Journal of Cellular Biochemistry 63:320–333 (1996)

Prostaglandin E_2 Up-Regulates Insulin-Like Growth Factor Binding Protein-3 Expression and Synthesis in Human Articular Chondrocytes by a c-AMP-Independent Pathway: Role of Calcium and Protein Kinase A and C

J.A. DiBattista, S. Doré, N. Morin, and T. Abribat

Department of Medicine, University of Montreal (J.A.D., T.A.), the Louis-Charles Simard Research Center, Notre-Dame Hospital (J.A.D., N.M., T.A.), and the Douglas Hospital Research Center (S.D.), Montreal, Quebec, Canada H4H-1R3

Abstract Insulin-like growth factor-1, IGF-1, is believed to be an important anabolic modulator of cartilage metabolism and its bioactivity and bioavailability is regulated, in part, by IGF-1 binding protein 3 (IGFBP-3). Prostaglandin E_2 (PGE_2) stimulates IGF-1 production by articular chondrocytes and we determined whether the eicosanoid could regulate IGFBP-3 and, as such, act as a modifier of IGF-1 action at a different level. Using human articular chondrocytes in high density primary culture, Western and Western ligand blotting to measure secreted IGFBP-3 protein, and Northern analysis to monitor IGFBP-3 mRNA levels, we demonstrated that PGE_2 provoked a 3.9 ± 1.1 ($n = 3$) fold increase in IGFBP-3 mRNA and protein. This effect was reversed by the Ca^{++} channel blockers, verapamil and nifedipine, and the Ca^{++}/calmodulin inhibitor, W-7. The Ca^{++} ionophore, ionomycin, mimicked the effects of PGE_2 as did the phorbol ester PMA, which activates Ca^{++}-phospholipid-dependent protein kinase C (PKC). Cyclic AMP mimetics, such as forskolin, IBMX, Ro-20-1724, and Sp-cAMP, inhibited the expression and synthesis of the binding protein. PGE_2 did not increase the levels of cAMP or protein kinase A (PKA) activity in chondrocytes. The PGE_2 secretagogue, IL-1β, down-regulated control levels of IGFBP-3 which could be completely abrogated by pre-incubation with the tyrosine kinase inhibitor, erbstatin, and partially reversed ($50 \pm 8\%$) by KT-5720, a PKA inhibitor. These observations suggested that PGE_2 does not mediate the effect of its secretagogue and that IL-1β signalling in chondrocytes may involve multiple kinases of diverse substrate specificities. Dexamethasone down-regulated control, constitutive levels of IGFBP-3 mRNA and protein eliminating the previously demonstrated possibility of cross-talk between glucocorticoid receptor (GR) and PGE_2 receptor signalling pathways. Taken together, our results suggest that PGE_2 modulates IGFBP-3 expression, protein synthesis, and secretion, and that such regulation may modify human chondrocyte responsiveness to IGF-1 and influence cartilage metabolism. © 1996 Wiley-Liss, Inc.

Key words: chondrocytes, calcium, protein kinase C, calphostin C, gene expression

Extracellular matrix (ECM) turnover is regulated by the intricate balance between the synthesis of new components and the degradation of existing structures. Matrix destruction of connective tissue, a hallmark of arthritic diseases, is believed to occur through the concerted action of a series of proteases, the most prominent of which are the family of Zn-dependent matrix metalloproteases (MMPs). These enzymes are induced by inflammatory mediators particularly cytokines like IL-1β and TNFα which are found in relative abundance at inflammatory sites [Evans, 1993; Mauviel, 1993; Harris, 1990; Pelletier et al., 1991].

In contrast to the putative catabolic effects of cytokines, growth factors like the insulin-like growth factor, IGF-1, are believed to be important anabolic modulators of cartilage metabolism [Malemud, 1993]. IGF-1 induces the expression and synthesis of collagen type II and proteoglycan core protein and, as such, can stabilize chondrocyte phenotype in situations where homeostasis may be perturbed [Tesch et al.,

Received May 13, 1996; accepted May 16, 1996.

Address reprint requests to John A. Di Battista, Ph.D., Rheumatic Disease Unit Room Y-2603, Notre-Dame Hospital, 1560 Sherbrooke St. East, Montreal (Que.), Canada H2L 4K8.

© 1996 Wiley-Liss, Inc.

1992; Franchimont and Bassler, 1991; Sandell and Dudek, 1988]. IGF-1 and IGF-1 receptor synthesis within cartilage (chondrocytes) have been shown to be under the control of a number of hormones that by themselves can modulate cartilage metabolism [Malemud, 1993; DiBattista et al., 1995a; Doré et al., 1994]. The action of IGF-1 on cellular metabolism is governed at several levels including the presence of extracellular, high affinity IGF-binding proteins (BPs, IGFBP-1 through to IGFBP-6) which modify the interaction of IGF-1 with its receptor [reviewed by Jones and Clemmons, 1995]. The circulating or local levels of IGFBPs are regulated ontogenetically, by various endocrine factors, and by specific cleavage with proteases that compromise the functionality of the IGFBPs [Lamson et al., 1993; Cohen et al., 1991].

Insulin-like growth factor binding protein-3 (IGFBP-3) is secreted in the greatest abundance of all IGFBPs by human chondrocytes in culture and indeed is present in dramatically increased amounts in osteoarthritic (OA) chondrocytes [Doré et al., 1994]. This observation has been cited as a possible reason why primary cultures of OA chondrocytes are refractive to exogenous IGF-1 stimulation. In fact the issue is somewhat more complex since IGFBP-3 can potentiate the IGF-1 stimulation of DNA synthesis in human fibroblasts when the binding protein is preincubated with the cells first but is inhibitory when added concurrently with IGF-1 [DeMellow and Baxter, 1988]. In addition IGFBPs may inhibit cellular growth independently of IGF-1 as has recently been shown in mouse embryo fibroblasts transfected with IGFBP-3 and having a targeted disruption of the IGF-1 receptor (null) gene [Valentinis et al., 1995]. Other studies have demonstrated that exogenous IGFBP-3 associates specifically with the cell surface of Hs578T human breast cancer cells and inhibits their proliferation in culture [Oh et al., 1993a]; IGF-1 and IGF-2 attenuate the inhibitory effect of IGFBP-3 by forming IGF/IGFBP-3 complexes. Specific receptors for IGFBP-3 have been convincingly demonstrated on Hs578T human breast cancer cells: IGF-1 blocks cell surface IGFBP-3 binding [Oh et al., 1993b].

