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INTERLEUKIN-1β STIMULATES PHOSPHOLIPASE A_2 mRNA SYNTHESIS IN RABBIT ARTICULAR CHONDROCYTES

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SUMMARY: A cDNA that codes for human rheumatoid synovial fluid phospholipase A_2 (PLA_2) hybridized with a RNA of the same size (900 base pairs) isolated from rabbit articular chondrocytes (RAC). Treatment of RAC with 100 ng/ml recombinant human interleukin-1β (IL-1) for 24 hours caused a 13-fold increase in mRNA for this PLA_2. Timecourse studies demonstrated that maximal induction of PLA_2 mRNA occurred by 16 hours post addition of IL-1 (100 ng/ml). Augmentation of RAC PLA_2 mRNA levels was dose-dependent; half-maximal induction was estimated to require 0.15 ng/ml IL-1. Actinomycin D inhibited IL-1 effects on PLA_2 mRNA levels. Coordinate effects of IL-1 on RAC PLA_2 activity were observed with respect to time and dose dependence as well as actinomycin D sensitivity.

Numerous findings have led to the hypothesis that many of the pro-inflammatory effects of IL-1 on synovial tissue may be mediated in part by its effects on cellular arachidonate metabolism. Synovial fluid from rheumatoid arthritis patients contains high levels of PLA_2 as well as IL-1 (1,2). Studies using cultured human synovial fibroblasts and rabbit articular chondrocytes (RAC) have shown that IL-1 stimulates the level of PLA_2 activity associated with and secreted by these cells as well as their synthesis of prostaglandin E_2 (3,4). The mechanism of IL-1 induction of PLA_2 activity, however, remains to be elucidated. The data presented herein provide the first evidence that RAC transcribe a gene encoding a PLA_2 of the rheumatoid arthritic synovial fluid type and that their steady-state mRNA levels for this PLA_2 are modulated by IL-1.

MATERIALS AND METHODS

Chondrocyte Culture: Articular cartilage was dissected from the knee joints of male New Zealand White rabbits and chondrocytes were isolated by

ABBREVIATIONS: RAC, rabbit articular chondrocytes; RSF, rheumatoid synovial fluid; PLA_2, phospholipase A_2; IL-1, human interleukin-1β; SCC, standard saline citrate; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PC, phosphatidylcholine.

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sequential enzymatic digestion with trypsin, hyaluronidase and collagenase as described previously (5). The cells were grown in Ham's F-12 media containing 10% fetal bovine serum, 2 mM glutamine, 110 ug/ml sodium pyruvate, 100 uM nonessential amino acids, and 1X antibiotics (100 units/ml penicillin, 100 ug/ml streptomycin) at 37°C in 5% CO_2 in air. Culture medium was changed every 2-3 days.

Chondrocyte Activation: Confluent primary chondrocytes were washed twice with Neuman Tytell serumless media containing 1X antibiotics and 0.25 ug/ml amphotericin B. The cells were then incubated with the same media with or without IL-1. The IL-1 used was a soluble, fully-active recombinant human IL-1β produced as previously described (6). One unit was defined as the amount of IL-1 that generated half-maximal activity in the thymocyte proliferation assay. The specific activity was 1 x 10^7 units/mg protein. For actinomycin D experiments, the cultures were treated with 200 ng/ml of the inhibitor concomitant with IL-1 stimulation.

RNA Preparation: At the end of the culture period, the test media were removed, the monolayers washed twice and the cells dissociated with 0.1% trypsin in PBS. Isolated cells were counted by hemocytometer. Total cellular RNA was prepared by the guanidinium isothiocyanate/cesium chloride method as previously described (7).

