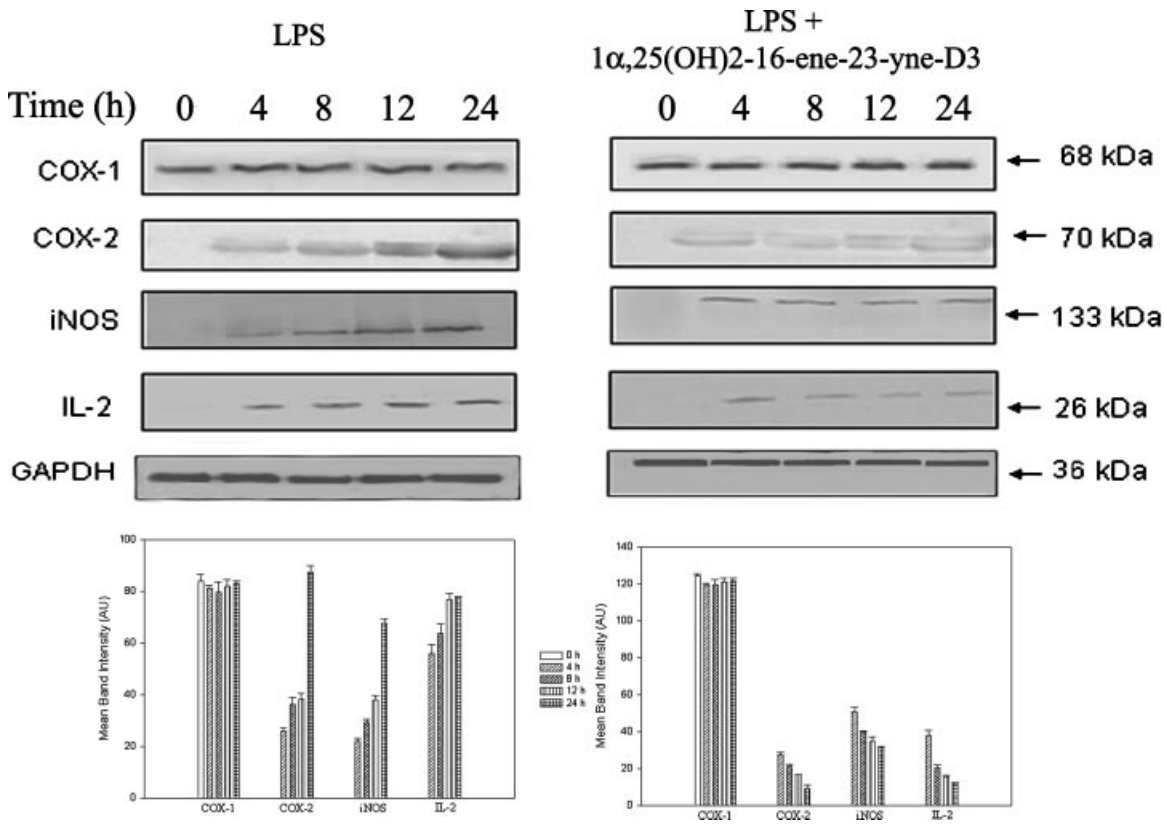

Fig. 4. Western blot analysis and Scion image analysis of band intensities showing the expression of COX-1, COX-2, iNOS, and IL-2proteins in RAW 264.7 cells treated LPS alone or LPS and 1α,25(OH)2-16-ene-23-yne-D3 (10 nM) for various time periods. GAPDH was used as an internal control for equal loading of proteins. Data represent the mean ± SEM values from three separate determinations. The values were significant at $P < 0.05$ in all the proteins (COX-2, iNOS, and IL-2) except COX-1.