Cellular activation by proinflammatory stimuli (like cytokines) results in, besides induction of matrix destructive MMPs, increased prostaglandin (PG) synthesis which has been assigned an important role in the pathogenesis of arthritic diseases [Robinson, 1985]. A growing body of evidence, however, suggests that perhaps PGs may also act as potent autocrine/paracrine feedback bioregulators with the ability to inhibit the expression of cytokines (e.g., IL-1β and TNFα) and MMPs [DiBattista et al., 1994, 1995b; Knudsen et al., 1986]. Interestingly, transgenic cyclooxygenase-2 (COX-2, enzyme responsible for PG synthesis) knockout mice models retain the inflammatory response under a number of stimulatory conditions [Morham et al., 1995] adding further support for the aforementioned hypothesis. Prostaglandin E_2 can exert anabolic effects to the extent that the eicosanoid stimulates the expression and synthesis of IGF-1 and the incorporation of proline into collagenase digestible proteins in fetal calvariae organ cultures and rat osteoblast-like cell cultures [McCarthy et al., 1991; Raisz et al., 1993; Bichell et al., 1993]. It was suggested that IGF-1 might mediate the effects of PGE_2 [Raisz et al., 1993]. We showed that PGE_2 also stimulates the expression and synthesis of IGF-1 and the incorporation of proline into collagenase digestible proteins in primary cultures of human articular chondrocyte cultures [DiBattista et al., 1995a]. Furthermore, PGE_2-stimulated proline incorporation is inhibited when antibodies to human IGF-1 are coincubated in the chondrocyte cultures suggesting that IGF-1 mediates the action of PGE_2 in this regard [DiBattista et al., in press).

Our intent in this study was to determine whether PGE_2 and substances known to modulate the production of PGE_2 (e.g., IL-1, glucocorticoids), could influence IGFBP-3 synthesis and expression and as such, act as a hormonal modifiers of IGF-1 action at a completely different level. In addition, since little is known about post-receptor intracellular signal transduction mechanisms controlling IGFBP-3 expression and synthesis in connective tissues, we investigated the role of serine-threonine and tyrosine protein kinases and serine-threonine protein phosphatases (PP-1, PP-2A) in this regard.

MATERIALS AND METHODS

Chemicals

Anti-IGF antibody (Mab), anti-IGFBP-3 (Polyclonal), cAMP-dependent protein kinase catalytic subunit, purified protein kinase C, cAMP-dependent protein kinase inhibitor peptide (PKI), and protein kinase C inhibitor peptide were from UBI (Lake Placid, NY). Prostaglandin E_2 (PGE_2), dibutyryl cAMP, forskolin, diethylpyrocarbonate (DEPC), polyvinylpyrrolidone

(PVP), Ficoll, salmon testes DNA, 2′-3′-dideoxy-adenosine (DDA), 3-isobutyl-1-methylxanthine (IBMX), ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), sodium acetate, trichloroacetic acid (TCA), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Staurosporine, Ro-20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone), calphostin-C, nifedipine, verapamil, W-7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfon-amide, HCl), Sp-cAMP, ionomycin, phorbol 12-myristate-13-acetate (PMA), and Okadaic acid (OKA) were the products of Calbiochem (San Diego, CA). Recombinant human interleukin-1β (rhIL-1β, SA: 1 U/10 pg protein) was obtained from Genzyme Corporation (Boston, MA). Dulbecco's modified Eagles' medium (DMEM), HEPES, heat-inactivated fetal calf serum (FCS), stock antibiotic/antimycotic mixture (10,000 U/mL of penicillin base, 10,000 μg/mL of streptomycin base, and 50 μg/mL of amphotericin-B), agarose, and phenol were products of GIBCO BRL (Gaithersburg, MD). TRIS (tris-(hydroxy-methyl)-aminomethane), NaCl, $MgCl_2$, $CaCl_2$, 8-hydroxyquinoline, formamide, formaldehyde, and ethanol were from Fisher Scientific (Montréal, Québec).

Specimen Selection and Cell Culture

Normal cartilage from tibial plateaus and femoral condyles was acquired by necropsy from the knee joints of adult human cadavers within 12 h of death (24 patients; mean age 61 years old, male/female ratio = 0.55) at Notre-Dame Hospital, Montreal. To ensure that only normal tissue was used, specimens were examined macroscopically and microscopically, as previously described [Mankin, 1974], and only those without any lesions or alterations were processed further. Chondrocytes were isolated by sequential enzymatic digestion as previously described [DiBattista et al., 1991a]. Cells were inoculated into 6- or 12-well cluster plates (1-52795A, Nunclon, Copenhagen, Denmark) at high density and all experiments were conducted at confluence when the cells reached a stationary phase. Cultures were incubated with DMEM containing 0.2% BSA and an antibiotic mixture for an additional 48 h prior to experimentation to establish basal conditions.

Western and Western Ligand Blotting of IGFBP-3

Twenty microliters of 20-fold concentrated medium (Centricon-3, Amicon, Danvers, MA) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel under nonreducing (Western ligand blot) and reducing (Western blot) conditions and separated proteins were transferred electrophoretically onto Hybond-N nitrocellulose filters (0.45 μm pore size). Filters were blocked and processed as previously described [Hossenlopp et al., 1986; DiBattista et al., 1991a] and then either labeled with [^{125}I]-IGF-1 (3.70×10^5 cpm/mL binding solution) overnight at 4°C or with rabbit anti-human IGFBP-3 (1:1,000; 0.5 μg/mL) for 2 h at RT. The ligand blots were washed and subjected to autoradiography at −70°C. Antibody-antigen complexes were revealed by chemiluminescence using a goat anti-rabbit IgG-HRP conjugate second antibody (ECL Amersham, Arlington Heights, IL). All blots were subjected to laser scanning densitometry (GS-300 Hoefer Scientific Instruments, San Francisco, CA) for semi-quantitative measurements. These two blotting procedures were performed in all experiments where warranted to establish the relationship between the amount of immunoreactive and functional (binds IGF-1) IGFBP-3. In addition the immunoblots provided some indication of proteolysis.

