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Effects of 1α,25-$(OH)_2D_3$ on Rat Growth Zone Chondrocytes are Mediated Via Cyclooxygenase-1 and Phospholipase A_2

V.L. Sylvia,[1] F. Del Toro,[1,4] D.D. Dean,[1] R.R. Hardin,[1] Z. Schwartz,[1,2,5] and B.D. Boyan[1,2,3]*

[1]Department of Orthopaedics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229
[2]Department of Periodontics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229
[3]Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229
[4]Department of Orthodontics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229
[5]Department of Periodontics, Hebrew University Hadassah Faculty of Dental Medicine, Jerusalem, Israel 91010

Abstract 1α,25-$(OH)_2D_3$ mediates its effects on growth zone chondrocytes via rapid membrane-associated events as well as through traditional nuclear receptor mechanisms. The membrane-associated signaling pathways include rapid production of diacylglycerol and activation of protein kinase C (PKC), as well as activation of phospholipase A_2 (PLA_2), increased production of arachidonic acid, and increased production of prostaglandins. This study examined the roles of PLA_2 and cyclooxygenase (Cox) in the mechanism of action of 1α,25-$(OH)_2D_3$ in these cells to determine whether one or both enzymes catalyze the rate limiting step and whether constitutive or inducible Cox is involved. Cultures were incubated with 1α,25-$(OH)_2D_3$ for 9 min to measure PKC or for 24 h to measure physiological responses ($[^3H]$-thymidine incorporation, alkaline phosphatase specific activity, $[^{35}S]$-sulfate incorporation). Based on RT-PCR and Northern blot analysis, growth zone chondrocytes expressed mRNAs for both Cox-1 and Cox-2 and neither Cox was modulated by 1α,25-$(OH)_2D_3$. To examine the role of Cox, the cultures were also treated with resveratrol (a specific inhibitor of Cox-1), NS-398 (a specific inhibitor of Cox-2), or indomethacin (a general Cox inhibitor). The results showed that Cox-1 inhibition reduced the 1α,25-$(OH)_2D_3$-dependent effects on proliferation, differentiation, and matrix production, whereas inhibition of Cox-2 only had an effect on proliferation. The effects of Cox inhibition were not rate limiting, based on experiments in which PLA_2 was activated with melittin or inhibited with quinacrine. However, at least part of the action of 1α,25-$(OH)_2D_3$ was regulated by metabolism of arachidonic acid to prostaglandins. This supports the hypothesis that 1α,25-$(OH)_2D_3$ exerts its effects via more than one signaling pathway and that these pathways are interrelated via the modulation of PLA_2 as a rate-limiting step. PKC regulation may occur at multiple stages in the signal transduction cascade. J. Cell. Biochem. Suppl. 36:32–45, 2001.

Key words: chondrocyte cultures; cyclooxygenase; Cox-1; Cox-2; 1,25-$(OH)_2D_3$; protein kinase C; PKC; alkaline phosphatase; membrane receptor

Recent evidence has shown that 1α,25-$(OH)_2D_3$ exerts its effects on rat growth plate chondrocytes through specific 1α,25-$(OH)_2D_3$-activated membrane-associated mechanisms in addition to traditional nuclear vitamin D receptor mechanisms [Pedrozo et al., 1999]. The signaling pathways involved in the membrane-mediated response include rapid changes in membrane fluidity [Swain et al., 1993] and Ca^{2+} ion flux [Langston et al., 1990]. Two interrelated pathways participate in the signal

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*Correspondence to: B.D. Boyan, Ph.D, Department of Orthopaedics (Mail Code 7774), The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900.
E-mail: BoyanB@uthscsa.edu

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transduction process, both of which depend on metabolism of membrane phospholipids. At least of part of the response to 1α,25-$(OH)_2D_3$ is mediated by rapid activation of protein kinase C alpha (PKCα) [Sylvia et al., 1993] via an increase in the production of diacylglycerol through the action of phospholipase C (PLC) and translocation of PKC to the membrane. In costochondral cartilage, this mechanism is specific to growth zone cells, since 1α,25-$(OH)_2D_3$ has no effect on PKC activity in chondrocytes from the resting zone.

Phospholipase A_2 (PLA_2) also plays a pivotal role in the rapid response of growth zone chondrocytes to 1α,25-$(OH)_2D_3$. There is a rapid increase in arachidonic acid release following exposure of these cells to the seco-steroid [Schwartz et al., 1990]. Specific activity of PLA_2 is increased due to the direct action of 1α,25-$(OH)_2D_3$ on membrane-associated enzyme, independently of any change in gene expression. When 1α,25-$(OH)_2D_3$ is incubated directly with plasma membranes and matrix vesicles isolated from growth zone chondrocyte cultures, specific activity of PLA_2 is stimulated [Schwartz et al., 1988]. Moreover, when purified PLA_2 is incubated with 1α,25-$(OH)_2D_3$, there is a dose-dependent increase in enzyme activity [Swain et al., 1992], suggesting that the vitamin D metabolite may act directly on the enzyme. Changes in PLA_2 activity also modulate the PKC response to 1α,25-$(OH)_2D_3$ [Boyan et al., 1998]. Activation of PLA_2 activity results in increased PKC activity and inhibition of PLA_2 activity results in decreased PKC activity.

Arachidonic acid, the product of PLA_2 action, exerts its own effects on growth zone cells. Arachidonic acid causes a dose-dependent stimulation of basal PKC and enhances the 1α,25-$(OH)_2D_3$-stimulated increase in PKC that is observed at nine minutes [Boyan et al., 1997a]. Arachidonic acid causes rapid effects in other cells, including increases in PKC [Giaume et al., 1997]. Part of the effect of arachidonic acid is likely via its own nuclear receptors. The fatty acid has been shown to regulate new gene expression via peroxisome proliferator-activated receptors (PPARs) [Tessier-Prigent et al., 1999]. However, arachidonic acid may also exert its effects on growth zone chondrocytes indirectly through its metabolites. 1α,25-$(OH)_2D_3$ causes a rise in the production of both prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2) [Schwartz et al., 1992], and inhibition of prostaglandin production by indomethacin reduces the stimulatory effects of 1α,25-$(OH)_2D_3$ on PKC activity. Exogenous PGE_2 regulates PKC activity through the EP1 receptor present on growth zone cells [Del Toro et al., 2000].

These studies suggest that modulation of PLA_2 by 1α,25-$(OH)_2D_3$ is a critical step in PKC activation. A second critical step in the mechanism of action of 1α,25-$(OH)_2D_3$ is the production of PGE_2. Cyclooxygenase (Cox) is the key enzyme in activating the synthesis of prostaglandins from arachidonic acid. These enzymes catalyze the cyclooxygenation and peroxidation of arachidonic acid, resulting in the formation of the intermediate prostaglandin H2. There are at least two isoforms of Cox, but it is not known which one is responsible for PGE_2 production in response to 1α,25-$(OH)_2D_3$. Cox-1, first cloned in 1988 [DeWitt and Smith, 1988], is constitutively expressed. Cox-2 is encoded by a separate gene [Otto and Smith, 1995]. This isoform is inducible, and the inducing stimuli include pro-inflammatory cytokines and growth factors, implying a role for Cox-2 in both inflammation and control of cell growth. Cox-1 and Cox-2 are similar in molecular weight and kinetics but vary in their expression and distribution [Vane et al., 1998].

The aim of this study was to examine whether 1α,25-$(OH)_2D_3$ mediates its effects on growth zone chondrocytes via Cox-1 or Cox-2, and if so, does regulation of this enzyme provide a critical point in the mechanism of action of the vitamin D metabolite. We also investigated the relationship of 1α,25-$(OH)_2D_3$-dependent cyclooxygenase with PLA_2, arachidonic acid and PGE_2 production. To do this, we examined the expression of Cox-1 and Cox-2 in growth zone chondrocytes and their regulation by 1α,25-$(OH)_2D_3$. The role of cyclooxygenases in the biological responses to 1α,25-$(OH)_2D_3$ was evaluated using the Cox-1 specific inhibitor resveratrol and the Cox-2 specific inhibitor NS-398 and comparing their effects to the general cyclooxygenase inhibitor indomethacin. The effects of cyclooxygenase inhibition were examined on [^{3}H]-thymidine incorporation, alkaline phosphatase specific activity, and proteoglycan sulfation. We evaluated the role of the cyclooxygenases in the mechanism of action of 1α,25-$(OH)_2D_3$ by assessing PKC activity in cultures treated with the vitamin D metabolite or arachidonic acid plus appropriate inhibitors.