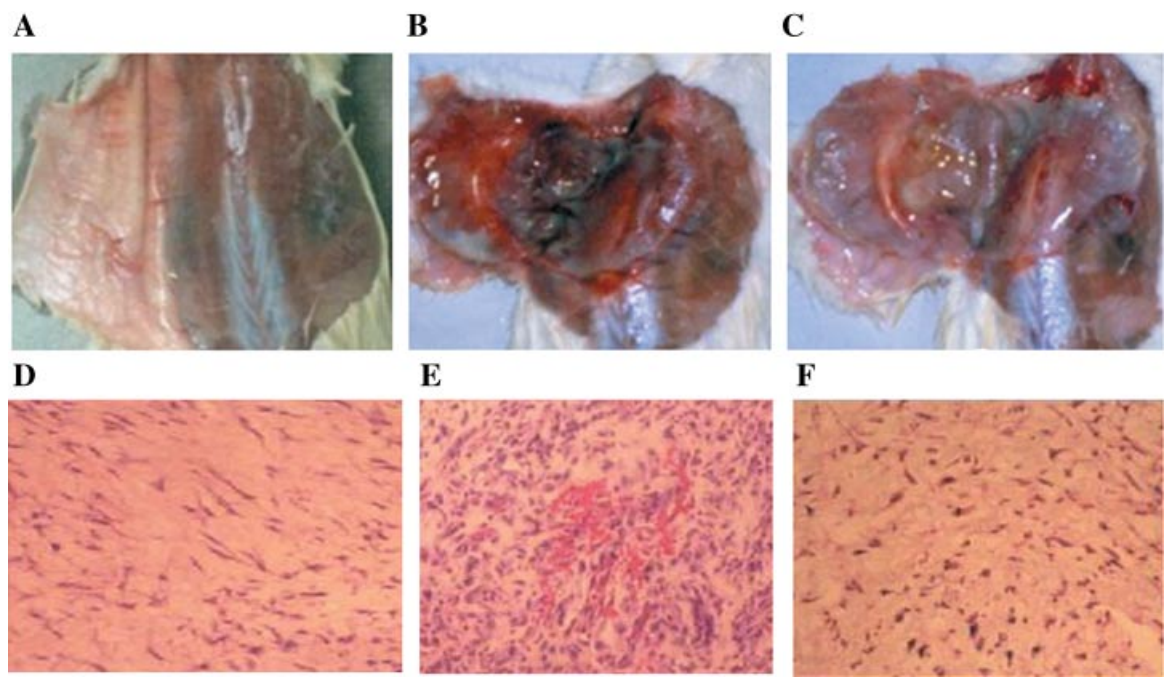

Fig. 5. Photographs (**A**–**C**) and photomicrographs of histological sections (400×) (**D**–**F**), of the air pouch tissues of rats treated with saline (A,D), carrageenan (B,E) and carrageenan + 1α,25(OH)2-16-ene-23-yne-D3 (10 μg/Kg body weight) (C,F). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pouches over a 24 h time period in the rats treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ was significantly less when compared to the volume of exudates collected in the air pouches of control rats (Fig. 6, lower panel). Inflammatory cells started infiltrating into the air pouches of the control rats as early as 4 h and their number increased gradually to reach maximum at 24 h. The number of cells infiltrated in to the air pouches of rats treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$, however, was significantly lower (Fig. 6, upper panel). These findings together indicate that the intensity of the inflammatory reaction is less in the air pouches of rats treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$.

Effect of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on Expression of COX-1, COX-2, iNOS, and IL-2 in Tissues of the Air Pouch

The effect of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on the expression of COX-1, COX-2, iNOS, and IL-2 was studied by Western Blot Analysis in the tissues of the air pouches of both the control rats treated with carrageenan alone and the experimental rats treated with carrageenan + 1α,25(OH)$_2$-16-ene-23-yne-D$_3$. The immunoblots and Scion image analysis of band intensities are shown in Figure 7. No change in the expression of COX-1 was noted in the tissues obtained from both control and experimental rats. The expression of COX-2, unlike COX-1, was not detected at 0 h in the tissues of the control rats as COX-2 is not expressed constitutively. However, the expression of COX-2 was induced within 4 h following carrageenan treatment and its intensity increased further at later time points to reach maximum at 24 h. On the contrary, the intensity of the expression of COX-2 in the tissues of the experimental rats treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ was more or less stabilized at all time points and was relatively less at 24 h.