Northern Blot Analysis of IGFBP-3 mRNA

Total cellular RNA was isolated using the TRI-REAGENT (Molecular Sciences, Cincinnati, OH), single-step extraction procedure. Following solubilization of the RNA pellet in DEPC-tretaed sterile H_2O, RNA was quantitated spectrophotometrically at 260 nm and the OD_{260}/OD_{280} was between 1.9–2.0 with no detectable genomic DNA contamination as judged by agarose gel electrophoresis. Ten micrograms of total RNA were resolved on 1.0% agarose-formaldehyde gels and transferred to Hybond-N™ nylon membranes (Amersham, Canada LTD, Oakville, Ont.) in 20 × SSC buffer, pH 7, by vacuum blotting. The RNA was cross-linked to the membranes by exposure for 5–10 min to UV light. Pre hybridization was performed for 18 h at 68°C in SET buffer (60 mM Tris, pH 7.4, 450 mM NaCl, 3 mM EDTA) containing 10 × Denhardt's solution, 250 μg/mL yeast RNA, 50 μg/mL denatured salmon testes DNA, 10 μg/mL polyadenylic acid, 0.1% SDS, and 0.1% sodium pyrophosphate. Hybridization was carried out in the same buffer containing random primed Digoxigenin (DIG)-deoxy UTP labeled cDNA probes for IGFBP-3 (180 bp PCR fragment cor-

responding to base pairs 400–579 of the published coding sequence [Wood et al., 1988] and cloned into pBS SK-) and 28S rRNA (re-probing; ATCC, 1.5 kb insert between EcoRI sites in pBS SK-) for 24 h at 50°C. All blots were subjected to laser scanning densitometry (GS-300 Hoefer Scientific Instruments, San Francisco, CA) for semiquantitative measurements with the relative amount of IGFBP-3 mRNA normalized to the level of 28S rRNA.

Analysis of Protein Kinase Activity

This nonradioisotopic technique is based on an enzyme linked immunosorbent assay (ELISA) that utilizes a synthetic peptide (corresponding to residues 3–13 of porcine glial fibrillary acidic protein, GFAP) as kinase substrate and a monoclonal antibody (YC-10) which recognizes the phosphorylated form of the peptide [Yano et al., 1991; Inagaki et al., 1990]. The assay measures total protein kinase A and C (PKA, PKC) activity and doesn't distinguish the activity of different isoforms. Standard curves were generated with increasing concentrations of purified PKC (0–100 ng: rat brain, mixture isozymes α, β, γ) and the catalytic subunit of PKA (0–500 ng: bovine heart). Results are expressed as ng equivalents per 100 μg of cellular protein; the change in optical density obtained with a fixed amount of cellular extract corresponding to an equivalent change in optical density of a given amount of purified enzyme.

Following experimentation, chondrocytes were scraped from the plates, pelleted, and extracted into RIPA buffer supplemented with 1 mM each of Na_3VO_4, NaF, and β-glycerophosphate. Generally 20–40 μg of protein was used per assay (linear range 0–100 μg) and specificity was controlled using specific inhibitors of PKA (peptide inhibitor and KT-5720) and PKC (peptide inhibitor, so-called pseudo-substrate/endogenous autoinhibitor and Calphostin C).

cAMP RIA

Cyclic AMP was measured in cellular extracts using a dual range (Biotrak, Amersham) enzyme immunoassay system. Following incubation with a biological effector for the appropriate time periods, cultures were snap frozen over a dry-ice/acetone mixture and the cells scraped into 1 mL of ice-cold 75% alcohol solution containing 0.5 mM IBMX. Following low-speed centrifugation to remove insoluble material, the supernatants were dried and redissolved in 1 mL of 50 mM sodium acetate buffer, pH 6.2. Data were expressed as pmols per 10^6 cells.

DNA, Protein, and Data Analysis

Values were expressed as mean ± SD. Cellular DNA content was determined by the method of Burton [1956] using salmon sperm DNA as a standard. Cellular protein was estimated by the BCA protein assay reagent (Pierce, Rockford, IL) using a mixture of gamma-globulin and BSA (80/20, respectively) as a standard.

Statistical significance was assessed by ANOVA or Student's *t*-test. Significant differences were confirmed only when the probability was less than or equal to 5%.

RESULTS

PGE_2 Up-Regulation of IGFBP-3 Protein and mRNA: Effect of cAMP Mimetics

Exposure of human articular chondrocytes to PGE_2 (1–1,000 ng/mL; 2.8 nM–2.8 μM) resulted in a dose-dependent increase (at 1,000 ng/mL, 3.6 ± 1.1) in the amount of IGFBP-3 (bands at 41 and 38 kDa) secreted into the culture medium as judged by Western ligand (^{125}I-IGF-1) blotting procedures (Fig. 1). Laser scanning densitometry of multiple blots (n = 3) revealed that the IC_{50} was 95 ± 36 ng/mL (i.e., 270 nM). Routinely we also detected bands at 34, 28, and 24 kDa, which we previously identified, using specific antibodies, as being IGFBP-2 and both the glycosylated IGFBP-4 and IGFBP-4, respectively [Doré et al., 1994]. IGFBP-3 is the predominant form secreted representing 60–70% of the total amount of IGFBPs secreted by human chondrocytes while IGFBP-4 accounts for about 25–40% and IGFBP-2 about 1–2% [Doré et al., 1994]. The increase in the 41 and 38 kDa doublet was verified by Western analysis and such verification also confirmed that there was little or no proteolytically induced fragmentation of IGFBP-3 either in treated or untreated culture medium (data not shown). In order to ascertain if the stimulatory pattern was manifested at the level of IGFBP-3 message, we measured the steady-state levels of IGFBP-3 mRNA. IGFBP-3 mRNA was upregulated by PGE_2 in a similar dose-dependent manner suggesting pre-translational control (see Fig. 1).

Because PGE_2 is known to elevate cAMP levels in many cell types including human synoviocytes [Robinson, 1985], we determined whether

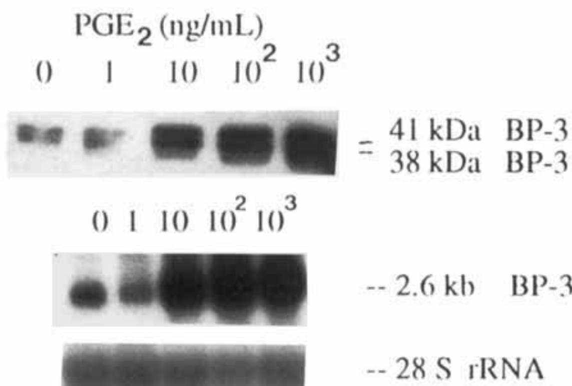

Fig. 1. Dose-dependent stimulation by PGE_2 of IGFBP-3 (BP-3) steady-state mRNA expression and synthesis using primary cultures of human articular chondrocytes. Cells were incubated with or without increasing concentrations of PGE_2 for 24 h. Conditioned medium was collected, concentrated, and analyzed by Western ligand blot analysis as described in Materials and Methods. Cell monolayers were extracted for total RNA and 10 μg was subjected to Northern analysis with probes for IGFBP-3 and 28S rRNA.