RNA Blot Hybridization: For Northern analysis, total cellular RNA samples were size-fractionated by electrophoresis through a 1.2% agarose gel containing formaldehyde and transferred to nitrocellulose (7). For quantitative analyses, slot blotting was performed using the procedure and Minifold II apparatus of Schleicher and Schuell, Inc. (8). The filters were then baked at 80°C for 2 hours. Pre-hybridizations were conducted at 42° C overnight. Hybridizations were conducted with 2 x 10^6 cpm/ml (10 ng/ml) of heat-denatured [^{32}P]-nick-translated DNA probes in 50% formamide, 5X SCC (1X SSC: 0.15M sodium chloride, 0.015M sodium citrate, pH 7.0), 1X Denhardt's solution (1X Denhardt's: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin) (9), 0.2% SDS, 5 mM EDTA, 10 mg/ml sheared salmon sperm DNA, 10 mg/ml yeast RNA and 10 ug/ml poly (A)n at 42° C overnight. The filters were washed twice in 2X SSC containing 1% SDS for 15 min at room temperature and then again at at 50°C. The final wash was performed in 0.2X SSC with 1% SDS at 50°C for 30 min. The filters were exposed to Kodak XAR-5 film with a DuPont Cronex Lightning Plus intensifying screen at -70°C for varying lengths of time. Radioactivity in the probes hybridizing to the RNA on the filters was quantitated by densitometric scanning of the autoradiograms. The levels of radioactivity were linear with respect to the total RNA applied to the filters. Subsequent hybridizations of the filters with a cDNA nick-translated probe specific for β-actin verified that equal amounts of RNA from control and IL-1-treated RAC were present on the filters.

cDNA Probes: A full-length cDNA encoding a transcript identical to that of synovial fluid PLA_2 (10,11) was isolated from a human placental cDNA library (G.L. Davis, manuscript submitted) using oligonucleotide probes based upon back translations of the amino-terminal amino acid sequence data (12) for the human synovial fluid (13) and rat platelet (14) PLA_2. The 821 bp insert isolated from lambda gt11 and subcloned into a plasmid vector was excised and gel purified prior to labeling. A gel-purified 2Kb DNA fragment encoding human β-actin was utilized to verify that equal amounts of RNA from control and IL-1-treated RAC were present on the filters. The DNAs were radiolabeled with [^{32}P]dCTP to a specific activity of 4 x 10^8 dpm/ug by nick-translation using a commercial kit (Promega, Madison WI).

PLA_2 Assay: PLA_2 activity in medium from control and IL-1-treated RAC was assessed using a phosphatidylcholine/sodium deoxycholate mixed-micelle assay. Samples were incubated for 2.5 h at 37°C in 25 mM Tris-HCl (pH 7.5) containing 75 mM sodium chloride, 2.5 mM calcium chloride, 0.02% deoxycholate and 4 uM phosphatidylcholine (PC) labeled with [^{14}C]-arachidonic acid at SN-2 (54 mCi/mmole, New England Nuclear, Boston, MA). Product was separated from unreacted substrate using silica gel columns as described previously (15).

RESULTS

To determine whether the IL-1-induced activation of PLA_2 in RAC is associated with greater levels of PLA_2 mRNA, equal amounts of total RNA from control cultures and RAC treated for 24 hours with 100 ng/ml IL-1 were probed by Northern analysis. As shown in Figure 1A, IL-1-treated RAC contained dramatically increased amounts of rheumatoid synovial fluid (RSF) PLA_2 transcript. Hybridization with a cDNA specific for actin showed no difference in the levels of actin message as a consequence of IL-1 treatment (Figure 1B). Thus, the effect of IL-1 on RSF PLA_2 transcript levels is specific. The size of the PLA_2 message was estimated to be 900 base pairs by comparison to size markers. RSF PLA_2 transcripts of similar size have recently been shown to be present in RNA from synovial tissue and peritoneal exudate cells (10) and other tissues (G. L. Davis, manuscript submitted).