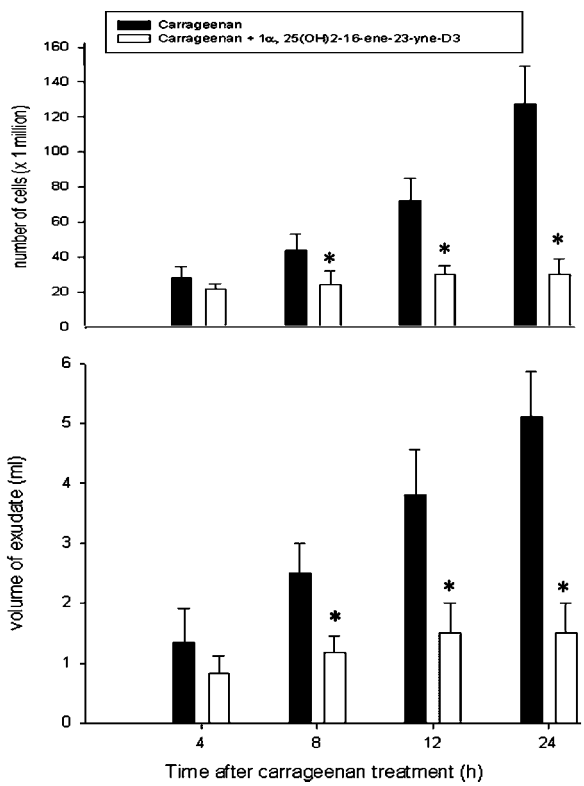

Fig. 6. Effect of 1α,25(OH)2-16-ene-23-yne-D3 on number of cells infiltrated (**upper panel**) and the volume of exudates accumulated (**lower panel**) in the air pouches of rats treated with either carrageenan alone or carrageenan + 1α,25(OH)2-16-ene-23-yne-D3 (10 μg/Kg body weight) for various time periods. Data represent the mean ± SEM values m three separate determinations; *$P < 0.05$.

Furthermore, it can also be seen in Figure 7 that the expression of both iNOS and IL-2, like COX-2, was also induced within 4 h following carrageenan treatment in the tissues of the control rats and the intensity of their expression increased gradually to reach maximum at 24 h. On the contrary, the intensity of the expression of both iNOS and IL-2 seen at 4 h time point decreased gradually with time in the tissues of the experimental rats treated with 1α,25(OH)$_2$-16-ene-23-yne-D$_3$.

Thus, these results together indicate that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ does not have any effect on COX-1 but inhibits the progressive increase in the intensity of the expression of COX-2, iNOS, and IL-2, in addition to its direct inhibitory effect on COX-2 activity and thereby reduces the intensity of inflammatory reaction generated by carrageenan in the air pouches.

DISCUSSION

The discovery that structural modifications of 1α,25(OH)$_2$D$_3$ can result in the dissociation of its non-calcemic actions such as regulation of cell growth and differentiation from its calcemic actions stimulated an active area of research leading to the synthesis of numerous vitamin D analogs with a wide array of biological actions. Out of many synthetic vitamin D analogs 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ is found to be one of the most promising analogs in vitamin D based drug discovery [Uskokovic et al., 2001]. It is 4- to 12-fold more potent than