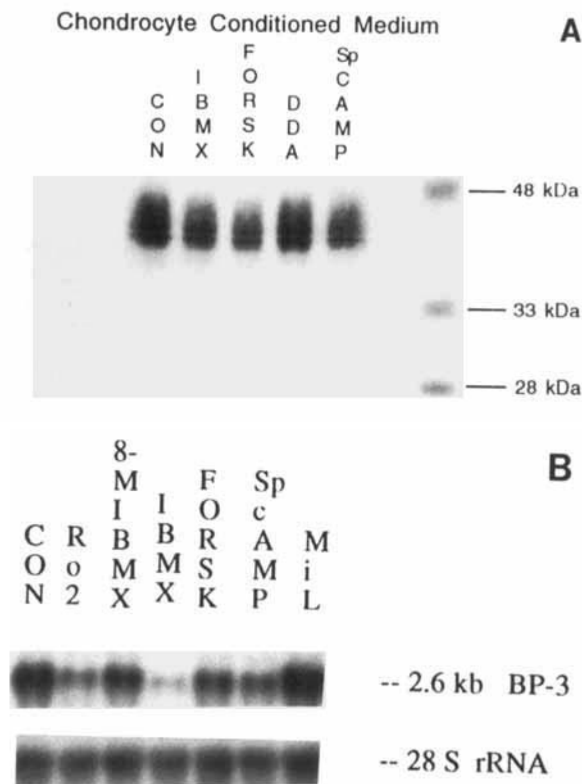

Fig. 2. **A:** Effect of cyclic AMP mimetics on constitutive IGFBP-3 (BP-3) production using primary cultures of human articular chondrocytes. Cells were incubated with or without the phosphodiesterase inhibitor IBMX (200 μM), cAMP mimetics forskolin (60 μM, FORSK), and Sp cAMP (250 nM), and the adenylate cyclase inhibitor 2′-3′ dideoxyadenosine (200 μM, DDA) for 24 h. Conditioned medium was collected, concentrated, and analyzed by Western analysis as described in Materials and Methods. **B:** Effect of cyclic AMP mimetics on constitutive IGFBP-3 (BP-3) steady-state mRNA expression using primary cultures of human articular chondrocytes. Cells were incubated with or without the phosphodiesterase inhibitors Ro-20-1724 (40 μM, RO2), 8 methoxy IBMX (40 μM, 8-MIBMX), IBMX (200 μM), Milrinone (20 μM, Mil), and cAMP mimetics forskolin (60 μM, FORSK) and Sp cAMP (250 nM) for 24 h. Cell monolayers were extracted for total RNA and 10 μg was subjected to Northern analysis with probes for IGFBP-3 and 28S rRNA.

the induction observed with the eicosanoid could be induced by substances which also putatively increase cellular levels of cAMP. In fact, however, cyclic AMP mimetics IBMX (200 μM), forskolin (60 μM), and Sp cAMP (250 μM) suppressed IGFBP-3 production by up to 73 ± 14% (n = 3, Sp cAMP) as judged by Western analysis. 2′-3′-dideoxyadenosine, an inhibitor of adenylate cyclase, had no discernible affect on IGFBP-3 production (see Fig. 2A). Since IBMX displays a broad range in terms of its specificity as an inhibitor of phosphodiesterase (PDE), we tested compounds that have far greater specificity. The specific PDE IVA inhibitor, Ro-20-1724 (40 μM), was essentially equipotent to IBMX with respect to the inhibition of IGFBP-3 mRNA expression. Milrinone (10 μM), an inhibitor of the cGMP stimulated PDE and 8-methoxy IBMX, an inhibitor of the Ca^{++}-calmodulin dependent PDE, did not inhibit the expression of IGFBP-3 to any great extent (Fig. 2B).

Given these unexpected results, we verified whether PGE_2 could increase intracellular levels of cAMP in human chondrocytes in culture. At the physiological level of 100 nmol/L, we could detect no significant changes in the cAMP levels for up to 8 h and no up-regulation of PKA activity (Table I). In contrast and as expected, there were massive elevations after only 2.5 min (ANOVA up to 40 min, $P < 0.001$) in synoviocytes to match the graded increases in PKA activity (Table I). However, when chondrocytes were preincubated for 30 min with 200 μM of IBMX prior to stimulation with PGE_2, there was a dramatic and sustained augmentation of cAMP levels (Table I) of which, on average and at each time point, 89 ± 17 pmol/10^6 cells (n = 3) of the total cAMP production was attributable to IBMX alone.

Since we interpreted these results to mean that PGE_2 may not signal chondrocytes primarily by inducing cAMP as a second messenger, we explored the role of Ca^{++}/calmodulin as a mediator of PGE_2 action [An et al., 1994; Irie et al., 1994]. The calcium ionophore, ionomycin (20

TABLE I. Effects of PGE_2 (100 nmol/L) on cAMP and Protein Kinase A Activity in Human Articular Connective Tissue Cells*

Time (min)	Synoviocytes	Chondrocytes	Chondrocytes ± IBMX
	cAMP formed pmol/10^6 cells		
0	31 ± 7[a]	37 ± 9	30 ± 9[b]
2.5	658 ± 53	53 ± 9	675 ± 41
5	520 ± 46	60 ± 7	817 ± 90
10	1174 ± 60	31 ± 3	835 ± 65
20	835 ± 37	75 ± 11	657 ± 61
40	408 ± 75	24 ± 9	408 ± 37
80	99 ± 23	69 ± 21	450 ± 66
160	82 ± 19	19 ± 5	395 ± 51
480	43 ± 18	18 ± 8	401 ± 53

Time (h)	Synoviocytes	Chondrocytes
	PKA activity (ng equivalents/100 μg protein)	
0	62 ± 11	41 ± 9
0, 25	65 ± 15	45 ± 10
1	89 ± 9	41 ± 5
4	191 ± 26[c]	42 ± 3
8	225 ± 35[c]	47 ± 8
18	46 ± 19	47 ± 9

*n = 3 separate experiments (duplicate determinations).
[a]ANOVA up to 40 min $P < 0.001$.
[b]ANOVA up to 480 min $P < 0.003$.
[c]Student's *t*-test $P < 0.01$ vs. 0 h (control).

nM) induced the steady-state expression and synthesis of IGFBP-3 mRNA by 2.9 ± 0.4-fold (n = 3) over controls, similar to the data with PGE_2 (also see above) (Fig. 3). The addition of W-7 (25 μM), a Ca^{++}/calmodulin inhibitor, of verapamil (50 μM) and nifedipine (10 μM), both Ca^{++} channel blockers and of Calphostin C (100 nM), a PKC inhibitor, abrogated the PGE_2 induced induction of IGFBP-3. Verapamil and nifedipine caused relatively small reductions in terms of basal IGFBP-3 expression and synthesis while Calphostin C and W7 were strong suppressors. The effects of ionomycin were also reversed by Calphostin C (Fig. 3).