Finally, to assess whether inhibition of cyclooxygenase might result in upregulation of lipoxygenase activity, leading to production of leukotrienes, we used NDGA and esculetin to inhibit lipoxygenase activity in cells treated with 1α,25-$(OH)_2D_3$.

MATERIALS AND METHODS

Reagents

The vitamin D_3 metabolite 1α,25-$(OH)_2D_3$ was purchased from BIO-MOL (Plymouth Meeting, MA). 1α,25-$(OH)_2D_3$ stock solutions were dissolved in ethanol and diluted at least 1:5000 (v/v) with culture medium before addition to the cultures. Arachidonic acid was obtained from Calbiochem (San Diego, CA). The Cox-1 specific inhibitor resveratrol [Jang et al., 1997] was obtained from Cayman Chemical (Ann Arbor, MI) and the Cox-2 specific inhibitor NS-398 [Futaki et al., 1994] was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). The general Cox inhibitor indomethacin [Vane, 1971] and the PLA_2 inhibitor quinacrine [Church et al., 1993] were obtained from Sigma Chemical Co. (St. Louis, MO). The lipoxygenase inhibitors, nordihydroguaiaretic acid (NDGA), which selectively inhibits 5-lipoxygenase [Hope et al., 1983], and esculetin, which preferentially inhibits 12- and 15-lipoxygenases [Neichi et al., 1983], were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). [^{3}H]-thymidine, [^{35}S]-sulfate and [^{32}P]-ATP were obtained from NEN-DuPont (Boston, MA). PKC assay reagents were obtained from GIBCO-BRL (Gaithersburg, MD). The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent [Smith et al., 1985] obtained from Pierce Chemical Co. (Rockford, IL).

Chondrocyte Cultures

The culture system has been previously described in detail [Boyan et al., 1988, 1992]. Chondrocytes were obtained from growth zone costochondral cartilage of eighteen 125 g male Sprague-Dawley rats after carbon dioxide asphyxiation and removal of the ribs according to a protocol approved by the Institutional Animal Care and Use Committee at The University of Texas Health Science Center at San Antonio. Growth zone chondrocyte cultures were seeded at an initial density of 25,000 cells/cm^2. Cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin-fungizone, and 50 μg/ml sodium ascorbate in an atmosphere of 5% CO_2 and 100% humidity for 7–10 days. At confluence, cells were subcultured using the same plating density and allowed to return to confluence. For all experiments, confluent, third passage cultures were subpassaged into 24-well microtiter plates and grown to confluence. Previous studies have demonstrated a retention of differential phenotypic markers through fourth passage [Boyan et al., 1997b].

To characterize the role of Cox in cell response to 1α,25-$(OH)_2D_3$, confluent cultures of fourth passage growth zone chondrocytes were treated with 10^{-8} or 10^{-7} M indomethacin, 10 or 100 μM Cox-1 inhibitor (resveratrol), or Cox-2 inhibitor (NS-398) in the absence or presence of 10^{-8} M 1α,25-$(OH)_2D_3$. After a 24-h incubation, [^{3}H]-thymidine incorporation, alkaline phosphatase specific activity, and [^{35}S]-sulfate incorporation were assessed. The dose of each inhibitor was chosen based on work in our own lab and previously published observations of others. The concentrations of indomethacin were chosen because they have been shown to significantly inhibit PGE_2 production by growth zone chondrocytes [Schwartz et al., 1992]. The dose of resveratrol used has been shown to selectively inhibit Cox-1 [Chen and Pace-Asciak, 1996; Jang et al., 1997; Knight et al., 1999]. However, resveratrol can also inhibit Cox-2 by suppressing Cox-2 mRNA production, although this requires much higher levels of resveratrol than used in the present study [Subbaramaiah et al., 1998]. NS-398 was added to the cultures at doses previously shown to be effective at inhibiting Cox-2 in a number of other cell culture systems [Asano et al., 1996; Murphy et al., 1998; Laufer et al., 1999; Crew et al., 2000]. Also, in unpublished work by ourselves, NS-398 has been shown to inhibit basal PGE_2 production by 42% in growth zone chondrocytes. The experimental protocol used in this study allows us to directly compare the effects of resveratrol with those of NS-398, a well-characterized Cox-2 inhibitor [Futaki et al., 1993, 1994].

To examine the mechanisms by which Cox modulates PKC activity, confluent cultures of growth zone cells were incubated with 0.1, 1, or 10 μM Cox-1 or Cox-2 inhibitors in the presence and absence of 10^{-8} M 1α,25-$(OH)_2D_3$ for nine

minutes. Alternatively, to establish whether production of leukotriene was involved, the cells were treated with the lipoxygenase inhibitors, NDGA (2, 20, and 40 μM) and esculetin (0.1, 1, and 10 μM). Cells were also treated with 10 μM arachidonic acid in the presence or absence of cyclooxygenase inhibitors.

Expression of Cox-1 and Cox-2

RT-PCR. To determine whether growth zone chondrocytes express one or both isoforms of Cox, cultures were screened by RT-PCR. Total RNA was isolated with TRIzol reagent (Gibco-BRL). For Cox-1 and Cox-2 sequence determination, total RNA from growth zone chondrocytes was reverse transcribed with the First-strand cDNA synthesis kit (Pharmacia) using the previously described rat primers [Kaplan et al., 1997] as indicated in Table I. Reaction products were gel purified (QIAquick gel extraction kit, Qiagen, Valencia, CA) and sequenced in the Center for Advanced DNA Technologies (University of Texas Health Science Center at San Antonio, San Antonio, TX).

Northern blot analysis. To quantitate the effects of 1α,25-$(OH)_2D_3$ on mRNA levels for Cox-1 and Cox-2, we performed Northern blot analysis. Cultures were treated for 24 h with 10^{-8}M 1α,25-$(OH)_2D_3$. In addition, we verified that levels of Cox-1 mRNA did not change as a function of time and in response to 1α,25-$(OH)_2D_3$. For these experiments, cultures were harvested at 1, 6, and 12 hours. Total RNA for untreated and 1α,25-$(OH)_2D_3$-treated growth zone chondrocytes was isolated with TRIzol. Total RNA was quantitated spectrophotometrically, separated on a 1% denaturing agarose gel and transferred to a positively charged nylon membrane (Ambion, Austin, TX) with the Turboblotter System (Schleicher and Schuell, Keen, NH). Total RNA from rat kidney was obtained from Ambion and used as a positive control. Northern blots were hybridized with Cox-1, Cox-2, and GAPDH strippable [^{32}P]-labeled anticoding RNA probes using the NorthernMax Kit (Ambion, Austin, TX). Cox-1 and Cox-2 anti-cRNA probes were synthesized from sequenced RT-PCR products amplified with modified antisense primers with the T7 promoter sequence, 5′-TAA TAG GAC TCA CTA TAG GGA GG-3′, attached to the 5′ end of the antisense primers. Anticoding RNA probes were synthesized with the Strip-EZ T7 Kit (Ambion, Austin, TX). Northern blots were analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Functional Assays

To determine whether Cox-1 or Cox-2 plays a role in mediating the effects of 1α,25-$(OH)_2D_3$ on cell function, we examined the effects of specific inhibitors of cyclooxygenase activity in confluent cultures of fourth passage growth zone cells treated with 10^{-8}M 1α,25-$(OH)_2D_3$.