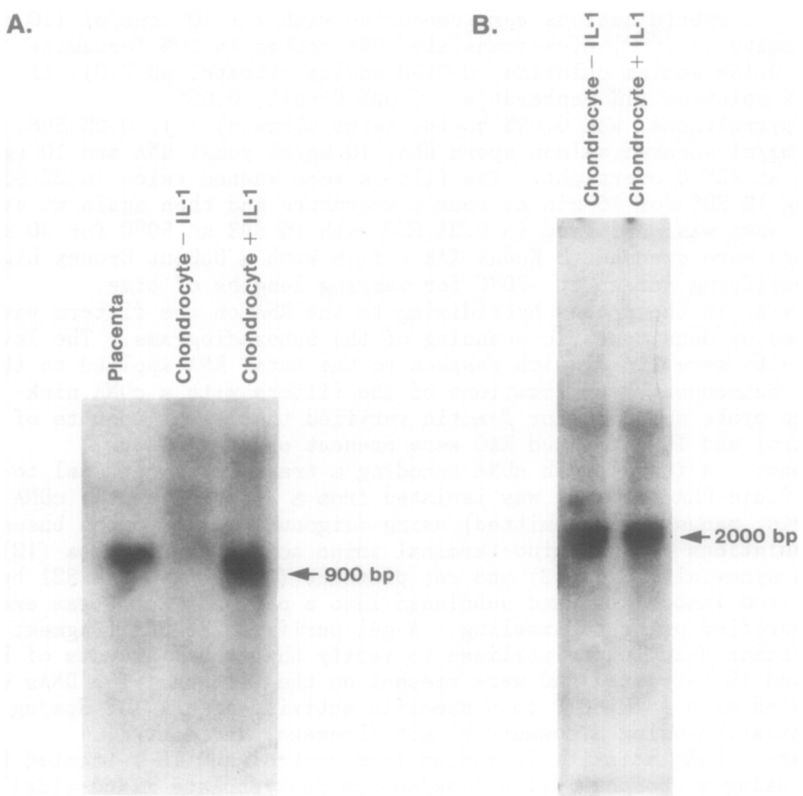

FIGURE 1. The effect of IL-1 on PLA_2 and actin mRNA levels in RAC. Ten ug of total cellular RNA from RAC incubated for 24 h with control media or IL-1 (100 ng/ml) was analyzed by Northern hybridization using a [^{32}P]-labeled cDNA specific for RSF PLA_2 (A) or for β-actin (B). Also shown for the purpose of a positive control are the results of hybridizations to 1 ug of poly $(A)^+$ RNA from placenta.

The concentration dependence of IL-1 induction of RSF PLA_2 mRNA was evaluated by slot blot hybridization of total RNA prepared from control