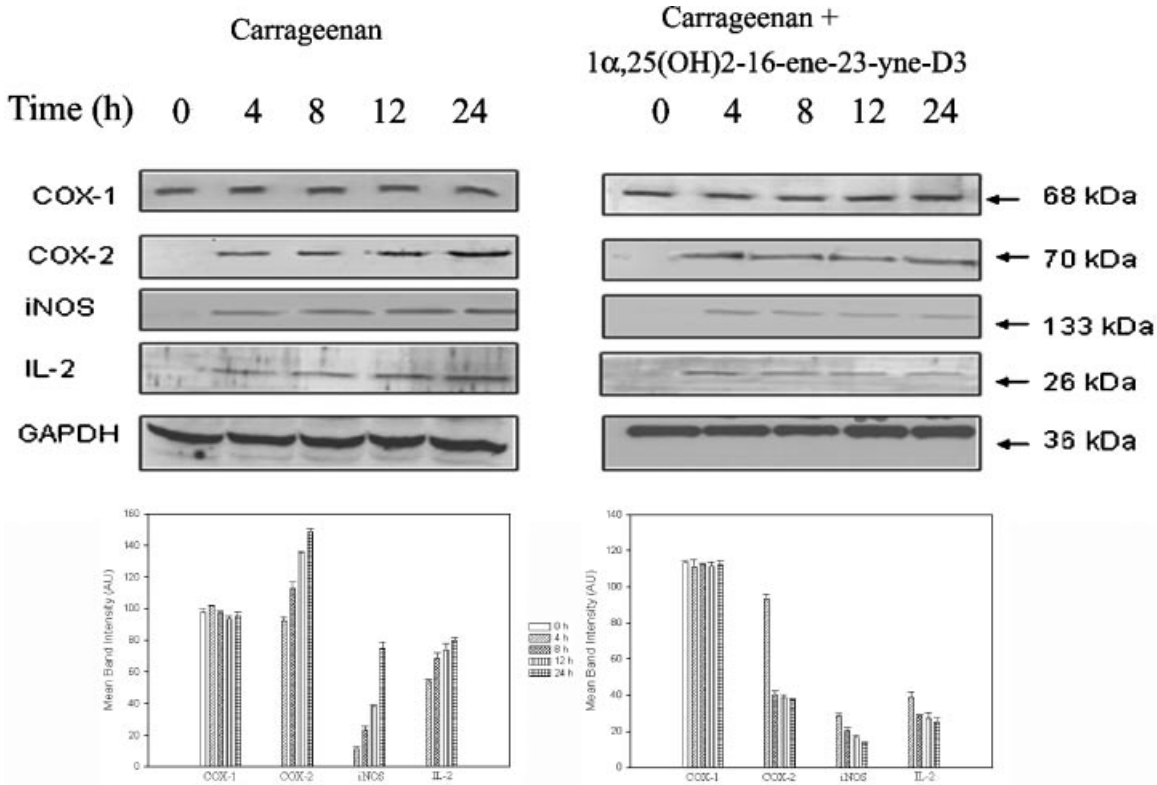

Fig. 7. Western blot analysis and Scion image analysis of band intensities showing the expression of COX-1, COX-2, iNOS, and IL-2 proteins in tissues of air pouches obtained from rats treated with carrageenan alone or carrageenan + 1α,25(OH)2-16-ene-23-yne-D3 (10 μg/Kg body weight) for various time periods. GAPDH was used as an internal control for equal loading of the proteins. Data represent the mean ± SEM values from three separate determinations. The values were significant at $P < 0.05$ in all the proteins (COX-2, iNOS, and IL-2) except COX-1.

1α,25(OH)$_2$D$_3$ in inhibiting clonal growth and inducing differentiation of human myeloid leukemia cells. However, 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ is found to be 30–50 times less potent than 1α,25(OH)$_2$D$_3$ in stimulating intestinal calcium absorption and bone calcium mobilization respectively [Zhou et al., 1989]. Thus 1α,25(OH)$_2$-16-ene-23-yne-D$_3$, when compared to 1α,25(OH)$_2$D$_3$ is less toxic and has a better therapeutic index. Also 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ increases the survival rate of leukemic mice [Zhou et al., 1990]. Furthermore, 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ has been shown to inhibit the growth of prostate [Schwartz et al., 1995] and colon cancer cells [Shabahang et al., 1994]. It was also shown that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ reduces PTH levels in uremic rats without producing hyper-calcemia [Lippuner et al., 2004]. At present, the mechanisms responsible for the dissociation of the calcemic from non-calcemic actions of various synthetic vitamin D analogs is an active area of research. This important issue is discussed in a recent review and it appears that different analogs work through different mechanisms [Bouillon et al., 2005]. Like most of the analogs, the mechanisms responsible for the dissociation of the calcemic actions from non-calcemic actions of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ have not been elucidated.