Effect of IL-1β and Dexamethasone on IGFBP-3 Expression and Synthesis

The synthesis of PGE_2 is induced by IL-1 in a rather dramatic fashion in human articular chondrocytes with quantities reaching up to 400 ng/10^6 cells in exceptional cases (average 100–200) [Chan et al., 1995]. Given the quantities and the potential of PGE_2 to act as an autocrine factor particularly in the avascular articular cartilage, we wondered whether the eicosanoid actually mediated the previously reported [Olney et al., 1995] effects of IL-1 on IGFBP-3 expression. Interestingly, rhIL-1β (0–1,000 U/mL) depressed the expression and synthesis of IGFBP-3 in a dose-dependent fashion (Fig. 4) in contrast to the effects of PGE_2. The broad spectrum serine-threonine kinase inhibitor, staurosporine, and the specific PKC inhibitor, Calphostin C, were unable to reverse the cytokine-induced effect while the tyrosine kinase inhibitor erbstatin (50 μM) could restablish the expression of IGFBP-3 mRNA to control levels. The PKA inhibitor, KT5720 (250 nM), also reversed 50 ± 8% (n = 3) of the suppressive activity of IL-1 (Fig. 5). With the exception of Calphostin C, the inhibitors alone had no consistent affects on control IGFBP-3 expression (data not shown).

The pleiotropic glucocorticoid receptor (GR) is a ligand inducible transacting factor that controls the expression at the transcriptional level of many genes involved in cartilage metabolism. We showed that PGE_2 can increase the levels of GR in connective tissue cells by at least 2-fold [DiBattista et al., 1991b] and, as such, it is possible that the GR may mediate some of the effects of the eicosanoid on IGFBP-3 expression. Given these facts and considering the role of the IGF-1/IGFBP-3 system on cartilage metabolism, we assessed the actions of the glucocorticoid dexamethasone on IGFBP-3 expression. Dexamethasone depressed the expression and synthesis of IGFBP-3 in an extremely potent and dose-dependent fashion in contrast to the effects of PGE_2 (Fig. 6).

Role of Protein Kinase C and Protein Phosphatases 1 and 2A on IGFBP-3 Expression and Synthesis

Considering the apparent importance of cAMP and PKA mediation in the regulation of IGFBP-3, we wondered about the role of PKC since the latter kinase often mediates effects opposing PKA. In addition, the activity of some isoforms of PKC is regulated by Ca^{++} directly [Kiley and Jaken, 1994] and our present results point to a role for Ca^{++} metabolism in the control of IGFBP-3. We explored this avenue by attempting to pharmacologically activate PKC. The phorbol ester, PMA, a tumour promoter and activator of protein kinase C (PKC), upregulated the expression and synthesis of IGFBP-3 by a maximum of 5.6 ± 1.1 fold (both mRNA and protein, n = 4) in a dose dependent manner with a zenith being rached at 200 nM (Fig. 7).

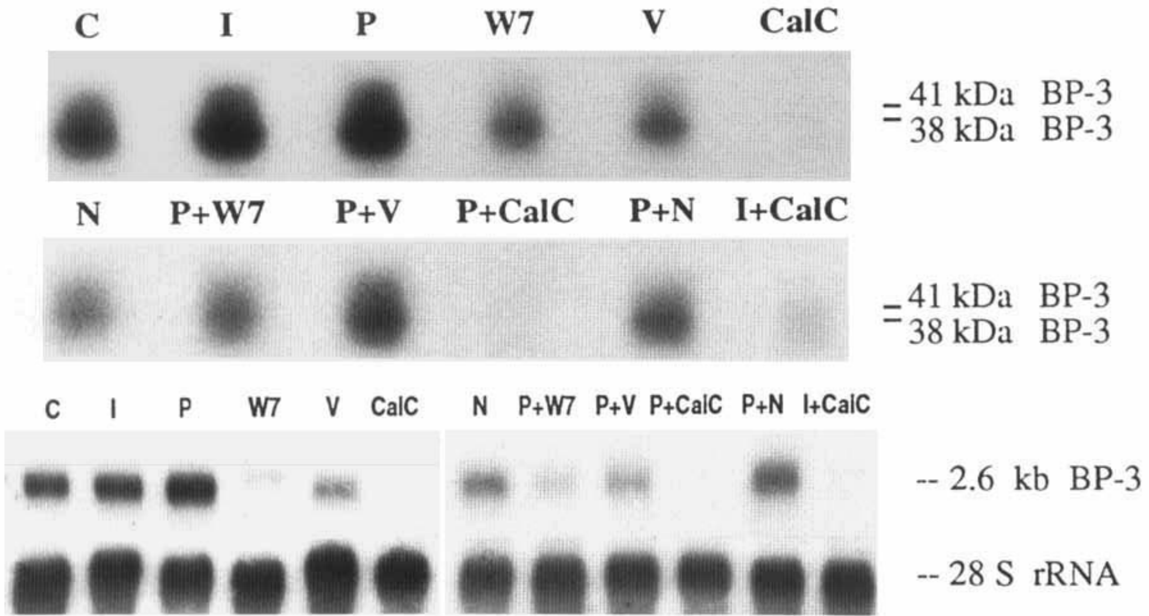

Fig. 3. Suppression of PGE_2 (P) stimulation of IGFBP-3 (BP-3) steady-state mRNA expression and synthesis in primary cultures of human articular chondrocytes by the Ca^{++}/calmodulin inhibitor W7, by the Ca^{++} channel blockers (verapamil, V; nifedipine, N), and by the Ca^{++}-phospholipid dependent protein kinase C inhibitor, Calphostin C (CalC). Cells were incubated without (control, C) or with PGE_2 (P, 100 ng/mL; 28.3×10^{-8} M) in the presence or absence of W7 (25 μM), V (50 μM), N (10 μM), and CalC (100 nM) for 24 h. The latter inhibitors were added 1 h before the addition of PGE_2. Ionomycin (I, 20 nM), a Ca^{++} channel ionophore, was added in the presence or absence of CalC under the same culture conditions. Conditioned medium was collected, concentrated, and analyzed by Western ligand blot analysis as described in Materials and Methods. Cell monolayers were extracted for total RNA and 10 μg was subjected to Northern analysis with probes for IGFBP-3 and 28S rRNA. A representative Northern blot is shown above.