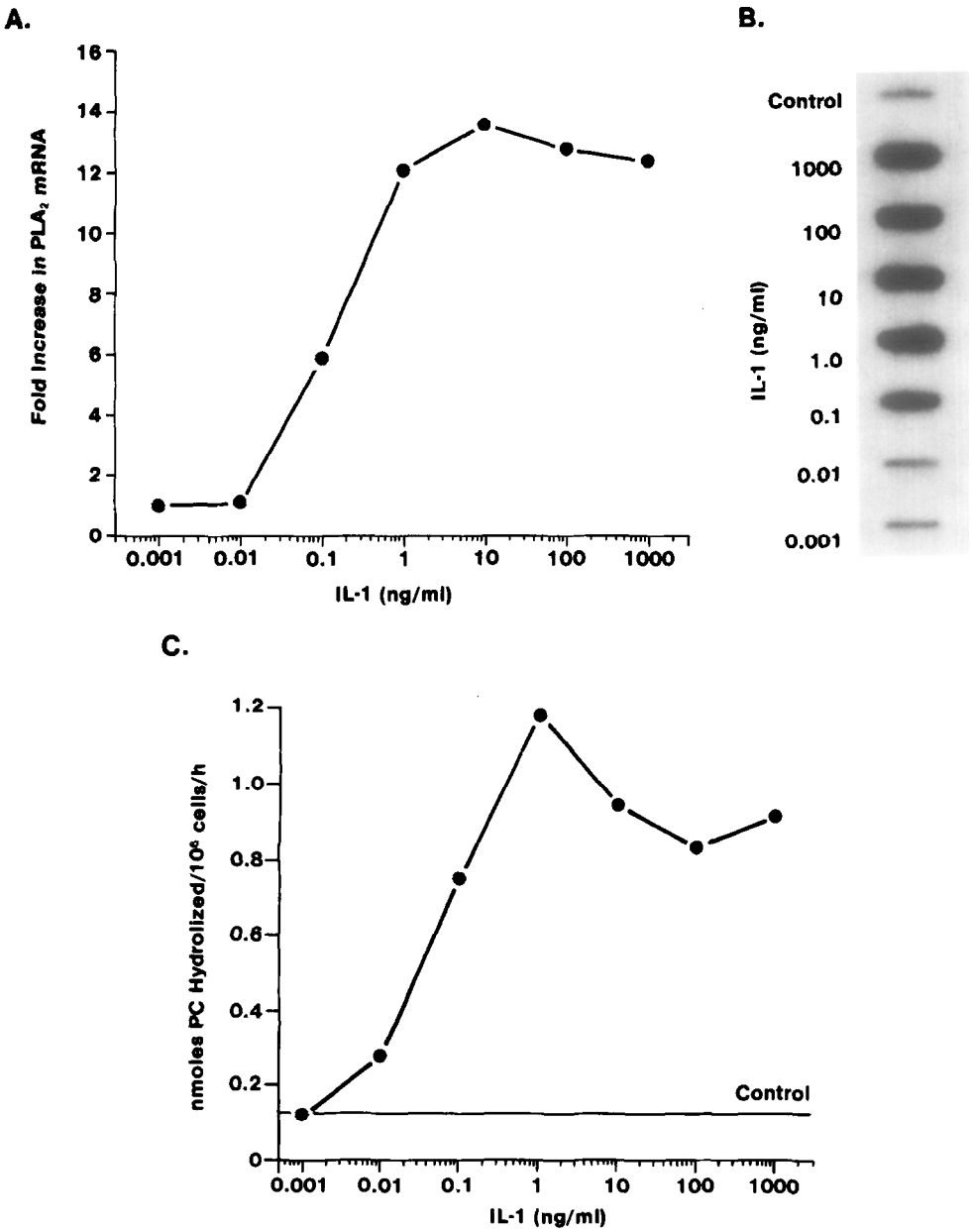

FIGURE 2. The concentration dependence of IL-1 effects on PLA_2 mRNA levels and enzymatic activity in RAC. Total cellular RNA (0.5-2.0 ug) from control cultures and cultures treated for 24h with IL-1 concentrations ranging from 0.001 to 1000 ng/ml was applied to nitrocellulose using a slot blot apparatus. The RNA was hybridized with a [^{32}P]-labeled cDNA probe for RSF PLA_2. PLA_2 activity in medium from control and IL-1-treated RAC was assessed using a phosphatidylcholine/sodium deoxycholate mixed-micelle assay. (A) Fold increase in PLA_2 mRNA in IL-1-treated cultures relative to that of control cultures. (B) A typical autoradiogram showing the concentration dependence of IL-1 effects on RSF PLA_2 mRNA levels in RAC where each slot contains 2 ug of total cellular RNA. (C) PLA_2 activity in RAC medium as a function of IL-1 concentration.