[^{3}H]-Thymidine incorporation. DNA synthesis was determined by measuring [^{3}H]-thymidine incorporation into trichloroacetic acid (TCA) insoluble cell precipitates as described previously [Schwartz et al., 1989]. Quiescence was induced by incubating confluent cultures for 48 h in DMEM containing 1% FBS. The medium was then replaced with DMEM containing 1% FBS alone (control), 1α,25-$(OH)_2D_3$ or 1α,25-$(OH)_2D_3$ plus cyclooxygenase inhibitors for 24 hours. Two hours prior to harvest, [^{3}H]-thymidine was added. Radioactivity in TCA-precipitable material was measured by liquid scintillation spectroscopy.

Alkaline phosphatase activity. Growth zone cells were cultured for 24 h with control medium (DMEM + 10% fetal bovine serum) or medium containing 10^{-8}M 1α,25-$(OH)_2D_3$ in the presence or absence of 10^{-8}M–10^{-7}M indomethacin (general cyclooxygenase inhibitor), 10 or 100 μM resveratrol (Cox-1 specific inhibitor), or 10 or 100 μM NS-398 (Cox-2 specific inhibitor). Cell layer lysates were prepared by a modification of a method described previously [Hale et al., 1986]. Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline (EC 3.1.3.1)] activity was measured as a function of release of *para-*

TABLE I. Nucleotide Sequences of Sense and Antisense Primers for Cox-1 and Cox-2

Isoform	Nucleotide Sequence	Primer
Cox-1	5′-TTC TGC CCT CTG TAC CCA AA-3′	Sense
	5′-AAG GAT GAG GCG AGT GGT CT-3′	Antisense
Cox-2	5′-GGT GTG AAA GGA AAT AAG GAA C-3′	Sense
	5′-GGA GGA TGG AGT TGT TGT AGA G-3′	Antisense

nitrophenol from *para*-nitrophenylphosphate at pH 10.2 [Bretaudiere and Spillman, 1984].

[^{35}S]-sulfate incorporation. To determine the role of Cox in mediating the effects of 1α,25-$(OH)_2D_3$ on extracellular matrix production, proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation as described previously [Nasatzky et al., 1994; O'Keefe et al., 1988]. Growth zone chondrocyte cultures were treated 24 h with control media (DMEM + 10% fetal bovine serum) or media containing 10^{-8}M 1α,25-$(OH)_2D_3$ in the presence or absence of 10^{-8}M–10^{-7}M indomethacin (general cyclooxygenase inhibitor), 10 or 100 μM resveratrol, or 10 or 100 μM NS-398. Four hours prior to harvest, 50 μl DMEM containing 18 μCi/ml [^{35}S]-sulfate and 0.814 mM carrier sulfate were added to each culture. At harvest, the conditioned media were removed, the cell layers (cells and matrix) collected, and the amount of [^{35}S]-sulfate incorporated determined by liquid scintillation spectrometry. The protein content of each sample was determined using the bicinchoninic acid (BCA) protein assay reagent [Smith et al., 1985] and the data expressed as disintegrations per minute per milligram protein in the cell layer.

Signal Transduction

To assess the role of cyclooxygenase in the activation of PKC by 1α,25-$(OH)_2D_3$, confluent fourth passage growth zone chondrocyte cultures were treated for nine minutes with control media, 10^{-8} M 1α,25-$(OH)_2D_3$, or 0.24 ng/ml PGE_2 in the presence or absence of 0.1, 1, or 10 μM of the Cox-1 inhibitor resveratrol or the Cox-2 inhibitor NS-398. Nine minutes was chosen as the optimal time-point for measurement of PKC activity since our previous reports have determined that 9 min is the time of maximal PKC stimulation by 1α,25-$(OH)_2D_3$ [Sylvia et al., 1993; Helm et al., 1996]. In one pair of experiments, the effects of Cox-1 and Cox-2 specific inhibitors were compared to that of the general Cox inhibitor indomethacin by inclusion of a 10^{-7} M indomethacin treatment group, also for nine minutes. PKC activity was measured in cell layer lysates as previously described [Bell et al., 1986; Sylvia et al., 1993].

Effect of arachidonic acid. To examine the effect of prostaglandin precursors on the PKC response to cyclooxygenase inhibition, growth zone chondrocyte cultures were treated with arachidonic acid. Confluent fourth passage growth zone chondrocyte cultures were treated with control media or with 10 μM or 100 μM arachidonic acid in the presence or absence of 0.1, 1, or 10 μM resveratrol or NS-398. PKC activity in cell layers was determined after 9 min of treatment. In one pair of experiments, the response to specific inhibition of Cox-1 or Cox-2 was compared to that of indomethacin by inclusion of a 10^{-7} M indomethacin treatment group, also for nine minutes.

Effect of phospholipase A_2. To examine whether PLA_2 or cyclooxygenase is the rate limiting step in the membrane-mediated response of growth zone cells to 1α,25-$(OH)_2D_3$, cultures were treated with the PLA_2 inhibitor quinacrine [Church et al., 1993] or the PLA_2 activator melittin [Habermann, 1972]. Confluent fourth passage growth zone chondrocytes were treated with control media, 10^{-8} M 1α,25-$(OH)_2D_3$, 10 μM quinacrine, or 1α,25-$(OH)_2D_3$ and quinacrine in combination with 1α,25-$(OH)_2D_3$ in the presence or absence of cyclooxygenase inhibitors. Melittin was tested in a similar manner at a final concentration of 3 μg/ml. PKC activity in cell layers was determined after 9 min of treatment.

Role of lipooxygenase. To examine whether metabolism of arachidonic acid via the lipoxygenase pathway plays a role in the response of chondrocytes to arachidonic acid, growth zone chondrocytes were treated for 9 min with control media or 10μM arachidonic acid in the presence or absence of 2, 20, or 40 μM NDGA or 0.1, 1, or 10 μM esculetin. PKC activity in cell layers was determined after 9 min of treatment.

Statistical Analysis

For each experiment, cells from the costochondral cartilages of 18 rats were pooled, expanded in culture through third passage, and then plated into the wells of 24-well plates. For each variable in each experiment, six individual culture wells were used. Each data point represents the mean ± SEM of these six individual cultures. Significance between groups was determined using Bonferroni's modification of Student's *t*-test for multiple comparisons. *P*-values < 0.05 were considered significant. Each experiment was repeated twice, and many experiments were repeated three or more times, to ensure the validity of the results. The data presented are from a single representative experiment. Data from all experiments for a particular variable are not

shown due to batch-to-batch variation in the cultures due to slight differences in culture media and confluence of the cultures. Calculation of treatment/control ratios and statistical analysis of the resulting data confirmed that the experiments shown are truly representative of the response of the cultures.

RESULTS

Expression of Cox Genes

Growth zone chondrocytes express transcripts for both Cox-1 and Cox-2 (Fig. 1). RT-PCR demonstrated amplification of a 556 bp Cox-1 cDNA fragment and a 753 bp Cox-2 cDNA fragment. Both fragments were sequenced and confirmed to be Cox-1 and Cox-2 by comparison with published sequences. In addition, reactions were run in the presence and absence of reverse transcriptase to eliminate the possibility of amplifying genomic DNA; no genomic DNA was found contaminating the RNA preparation. Significantly higher levels of Cox-1 were evident when bands were normalized to background and GAPDH levels.

Northern blot analysis also showed expression of Cox-1 and Cox-2 mRNA in the chondrocytes (Fig. 2). Cox-1 and Cox-2-specific probes hybridized to the 1.9 kb Cox-1 mRNA and the 4.2 kb Cox-2 mRNA from control and 1α,25-$(OH)_2D_3$-treated growth zone chondrocyte cultures. Significantly higher levels of Cox-1 compared to Cox-2 were evident when the bands were normalized to background and GAPDH levels. There was no significant alteration in Cox-1 or Cox-2 mRNA levels in growth zone cells treated with 1α,25-$(OH)_2D_3$ for 24 hours (Fig. 2). In addition, Cox-1 mRNA levels were unchanged when the cultures were treated for 1, 6, or 12 hours (Fig. 3).