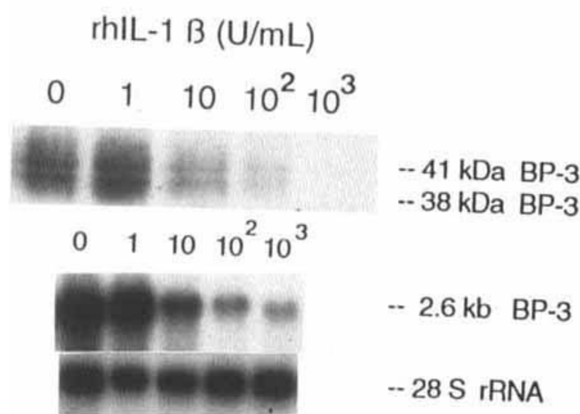

Fig. 4. Dose-dependent suppression by rhIL-1β of IGFBP-3 (BP-3) steady-state mRNA expression and synthesis using primary cultures of human articular chondrocytes. Cells were incubated with or without increasing concentrations of rhIL-1β for 24 h. Conditioned medium was collected, concentrated, and analyzed by Western analysis as described in Materials and Methods. Cell monolayers were extracted for total RNA and 10 μg was subjected to Northern analysis with probes for IGFBP-3 and 28S rRNA.

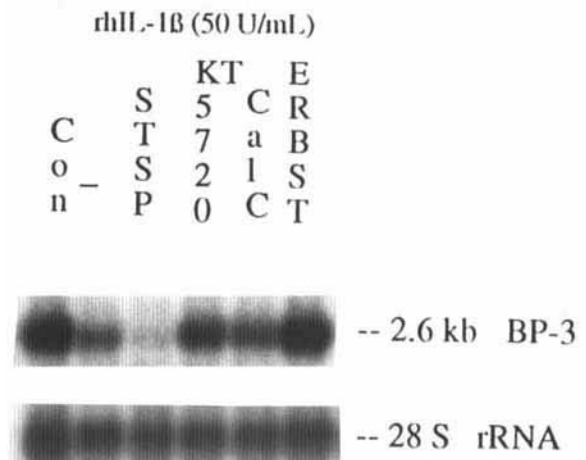

Fig. 5. Effect of serine-threonine and tyrosine kinase inhibitors on the rhIL-1β induced suppression of IGFBP-3 steady-state mRNA expression (BP-3) in primary cultures of human articular chondrocytes. Cells were incubated with or without (Con) rhIL-1β in the presence or absence of staurosporine (50 nM, STSP), KT-5720 (250 nM), Calphostin C (250 nM, Cal C), and erbstatin (50 μM, ERBST) for 24 h. Cells were extracted for total RNA and 10 μg was subjected to Northern analysis (see Materials and Methods) with probes for IGFBP-3 and 28S rRNA. The size of the hybridization band was estimated from the relative migration rate of 18S and 28S rRNA.

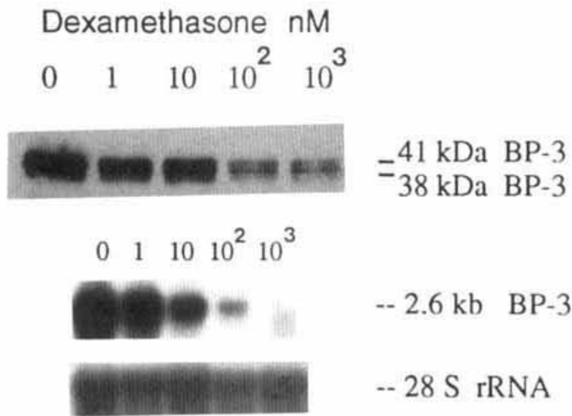

Fig. 6. Dose-response suppression by dexamethasone (DEX) of IGFBP-3 (BP-3) steady-state mRNA expression and synthesis using primary cultures of human articular chondrocytes. Cells were incubated with or without increasing concentrations of DEX for 24 h. Conditioned medium was collected, concentrated, and analyzed by Western analysis as described in Materials and Methods. Cell monolayers were extracted for total RNA and 10 μg was subjected to Northern analysis with probes for IGFBP-3 and 28S rRNA.

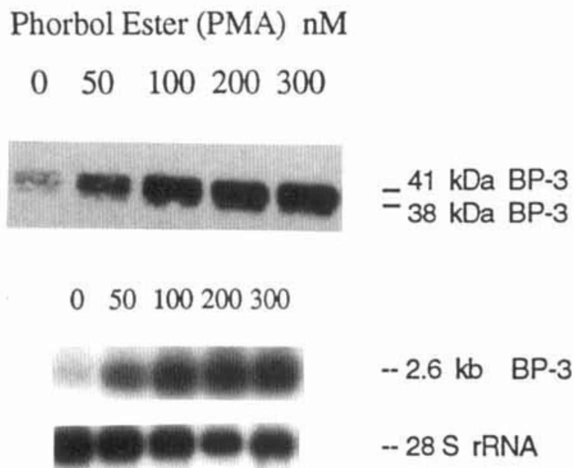

Fig. 7. Dose-response stimulation by the phorbol ester, PMA, of IGFBP-3 (BP-3) steady-state mRNA expression and synthesis using primary cultures of human articular chondrocytes. Cells were incubated with or without increasing concentrations of PMA for 18 h. Conditioned medium was collected, concentrated, and analyzed by Western ligand blot analysis as described in Materials and Methods. Cell monolayers were extracted for total RNA and 10 μg was subjected to Northern analysis with probes for IGFBP-3 and 28S rRNA.