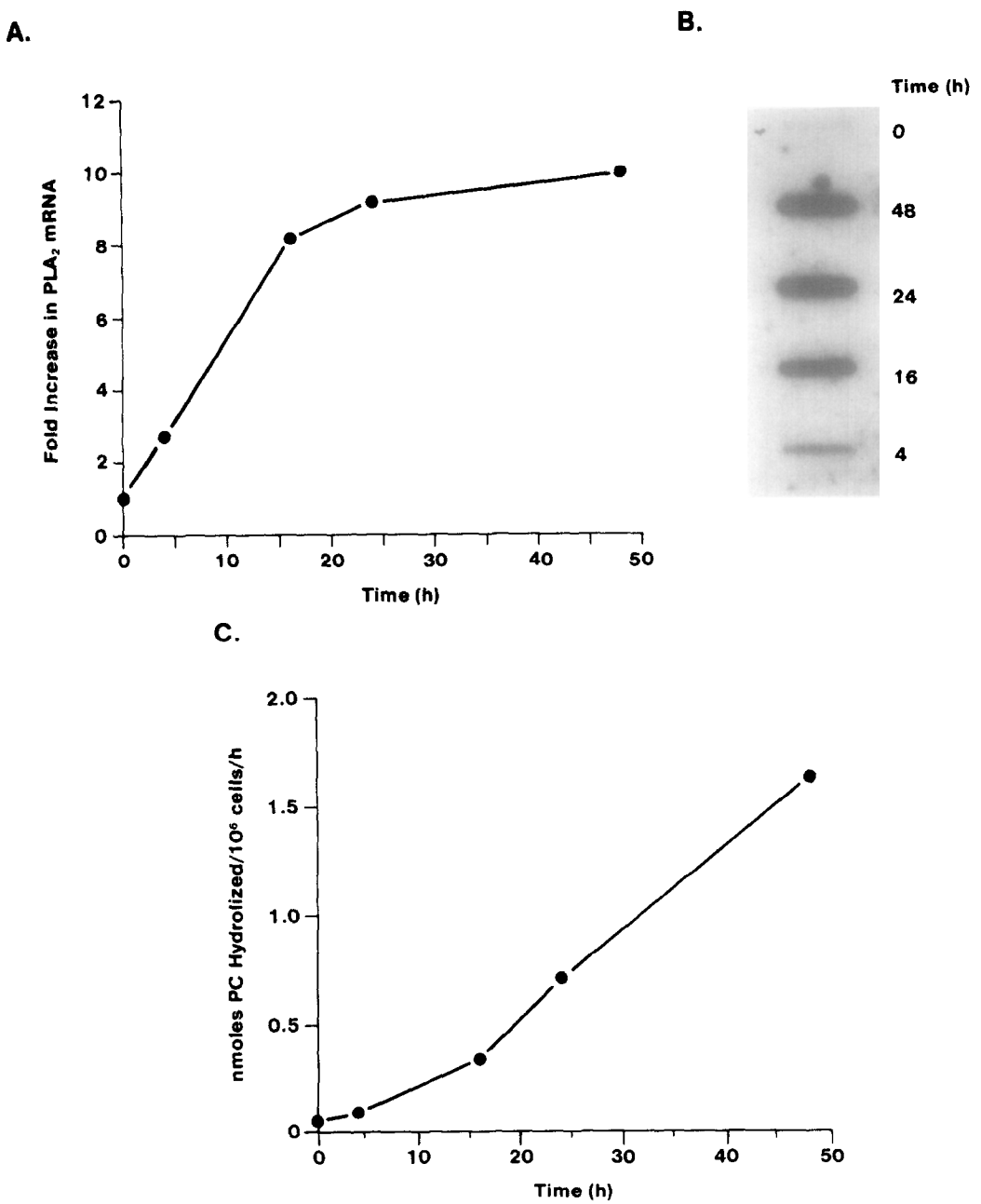

FIGURE 3. The timecourse of IL-1 effects on RSF PLA_2 mRNA levels and PLA_2 enzymatic activity in RAC. Total cellular RNA (0.5-2.0 ug) from control cultures and cultures treated for 1-48 h with 100 ng/ml IL-1 was applied to nitrocellulose using a slot blot apparatus and hybridized to a [^{32}P]-labeled cDNA specific for RSF PLA_2. PLA_2 activity in medium from IL-1-treated RAC was assessed using a phosphatidylcholine/sodium deoxycholate mixed-micelle assay. (A) Fold increase in PLA_2 mRNA levels in IL-1-treated RAC relative to that of control cultures for each time point. (B) A typical autoradiogram showing the time dependent increase in RSF PLA_2 mRNA levels induced by IL-1 where each slot contains 2 ug of total cellular RNA. (C) Accumulation of PLA_2 activity in the medium of IL-1-treated cultures as a function of time. Control PLA_2 activity did not exceed 0.22 nmoles PC hydrolyzed/10^6 cells/h throughout the timecourse.

cultures and RAC treated for 24 hours with IL-1 concentrations ranging from 0.001 to 1000 ng/ml. As shown in Figure 2A, IL-1 caused a concentration-dependent increase in PLA_2 mRNA levels. The maximal increase in RSF PLA2 transcript levels was approximately 13 fold as evidenced in cells treated with 1-1000 ng/ml IL-1. Half-maximal induction of RAC PLA_2 transcript was estimated to require 0.15 ng/ml IL-1 by interpolation. For comparison, the concentration dependence of IL-1 effects on RAC PLA_2 activity were evaluated. As shown in Figure 2C, IL-1-induced increases in PLA_2 activity were coordinate with its effect on RSF transcript levels. Maximal induction of PLA_2 activity was evidenced in RAC treated with 1-1000 ng/ml IL-1. Half-maximal induction of RAC PLA_2 activity was estimated to require 0.15 ng/ml IL-1.