Role of Cyclooxygenase in Mediating Response to 1α,25-$(OH)_2D_3$

[^{3}H]-thymidine incorporation. 1α,25-$(OH)_2D_3$ significantly decreased [^{3}H]-thymidine incorporation in growth zone chondrocyte cultures (Fig. 4). Indomethacin, as well as the Cox-1 and Cox-2 specific inhibitors, decreased [^{3}H]-thymidine incorporation in control cul-

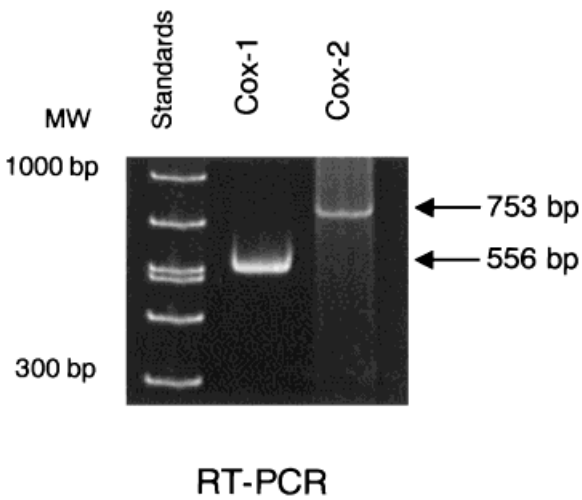

Fig. 1. RT-PCR analysis of Cox-1 and Cox-2 expression in growth zone chondrocytes. Total RNA was extracted from confluent, fourth passage growth zone chondrocytes. The expected 556 bp Cox-1 and 753 bp Cox-2 cDNA fragments were amplified from the total RNA and separated by 5% polyacrylamide gel electrophoresis. The Cox-1 band was a reamplification of an RT-PCR product after gel extraction and purification. The Cox-2 band is the original RT-PCR product.

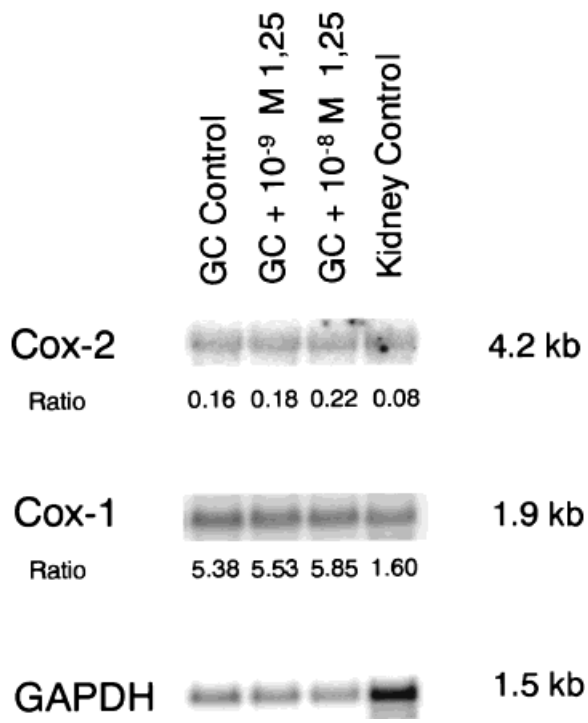

Fig. 2. Northern blot analysis of Cox-1 and Cox-2 expression in growth zone chondrocytes. 5 μg total RNA was extracted from confluent, fourth passage growth zone chondrocytes (GC), separated on a denaturing agarose gel, transferred to an Ambion BrightStar positively-charged membrane, and then hybridized with [α^{32}P-UTP]-labelled Ambion Strip-Eze RNA probes. Cultures were treated with control media or media containing 10^{-9} M or 10^{-8} M 1α,25-$(OH)_2D_3$ for 24 h prior to RNA extraction. Expression of Cox mRNA was normalized to mRNA levels for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Rat kidney total RNA was used as a positive control.

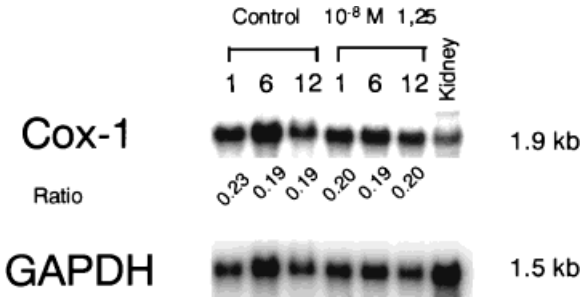

Fig. 3. Northern blot analysis of Cox-1 expression in growth zone chondrocytes treated with 10^{-8} M 1α,25-$(OH)_2D_3$ for 1, 6, or 12 hours. 5 μg total RNA was extracted from confluent, fourth passage growth zone chondrocytes, separated on a denaturing agarose gel, transferred to an Ambion BrightStar positively charged membrane, and then hybrized with [α^{32}P-UTP]-labeled Ambion Strip-Eze RNA probes. Expression of Cox-1 mRNA was normalized to mRNA levels glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Rat kidney total RNA was used as a positive control.

tures to levels seen in the 1α,25-$(OH)_2D_3$-treated cultures.

Alkaline phosphatase. The 1α,25-$(OH)_2D_3$-dependent increase in alkaline phosphatase was sensitive to inhibitors of cyclooxygenase activity (Fig. 5). 1α,25-$(OH)_2D_3$ increased alkaline phosphatase specific activity in growth zone chondrocytes as expected. Indomethacin caused a significant decrease in enzyme activity in both the control and 1α,25-$(OH)_2D_3$-treated cultures. After treatment with indomethacin, enzyme activity was similar in both the control and 1α,25-$(OH)_2D_3$-treated groups. Inhibition of Cox-1 with resveratrol also decreased enzyme activity to levels which were significantly less than the controls, and maximal effects were noted in cultures treated with 10 μM resveratrol. No further decrease in either the control or 1α,25-$(OH)_2D_3$-treated cultures was seen at 100 μM resveratrol. Inhibition of Cox-2 with NS-398 had no effect on alkaline phosphatase whether the cells were treated with 1α,25-$(OH)_2D_3$ or not.

[^{35}S]-sulfate incorporation. 1α,25-$(OH)_2D_3$-dependent increases in proteoglycan sulfation were sensitive to inhibition of cyclooxygenase activity (Fig. 6). Indomethacin reduced [^{35}S]-sulfate incorporation in control growth zone cells and blocked the stimulatory effect of 1α,25-$(OH)_2D_3$ on proteoglycan sulfation. The Cox-1 inhibitor, resveratrol, had no significant effect on [^{35}S]-sulfate incorporation in control cultures, but blocked the 1α,25-$(OH)_2D_3$-dependent increase in [^{35}S]-sulfate incorporation by