Induction required a maximum of 8 h (Fig. 8) and was preceded by a major up-regulation of total cellular PKC activity (particulate and soluble) (Fig. 9) which began after 30 min, reached a maximum at 4 h, and declined rapidly to control values at about 18 h. Maximal PMA induced PKC activity was suppressed by 91 ± 7%

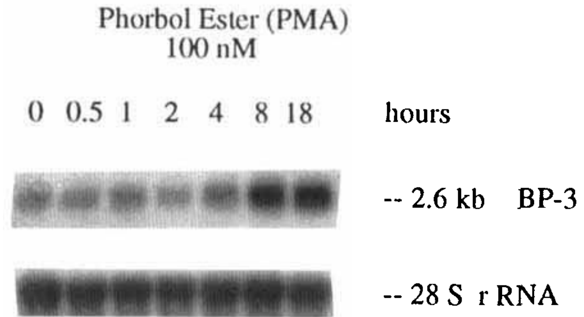

Fig. 8. Time course of PMA induction of IGFBP-3 (BP-3) mRNA expression using primary cultures of human articular chondrocytes. Cells were incubated with or without 100 nM of PMA for up to 18 h. Cell monolayers were extracted for total RNA and 10 μg was subjected to Northern analysis with probes for IGFBP-3 and 28S rRNA.

(n = 7 determinations) when chondrocytes were pre-incubated with 100 nM of Calphostin C.

In order to ascertain whether protein phosphatases, which play a role in the overall regulation of cellular phosphorylation, could modulate IGFBP-3 in human chondrocytes, we performed time-course experiments with okadaic acid (OKA), a potent inhibitor of the serine-threonine protein phosphatases 1 and 2A. Surprisingly, we found that it had no discernible effect either at the level of message or protein (Fig. 10).

DISCUSSION

This study was prompted by, among other interests, our recent observation that PGE_2 can stimulate the expression and synthesis of IGF-1 and the incorporation of proline into collagenase-digestible proteins in human articular chondrocytes. Furthermore, the eicosanoid-stimulated incorporation was reversed by co-incubations with anti-IGF-1 antibodies suggesting that perhaps IGF-1 actually mediated the response. Since IGFBPs are important modifiers of IGF-1 action, we wondered whether PGE_2 could influence the expression and synthesis of IGFBP-3 and modify the actions of IGF-1 on another level. The maintenance of human chondrocyte phenotype in terms of collagen type II synthesis by PGE_2 has been suspected for some time. PGE_2 stimulates COL2A1 promoter activity in transfected human chondrocytes [Goldring and Suen, 1994] and analysis of deletion constructs indicated that elements responsive to IL-1 and PGE_2 resided in the upstream promoter region of COL2A1. However, no canonical PGE_2 (i.e., cAMP) response elements have been identified in the promoter region of the gene [Ryan et al.,

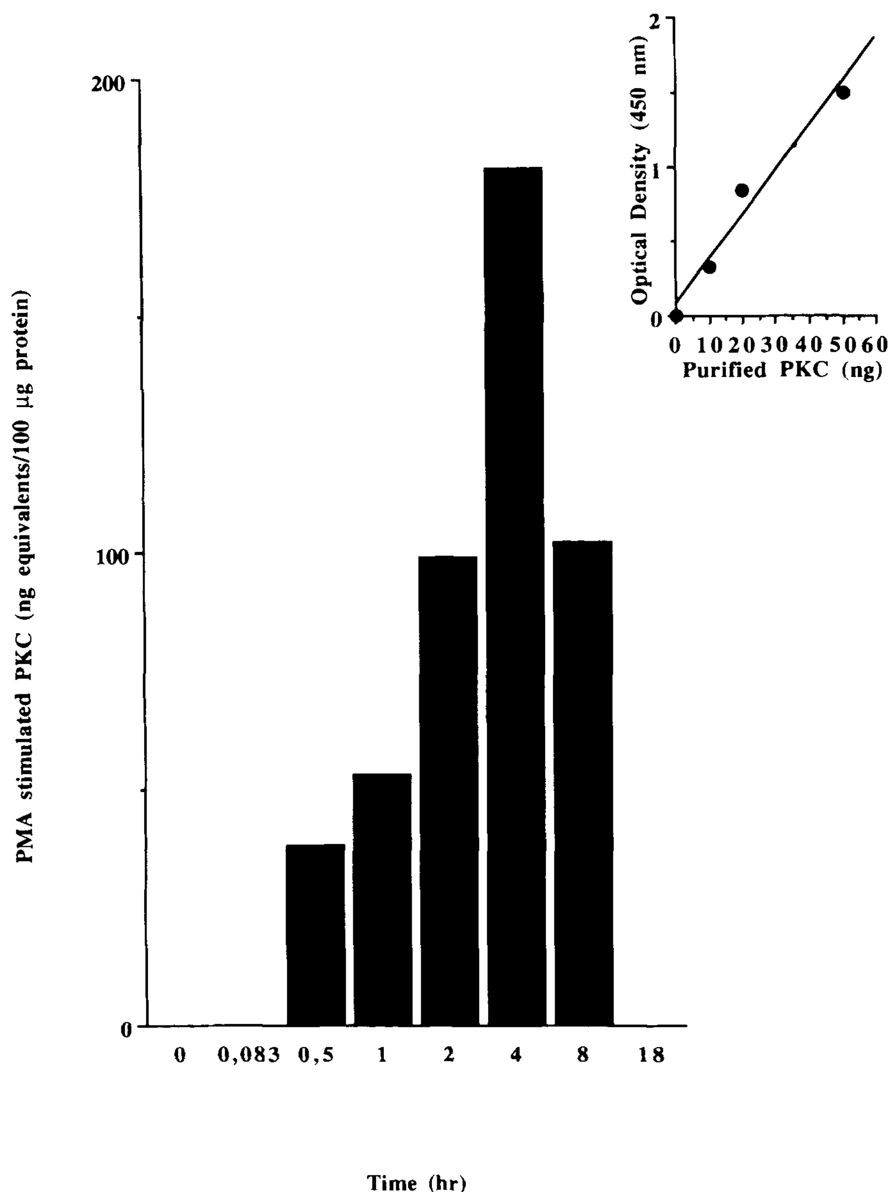

Fig. 9. Time course of PMA activation of human chondrocyte PKC. Cells were incubated with or without 100 nM of PMA for up to 18 h. Cell monolayers were extracted for total cellular protein at the appropriate times and the PKC activity estimated as described in Materials and Methods.

1990; Wang et al., 1991]. Inasmuch as IGF-1 favours collagen type II expression in human cartilage [Sandell and Dudek, 1988] while IGFBP-3 could modulate this effect by sequestering IGF-1, chondrocyte phenotype may be influenced by the ability of the eicosanoid to influence the synthesis of IGF-1 and IGFBP-3. Given the avascular nature of cartilage, there is limited ability for repair and thus local factors that maintain homeostasis are extremely important to the integrity and quality of cartilage [Malemud, 1993]. Indeed, we envisage PGE_2 modulation of cartilage anabolism largely by autocrine/paracrine mechanisms.