In the present study, we report another important non-calcemic action of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$. We found that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ selectively inhibits the COX-2 activity and it has a better IC$_{50}$ value when compared with natural hormone 1α25(OH)$_2$D$_3$. Furthermore, the efficacy of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ is even better than Celecoxib, the well known COX-2 inhibitor (Table I). Also, It is highly significant to note that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ did not have any effect on COX-1 activity. The docking scores calculated for vitamin D and its analogs, using GOD (CDDC) docking program and employing

COX-1 and COX-2 crystal structures, have also supported the selective inhibition of COX-2 by 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ (unpublished observations).

Macrophages play an important role in the regulation of inflammation and immune response. Activated macrophages produce and release growth factors, cytokines and lipid mediators such as prostaglandins and leukotrienes which promote inflammation. Also, COX-2 expression is induced in macrophages upon activation with bacterial endotoxin like LPS. It is now well established that 1α,25(OH)$_2$D$_3$ plays an important role in regulating immune response at inflammatory sites and the activated macrophages, including peritoneal macrophages can produce 1α,25 (OH)$_2$D$_3$ [Adams et al., 1983; Shany et al., 1991; Cohen-Lahav et al., 2007]. In the present study we showed that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ effectively inhibits the growth of murine macrophage cells (RAW 264.7) exposed to bacterial endotoxin (LPS) with IC$_{50}$ value of 10 nM (Fig. 2). In the same cells we also showed that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ down regulated the expression of COX-2. Similar down regulation of COX-2 expression by 1α,25(OH)$_2$D$_3$ was recently reported in prostate cancer cells [Krishnan et al., 2007].

Inflammation, which was initially recognized as a simple allergic reaction for centuries is now understood to be extremely important in pathogenesis of a large number of diseases. The process of inflammatory reaction, initiated by carrageenan, begins by utilizing the locally released population of mediators. The stimulus and the low level of released mediators specifically LTB$_4$ lead to chemotactic attraction of leukocytes into the area of stimulus. This in turn leads to induction of COX-2, and other cytokines. Furthermore, the action of small amounts of Reactive Oxygen Species (ROS) released by the local neutrophils additionally induce cytokines and growth factors. Monocytes and macrophages arrive within few hours to ingest and phagocytose the foreign substance, and finally undergo apoptosis. If the stimulus persists, apoptosis of the macrophages is discouraged, infiltration of cells continues and finally the release of mediators cause tissue damage leading to a severe inflammatory reaction. In the present study, we tested the effect of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ on carrageenan induced inflammatory reaction in a rat air pouch. We noted that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ decreased not only the volume of the exudate accumulated in the pouch but also the number of cells in the exudate. These findings indicate that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ is an anti-inflammatory agent. In a previous study, another vitamin D analog namely 22-oxa-1α,25(OH)$_2$-D$_3$ was also shown to produce similar results [Hirata et al., 1994]. However, in our present study, we not only identified 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ as an anti-inflammatory agent but also found that it down regulates the expression of COX-2 and other pro-inflammatory molecules such as iNOS and IL-2. Furthermore, it is also significant to note that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$, however, does not show any effect on the expression of COX-1. The finding of certain vitamin D compounds having inhibitory effect on COX-2 expression is further supported by another study which was published while our paper was in review. In this study, it was shown that another Roche vitamin D analog (RO26-2198) also inhibited the expression of COX-2 in an AOM/DSS model of colitis associated carcinogenesis [Fichera et al., 2007].

In summary, the results of our study indicate that 1α,25(OH)$_2$-16-ene-23-yne-D$_3$, a less calcemic vitamin D analog, is a selective COX-2 inhibitor exhibits anti-proliferative and anti-inflammatory effects on LPS treated mouse macrophage cells (RAW 264.7) and anti-inflammatory effect on carrageenan induced inflammation in a rat air pouch. In both situations, 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ down regulated the expression of COX-2 without effecting the expression of COX-1, the inhibition of which is known to cause renal and other complications. Based on these results, we propose that 1α, 25(OH)$_2$-16-ene-23-yne-D$_3$ may be used as an effective anti-inflammatory agent in addition to its well established use as an anti-proliferative agent.

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