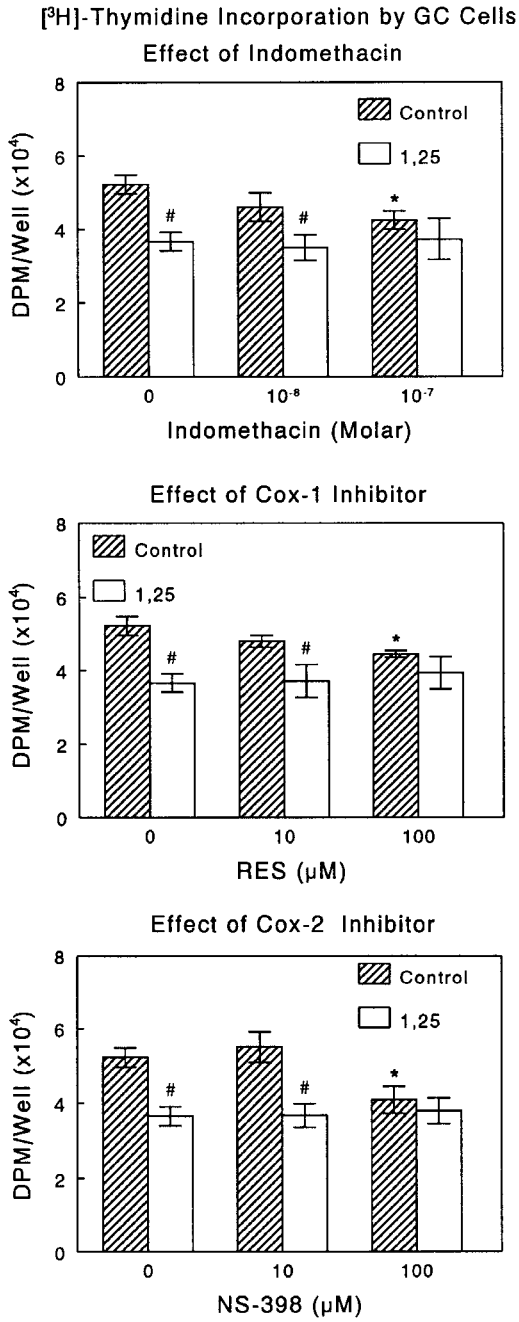

Fig. 4. Effect of Cox inhibition on 1α,25-$(OH)_2D_3$-induced [^{3}H]-thymidine incorporation by confluent, fourth passage growth zone chondrocytes. Cells were treated for 24 h with varying concentrations of indomethacin (top panel), Cox-1 inhibitor resveratrol (RES, middle panel) or Cox-2 inhibitor NS-398 (bottom panel) in the presence or absence of 10^{-8} M 1α,25-$(OH)_2D_3$ and then assayed for [^{3}H]-thymidine incorporation as described in the Methods. Each bar is the mean ± SEM of six cultures from one representative experiment; the experiment was repeated three separate times, all with comparable results. #$P < 0.05$, treatment vs. control; *$P < 0.05$, inhibitor vs. no inhibitor.

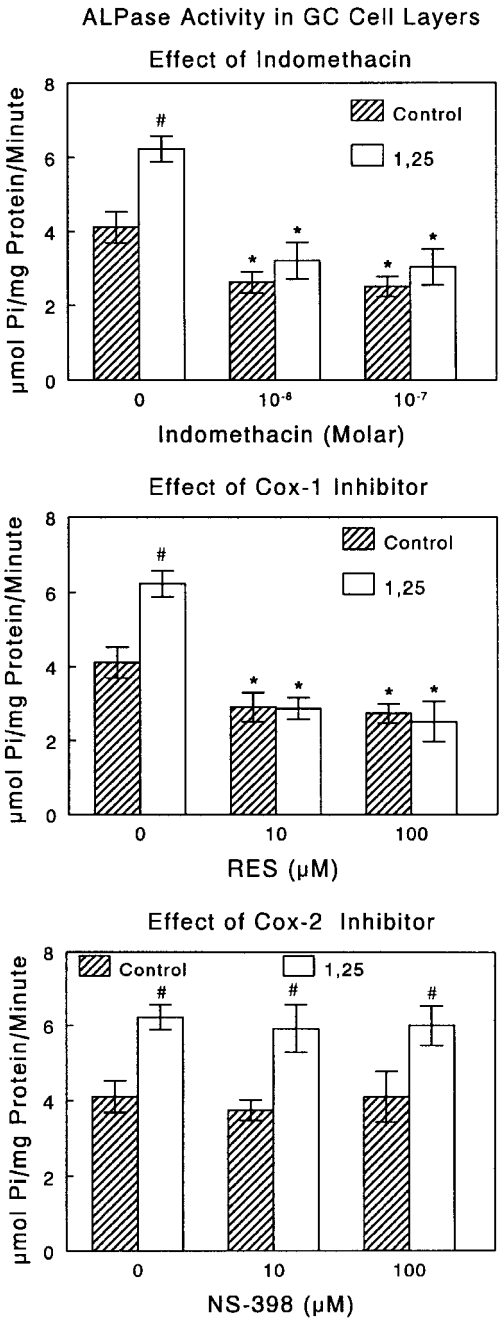

Fig. 5. Effect of Cox inhibition on 1α,25-$(OH)_2D_3$-induced alkaline phosphatase (ALPase) specific activity of confluent, fourth passage growth zone chondrocyte cell layers. Cells were treated for 24 h with varying concentrations of indomethacin (top panel), Cox-1 inhibitor resveratrol (RES, middle panel) or Cox-2 inhibitor NS-398 (bottom panel) in the presence or absence of 10^{-8} M 1α,25-$(OH)_2D_3$ and then assayed for alkaline phosphatase specific activity as described in the Methods. Each bar is the mean ± SEM of six cultures from one representative experiment; the experiment was repeated three separate times, all with comparable results. #$P < 0.05$, treatment vs. control; *$P < 0.05$, inhibitor vs. no inhibitor.

the cultures. The Cox-2 inhibitor, NS-398, had no effect on basal or 1α,25-$(OH)_2D_3$-stimulated incorporation of [^{35}S]-sulfate by growth zone chondrocyte cultures.

Signal Transduction

Protein kinase C. Both 1α,25-$(OH)_2D_3$ and prostaglandin E_2 (PGE_2) significantly increased PKC activity in growth zone chondrocyte cultures (Fig. 7). Inhibition of Cox-1 with resveratrol had a small inhibitory effect on basal PKC activity that was significant in some, but not all, experiments (Fig. 7A). The inhibitor had no effect on PKC activity in cultures treated with PGE_2. However, resveratrol caused a dose-dependent decrease in PKC activity, significant at 0.1, 1, and 10 μM, in cultures treated with 10^{-8} M 1α,25-$(OH)_2D_3$. In contrast, inhibition of Cox-2 with NS-398 had no effect on basal PKC activity or on activity in PGE_2-treated or 1α,25-$(OH)_2D_3$-treated cultures (Fig. 7B). The Cox-1 inhibitor resveratrol, at a final concentration of 10 μM, reduced PKC activity of growth zone cells to a level comparable with that seen in cultures treated with 10^{-7} M indomethacin and reduced PKC of 1α,25-$(OH)_2D_3$-treated cultures to that of controls (Fig. 8A). In contrast, the Cox-2 inhibitor, NS-398, did not display the same inhibitory effect seen with indomethacin (Fig. 8B).

Some of the effect of arachidonic acid on PKC activity is due to cyclooxygenase mediated prostaglandin production. Arachidonic acid increased PKC activity of growth zone cells, and inhibition of Cox-1 by resveratrol enhanced the stimulatory effect of arachidonic acid on PKC (Fig. 9A). In contrast, NS-398 had no effect on basal PKC nor on the arachidonic acid-dependent stimulation of the enzyme (Fig. 9B).

Role of Phospholipase A_2

PLA_2 is a rate-limiting step in the action of 1α,25-$(OH)_2D_3$ on PKC. Inhibition of PLA_2 with quinacrine caused a decrease in basal PKC, and this decrease was also evident in cultures treated with 1α,25-$(OH)_2D_3$ (Fig. 10A). Inhibition of Cox-1 with resveratrol resulted in a further decrease in PKC activity. In contrast, inhibition of Cox-2 had no effect (Fig. 10B).