The timecourse of IL-1 induction of RSF PLA_2 mRNA levels was investigated. Total RNA from control cultures and cultures treated for 1 to 48 hours with 100 ng/ml IL-1 was collected for analysis by slot blot hybridization. IL-1 caused a time-dependent increase in steady state RSF PLA_2 mRNA levels. RSF PLA_2 mRNA in IL-1-treated RAC was increased by 4 h post addition of IL-1. Maximal augmentation of RSF PLA_2 message was evidenced 16 hours post-addition of IL-1 to the cultures (Figure 3A and 3B). Figure 3C shows the timecourse of the IL-1 effect on RAC PLA_2 activity. The timecourse of IL-1-induced accumulation of PLA_2 enzymatic activity paralleled the timecourse of IL-1-induced increases in RSF PLA_2 transcript levels from 0-16

Table 1. The effect of actinomycin D on IL-1-induced increases in RSF PLA_2 mRNA and PLA_2 activity [a]

Treatment	mRNA[b]	PLA_2 Activity[c]
Control	1	0.09
IL-1	13	0.88
Control + actinomycin D	1	0.14
IL-1 + actinomycin D	1	0.12

[a] Control RAC cultures +/- actinomycin D (200 ng/ml) and RAC treated with 100 ng/ml IL-1 +/- actinomycin D (200 ng/ml) were incubated for 24 hours. Total cellular RNA (0.5 - 2.0ug) was applied to nitrocellulose using a slot blot apparatus. The RNA was hybridized with a [^{32}P]-labeled cDNA probe for RSF PLA_2. PLA_2 activity in the medium from these cultures was assessed using a phosphatidylcholine/sodium deoxycholate mixed micelle assay.

[b] Fold increase in PLA_2 mRNA relative to that of control RAC without actinomycin D.

[c] PLA_2 activity as nmoles PC hydrolyzed/10^6 cells/h.

h. From 16-48 h, however, PLA_2 activity continued to increase suggesting an accumulation of the protein at an increased rate, corresponding to the maximal induction of RSF PLA_2 transcript levels by IL-1.

An increase in steady-state mRNA may reflect an increase in the rate of gene transcription and/or greater stability of previously transcribed message. To determine whether the IL-1 induction of RSF PLA_2 mRNA levels required new synthesis of RNA, the ability of actinomycin D to block IL-1 effects on RSF PLA_2 mRNA levels was assessed. As shown in Table 1, RSF PLA_2 mRNA levels in RAC stimulated with 100 ng/ml IL-1 for 24 hours in the presence of 200 ng/ml actinomycin D were not elevated relative to actinomycin D treated control cultures. Actinomycin D treatment alone did not modulate basal RSF PLA_2 mRNA expression over the course of 24 hours. The effects of actinomycin D on IL-1-induced PLA_2 activity were also investigated. Actinomycin D (200 ng/ml) completely inhibited the induction of PLA_2 activity by IL-1 (100 ng/ml). Actinomycin D alone did not reduce PLA_2 activity in control RAC. Thus, enhancement of both RAC PLA_2 activity and RSF PLA_2 transcript levels by IL-1 was dependent upon de novo mRNA synthesis.

DISCUSSION

The synovial fluid from patients with rheumatoid arthritis is known to contain high levels of PLA_2 and IL-1 (1,2). That synovial fibroblasts and chondrocytes contribute to the accumulation of RSF PLA_2 in the joint space has been inferred from the findings that IL-1 causes increased secretion of PLA_2 activity by these cells (3,4). The ability of RAC to synthesize message that cross-hybridizes at high stringency to a probe which is specific for human RSF PLA_2 provides the first direct evidence that chondrocytes transcribe a gene encoding a PLA_2 of this type.

The concentration and time dependence of IL-1 effects on RAC PLA_2 activity reported herein confirm earlier work by Chang et al.(3). In addition, we provide the first insight into the mechanism of IL-1 induction of RAC PLA_2 activity. Using Northern and slot blot analyses, we have demonstrated that IL-1 causes a dramatic increase in RAC RSF PLA_2 mRNA levels. The concentration and time dependence of IL-1 augmentation of steady-state mRNA levels for RSF PLA_2 correlate well with the concentration and time dependence of IL-1 induction of RAC PLA_2 activity. Likewise, both IL-1 effects on PLA_2 activity and RSF PLA_2 transcript levels are sensitive to inhibition by actinomycin D. These data suggest that modulation of RAC PLA_2 activity by IL-1 involves transcriptional regulation of the synthesis of RSF PLA_2. They do not preclude the possibility that IL-1 induction of PLA_2 activity reflects IL-1 effects on the levels of PLA_2 activators or inhibitors

in the cultures or on the levels of other types of PLA_2 enzymes which RAC may synthesize.

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