Activation of PLA_2 with melittin stimulated basal PKC activity and enhanced the 1α,25-$(OH)_2D_3$-stimulated increase in enzyme activity (Fig. 11A). Inhibition of Cox-1 with 10 μM resveratrol did not alter the effect of melittin,

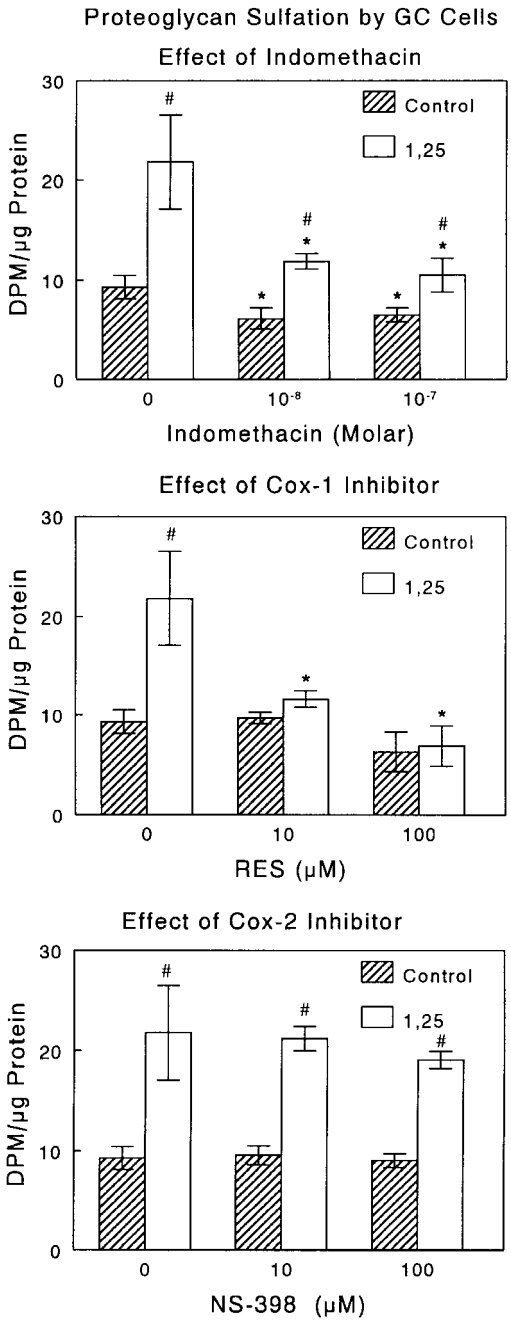

Fig. 6. Effect of Cox inhibition on 1α,25-$(OH)_2D_3$-induced proteoglycan sulfation of confluent, fourth passage growth zone chondrocyte cell layers. Cells were treated for 24 h with varying concentrations of indomethacin (top panel), Cox-1 inhibitor resveratrol (RES, middle panel) or Cox-2 inhibitor NS-398 (bottom panel) in the presence or absence of 10^{-8} M 1α,25-$(OH)_2D_3$ and then assayed for [^{35}S]-sulfate incorporation as described in the Materials and Methods. Each bar is the mean± SEM of six cultures from one representative experiment; the experiment was repeated three separate times, all with comparable results. #$P < 0.05$, treatment vs. control; *$P < 0.05$, inhibitor vs. no inhibitor.

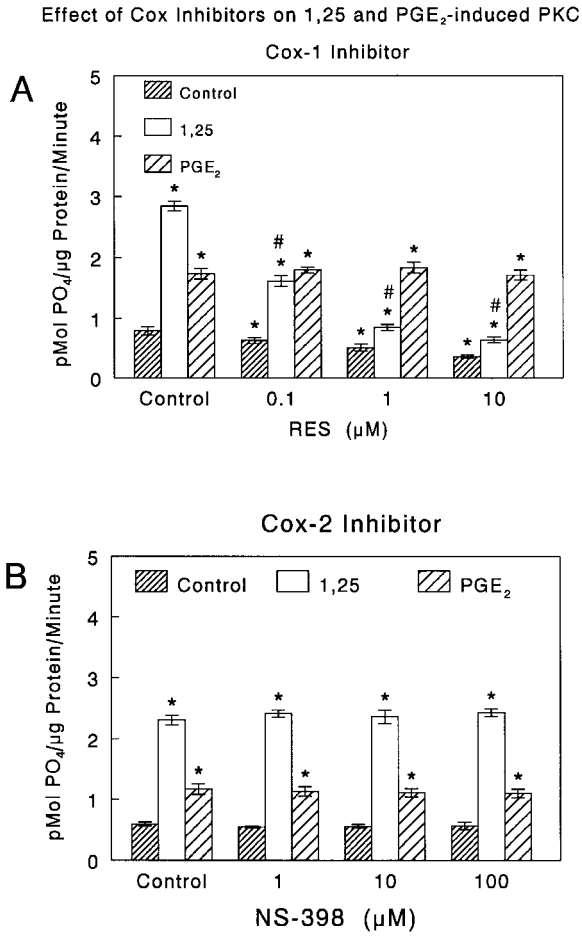

Fig. 7. Effect of Cox inhibitors on 1α,25-$(OH)_2D_3$- and PGE_2-induced protein kinase C (PKC) activity in growth zone chondrocytes. Confluent, fourth passage growth zone chondrocyte cultures were treated with control media, 10^{-8} M 1α,25-$(OH)_2D_3$, or 0.24 ng/ml PGE_2 in the presence or absence of 0.1, 1, or 10 µM of the Cox-1 inhibitor resveratrol (RES; **Panel A**) or the Cox-2 inhibitor NS-398 (**Panel B**) for nine minutes. At harvest, the cell layers were assayed for PKC specific activity. Each bar is the mean±SEM of six cultures from one representative experiment; the experiment was repeated three separate time, all with comparable results. *$P < 0.05$, treatment vs. control; #$P < 0.05$, Cox inhibitor vs. no inhibitor.

but 100 µM resveratrol brought PKC back to control levels. However, when resveratrol was added to the cells together with 1α,25-$(OH)_2D_3$, the melittin-dependent stimulation of PKC activity was reduced to below that of 1α,25-$(OH)_2D_3$ alone. Inhibition of Cox-2 had no effect on basal PKC, melittin-stimulated PKC, or PKC in cells treated with melittin and 1α,25-$(OH)_2D_3$ (Fig. 11B).

Role of Lipoxygenase

The effect of 1α,25-$(OH)_2D_3$ and arachidonic acid on PKC activity does not involve leuko-

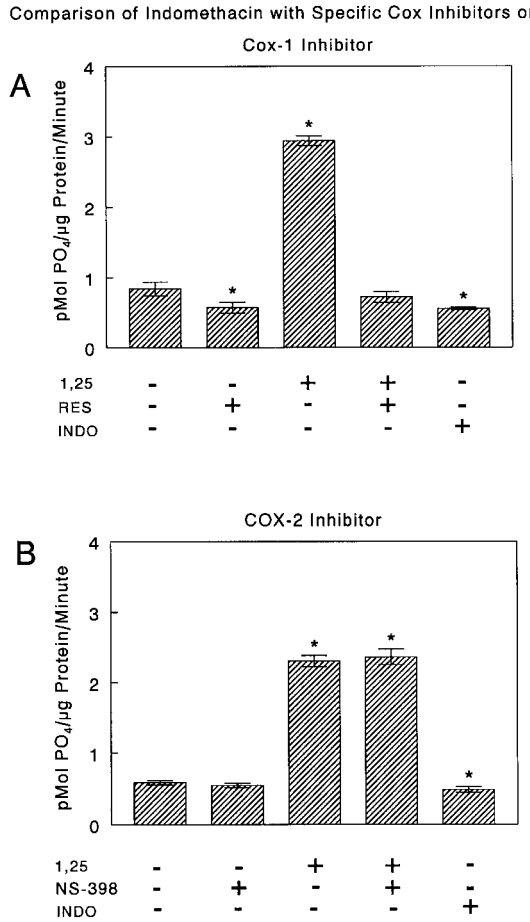

Fig. 8. Comparison of the effect of specific Cox-1 and Cox-2 inhibitors with a general Cox inhibitor on activation of protein kinase C (PKC) activity in growth zone chondrocytes treated with 1α,25-$(OH)_2D_3$. Confluent, fourth passage growth zone chondrocyte cultures were treated with control media, 10^{-8} M 1α,25-$(OH)_2D_3$ or 10^{-7} M indomethacin in the presence or absence of the Cox-1 inhibitor resveratrol (RES, **Panel A**) or the Cox-2 inhibitor NS-398 (**Panel B**) at a final concentration of 10 µM for nine minutes. At harvest, the cell layers were assayed for PKC specific activity. Each bar is the mean±SEM of six cultures from one representative experiment; the experiment was repeated three separate times, all with comparable results. $^*P < 0.05$, treatment vs. control.

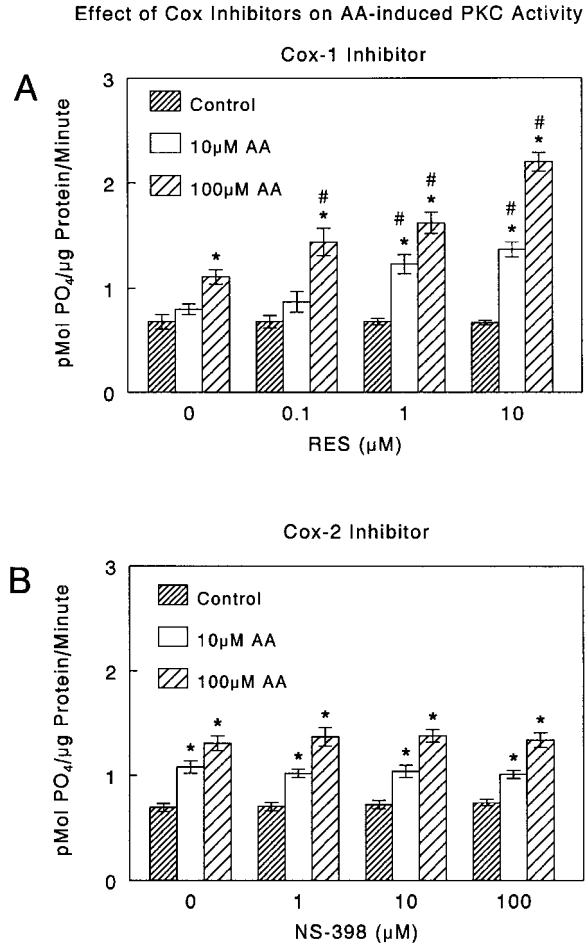

Fig. 9. Effect of Cox inhibitors on arachidonic acid- (AA) induced protein kinase C (PKC) activity in growth zone chondrocytes. Confluent, fourth passage growth zone chondrocytes were treated with control media, 10 µM AA or 100 µM AA in the presence or absence of 0.1, 1 or 10µM resveratrol (RES, **Panel A**) or 1, 10, or 100 µM NS-398 (**Panel B**) for nine minutes. At harvest, the cell layers were assayed for PKC specific activity. Each bar is the mean±SEM of six cultures from one representative experiment; the experiment was repeated three separate times, all with comparable results. $^*P < 0.05$, treatment vs. control; $\#P < 0.05$, Cox inhibitor vs. no inhibitor.

trienes (Table II). Neither NDGA nor esculetin, both inhibitors of lipoxygenase, had any significant effect on PKC activity in control cultures or cultures treated with 1α,25-$(OH)_2D_3$. When 10 µM arachidonic acid was added to the cultures in the presence or absence of the same lipoxygenase inhibitor, no effect on PKC activity was observed.

DISCUSSION

We had previously shown that the effects of 1α,25-$(OH)_2D_3$ on growth zone chondrocytes are mediated in part through modulation of prostaglandin production [Schwartz et al., 1992]. This suggested that cyclooxygenase might play a role. Here we show that growth zone chondrocytes express both Cox-1 and Cox-2 but it is primarily the Cox-1 isoform that participates in the 1α,25-$(OH)_2D_3$ dependent regulation of cell proliferation, alkaline phosphatase, and proteoglycan sulfation. These results confirm our earlier observations using a general inhibitor of cyclooxygenase, indomethacin. The lack of an effect of the Cox-2 inhibitor NS-398, except on cell proliferation, supports this conclusion.

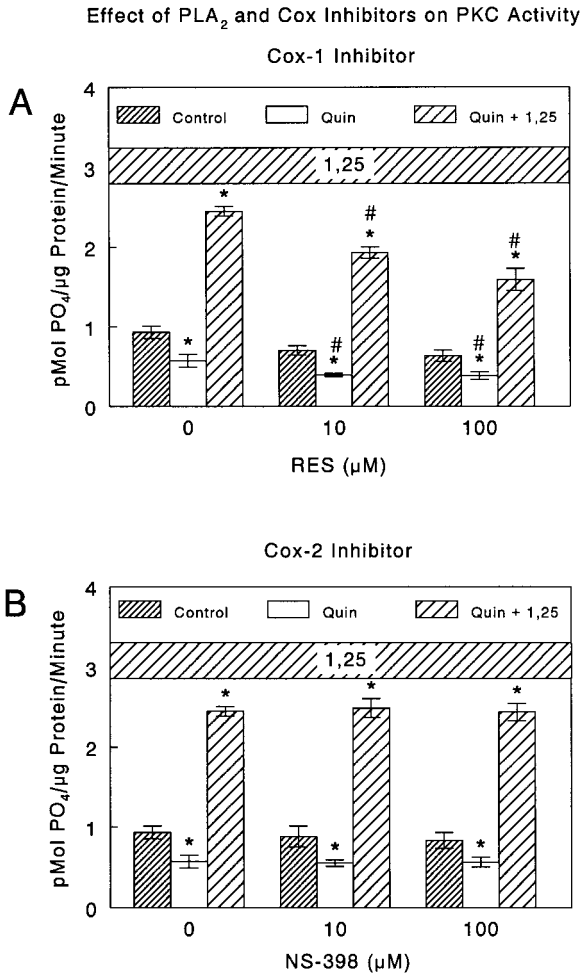

Fig. 10. Effect of phospholipase A_2 (PLA_2) and Cox inhibitors on protein kinase C (PKC) activity in growth zone chondrocytes. Confluent, fourth passage growth zone chondrocytes were treated with control media, 10^{-8} M 1α,25-$(OH)_2D_3$ (1,25), 10 µM PLA_2 inhibitor quinacrine (Quin), or 1α,25-$(OH)_2D_3$ and quinacrine together, in the presence or absence of 10 or 100 µM resveratrol (RES, **Panel A**) or NS-398 (**Panel B**) for nine minutes. At harvest, the cell layers were assayed for PKC specific activity. Each bar is the mean±SEM of six cultures from one representative experiment; the experiment was repeated three separate times, all with comparable results. The 1,25 band is the mean ± SEM for the effect of this vitamin D metabolite alone. *$P < 0.05$, treatment vs.control; #$P < 0.05$, Cox inhibitor vs. no inhibitor.

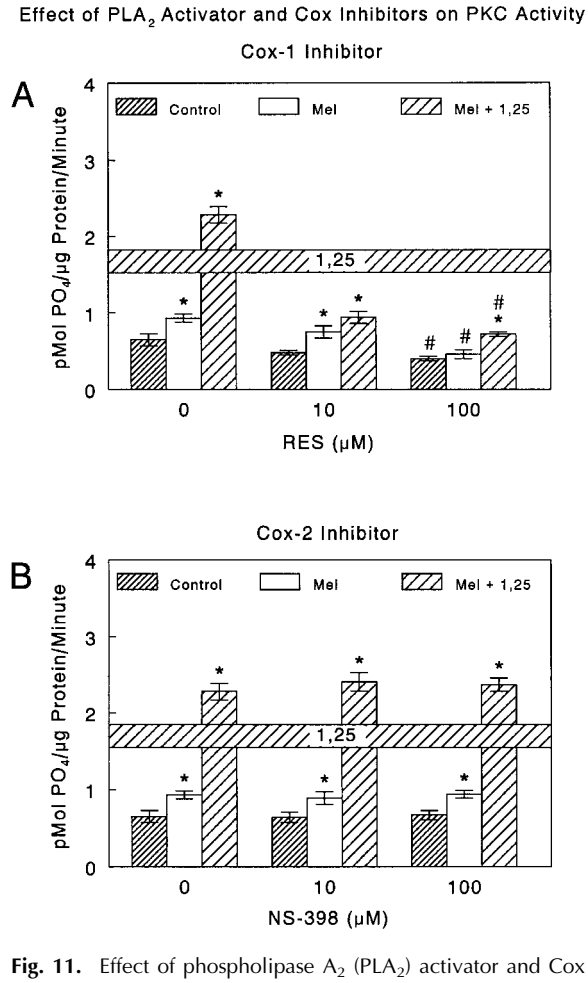

Fig. 11. Effect of phospholipase A_2 (PLA_2) activator and Cox inhibitors on protein kinase C (PKC) activity in growth zone chondrocytes. Confluent, fourth passage growth zone chondrocytes were treated with control media, 10^{-8} M 1α,25-$(OH)_2D_3$ (1,25), 10 µM PLA_2 activator melittin (Mel), or 1α,25-$(OH)_2D_3$ and melittin together, in the presence or absence of 10 or 100 µM resveratrol (RES, **Panel A**) or NS-398 (**Panel B**) for nine minutes. At harvest, the cell layers were assayed for PKC specific activity. Each bar is the mean±SEM of six cultures from one representative experiment; the experiment was repeated three times, all with comparable results. The 1,25 band is the mean ± SEM for the effect of this vitamin D metabolite alone. *$P < 0.05$, treatment vs.control; #$P < 0.05$, Cox inhibitor vs. no inhibitor; ($P < 0.05$, treatment vs. 1α,25-$(OH)_2D_3$ alone.

Moreover, it is only Cox-1 that participates in the 1α,25-$(OH)_2D_3$ dependent regulation of its major signal transduction pathways via PKC and PLA_2. If resveratrol exerted its inhibitory effects via Cox-2 [Subbaramaiah et al., 1998], this would have been evident in cultures treated with NS-398, which was not the case.

Cox-1 is by definition a constitutive enzyme and, in other tissues, it is unregulated by hormones or other factors [Vane et al., 1998]. In contrast, Cox-2 is inducible, suggesting that if cyclooxygenase did participate in the mechanism of 1α,25-$(OH)_2D_3$ action as a rate-limiting step, it would be via Cox-2. However, 1α,25-$(OH)_2D_3$ had no effect on gene expression for either enzyme. Moreover, inhibition of Cox-2 had no effect on any of the parameters examined in this study except cell proliferation. The possibility that the Cox-2 inhibitor, NS-398, failed to penetrate the cells was also unlikely,

TABLE II. The Effect of Lipoxygenase Inhibitors on 1α,25-$(OH)_2D_3$ and Arachidonic Acid Regulation of Protein Kinase C Activity in Growth Zone Chondrocytes

Treatment	Protein Kinase C Specific Activity(pmol Pi/μg protein/minute)		
NDGA(μM)	Control	1α,25-$(OH)_2D_3$	Arachidonic Acid
0.0	0.88±0.07	2.37±0.11*	1.56±0.06*
0.1	0.80±0.04	2.40±0.12*	1.60±0.09*
1.0	0.79±0.04	2.44±0.13*	1.59±0.09*
10	0.81±0.05	2.42±0.08*	1.58±0.06*
Esculetin(μM)	Control	1,25-$(OH)_2D_3$	Arachidonic Acid
0.0	0.96±0.02	2.37±0.11*	1.56±0.06*
0.1	0.86±0.07	2.25±0.16*	1.51±0.08*
1.0	0.83±0.07	2.28±0.07*	1.56±0.05*
10	0.85±0.05	2.37±0.04*	1.58±0.08*

Confluent, fourth passage growth zone chondrocyte cultures were treated with control media, 10^{-8} M 1α,25-$(OH)_2D_3$, or 10 μM arachidonic acid in the presence or absence of 2, 20, or 40 μM NDGA or 0.1, 1 or 10 μM esculetin for nine minutes. At harvest, the cell layers were assayed for PKC specific activity. Data represent the mean ± SEM of six independent cultures.
*$P < 0.05$, treatment vs. control.

since preliminary studies indicate that NS-398 reduces basal PGE_2 production by 42% in growth zone cells (unpublished data). In contrast, inhibition of Cox-1 activity had profound effects on response to 1α,25-$(OH)_2D_3$, although the results do not support a role for Cox-1 as a rate-limiting factor.

While the mRNA for Cox-1 was not modulated by 1α,25-$(OH)_2D_3$, it is not clear that the activity of this constitutively expressed enzyme was unaffected. The decrease in PKC activity in cultures treated with resveratrol and 1α,25-$(OH)_2D_3$ demonstrates a role for Cox-1 in 1α,25-$(OH)_2D_3$ regulation of PKC activity. These observations suggest that the point of regulation in the mechanism of 1α,25-$(OH)_2D_3$ stimulation of PKC is PLA_2, which controls the availability of substrate for Cox-1. This hypothesis is supported by the fact that Cox-1 modulates the effects of PLA_2 activation or inhibition on PKC activity, both in the presence and in the absence of 1α,25-$(OH)_2D_3$. Moreover, the effects of arachidonic acid are not modulated by inhibition of lipoxygenase, making it unlikely that leukotrienes play a role in the mechanism of 1α,25-$(OH)_2D_3$ action.

Arachidonic acid released by PLA_2 from membrane phospholipids may also modulate 1α,25-$(OH)_2D_3$ effects by PPAR-dependent regulation of gene expression [Keller et al., 1993; Bocos et al., 1995; Tessier-Prigent et al., 1999]. Interestingly, the lack of an effect of leukotriene inhibition on 1α,25-$(OH)_2D_3$-stimulated PKC implicates PPARγ, and not PPARα, in the rapid response of this enzyme to the vitamin D metabolite. Activation of PPARα by the leukotriene LTB4 has been shown to stimulate Cox-2 mRNA production [Bonazzi et al., 2000], but 1α,25-$(OH)_2D_3$ had no effect on Cox-2 mRNA, and inhibition of Cox-2 had no effect on cell responses to 1α,25-$(OH)_2D_3$, other than [^{3}H]-thymidine incorporation. Arachidonic acid does bind PPARα [Lin et al., 1999], however, so a contribution of this receptor to the physiological responses of the cells to 1α,25-$(OH)_2D_3$ cannot be ruled out.

In this study, we measured the effects of 1α,25-$(OH)_2D_3$ or arachidonic acid on PKC activity at 9 min and effects of 1α,25-$(OH)_2D_3$ on phenotypic expression at 24 hours. Because of the time course involved, it could not be determined if Cox-1 was playing a role at multiple points in the signal transduction cascade. By decreasing the amount of arachidonic acid, Cox-1 limits the stimulatory effect of this fatty acid on PKC activity, while at the same time catalyzing the key step in prostaglandin production. Thus, Cox-1 may contribute to the rapid increase in PKC that occurs in response to 1α,25-$(OH)_2D_3$ activation of the 1α,25-$(OH)_2D_3$ membrane vitamin D receptor by metabolizing arachidonic acid released by PLA_2 over the first 5 min [Schwartz and Boyan, 1988] and at the same time contributing to the downregulation of the rapid response. The PGE_2 produced as a consequence of the action of Cox-1 can then act through its EP-1 receptor [Del Toro et al., 2000] to amplify the rapid effects of 1α,25-$(OH)_2D_3$ on PKC. 1α,25-$(OH)_2D_3$ also induces new PKC expression and incorporation of PKCα into plasma membranes and PKCξ into matrix vesicles within 24 h of exposure to the hormone [Sylvia et al., 1996]. Others have observed that 1α,25-$(OH)_2D_3$ causes an increase

in PKCα activity in the plasma membranes of kidney cells, due in part to translocation of cytosolic PKCα [Simboli-Campbell et al., 1994]. In addition, PKCβ translocates to the nuclear membrane. Both of these effects in kidney cells are evident at 24 h, consistent with a nuclear vitamin D receptor-mediated mechanism. Arachidonic acid may contribute to these delayed effects of 1α,25-$(OH)_2D_3$ by promoting new gene expression. The continuous action of Cox-1 on arachidonic acid may contribute to the long-term control of PKC production through down-regulation of arachidonic acid and production of prostaglandin.

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