Prostaglandin E_2 may modulate IGFBP-3 production in human chondrocytes by transcriptional means since changes in steady-state message levels were matched by changes in the amount of IGFBP-3 released into the medium. Furthermore, we detected no extracellular IGFBP-3-protease activity (as judged by comparing data from Western and Western ligand blots) that might influence the levels found in the conditioned medium both in control or experi-

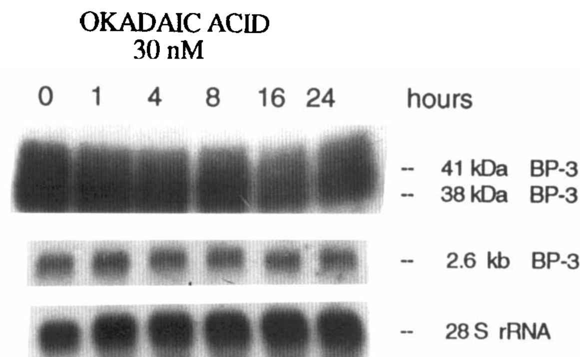

Fig. 10. Effect of okadaic acid on IGFBP-3 (BP-3) steady-state mRNA expression and synthesis using primary cultures of human articular chondrocytes. Cells were incubated with or without okadaic acid (30 nM) for 24 h. Conditioned medium was collected, concentrated, and analyzed by Western analysis as described in Materials and Methods. Cell monolayers were extracted for total RNA and 10 μg was subjected to Northern analysis with probes for IGFBP-3 and 28S rRNA.

mental cultures. Most of the IGFBP-3 specific proteolytic activity has been identified as being serine and/or metal (e.g., metalloproteases) dependent enzymes [Binoux et al., 1991] and PGE_2 is a powerful inhibitor of the expression and synthesis of metalloproteases produced by connective tissue cells [DiBattista et al., 1994].

Through the manipulation of cellular cAMP levels by different pharmacological means, it would appear that the expression of IGFBP-3 is negatively regulated by elevations in the levels of cAMP. No canonical cAMP response elements (CRE) have been identified in the promoter region of the human IGFBP-3 gene but potential AP-2 and SP-1 sites have been found and it is known that the AP-2 consensus sequence can act as a cAMP-responsive element [Cubbage et al., 1990]. In addition, growth factor, IGF-1, and insulin response elements that may mediate the stimulatory effects of these substances have also not been identified. The use of more specific inhibitors of phosphodiesterase (PDE) such as R0-20-1724, and milrinone has permitted us to highlight the importance of cAMP-specific PDE (cAMP-PDE) in the signalling cascade initiated by the elevation in cAMP in human chondrocytes [Conti et al., 1991; Sette et al., 1994]. Milrinone, a potent inhibitor of cGMP-inhibited PDE, had no discernible effect on IGFBP-3 expression despite the fact that the latter PDE has a higher affinity for cAMP (i.e., hydrolysis) than the cAMP-PDE [Beavo and Reifsnyder, 1990; Nicholson et al., 1991]. Cyclic AMP-PDE is hormonally regulated in certain cell types and its activity is dependent on phosphorylation by PKA [Conti et al., 1991]. The situation is less clear in human chondrocytes but our results, though circumstantial, suggest that cAMP dependent PDEIV is constitutively activated and surface signalling (e.g., by PGE_2) through G protein-adenylate cyclase associated receptors may not result in elevations in cAMP. In synoviocytes by contrast, gradual metabolism of cAMP occurs after 10–20 min (see Table I) suggesting that PKA activation of c-AMP dependent PDEIV is inducible by extracellular signalling and not "constitutive." We explored the possibility that PGE_2 may signal chondrocytes by Ca^{++}/calmodulin mediated mechanisms. Ionomycin, a divalent cation ionophore, strongly upregulated IGFBP-3 expression and synthesis, possibly the result of changes in intracellular Ca^{++}/calmodulin activity and the subsequent diacylglycerol induced upregulation of PKC activity [Kiley and Jaken, 1994]. This response was quantitatively very similar to that brought about by incubations with PGE_2. IGFBP-3 induction by PGE_2 was reversed by W7 and also verapamil and nifedipine, both blockers of voltage-regulated Ca^{++} channels and Calphositn C. They altered, to one degree or another, the basal control production of IGFBP-3 and indeed the marked response induced by Calphostin C suggests a major dependence on PKC activity in this regard.

Our data show that dexamethasone (DEX), a potent antinflammatory glucocorticoid, substantially diminished the expression and synthesis of IGFBP-3. Similar results were obtained in normal human osteoblast-like cells [Okazaki et al., 1994]. Glucocorticoids have been shown to potentiate the effects of IGF-1 in terms of matrix protein synthesis (e.g., collagen) in bone cells and certain organ culture systems and these effects have been ascribed to the ability of these steroids to inhibit the expression and synthesis of IGFBP-3 and other binding proteins [Kream et al., 1990; Chen et al., 1991]. It would thus appear that this inhibitory effect is manifested at the pre-translational level. We showed that PGE_2 could induce a progressive upregulation in the levels of GR in connective tissue cells [DiBattista et al., 1991b] and it was thought that this would render the cells more sensitive to glucocorticoids [DiBattista et al., 1993] in terms of the expression of glucocorticoid sensitive genes like collagenase (MMP-1). Thus we speculated that there may cross-talk between the two signalling systems and that for some genes the GR medi-

ated the effect of PGE_2. The latter scenario would not seem to be the case for the IGFBP-3 gene as the two signals function in opposing directions. Still, glucocorticoids apparently exert protective effects on cartilage integrity in canine models of osteoarthritis [Pelletier and Martel-Pelletier, 1989] as does PGE_2 so that more work is needed to rationalize some inconsistencies. It should be added that glucocorticoids are known to affect other components of the IGF-1 signalling cascade as, for example, the number of IGF-1-receptor sites [Bennett et al., 1984] which could conceivably influence IGF-1 action as much as modulation of IGFBPs.

While it would appear that IGFBP-3 expression is negatively regulated by cAMP intracellular signalling, our results show that the tumour-promoting phorbol ester, PMA, upregulates the expression and synthesis of the BP in human chondrocytes. In human articular chondrocytes PMA stimulates an increase in PKC activity after about 15 min, peaks at 4 h, and then declines to control values after 18 h. The increase is probably due to a PMA induced post-translational modification of PKC given the rapidity of the response. As in other cell types, there is a rapid activation of *c-jun* expression which is evident after 15 min but declines to control levels in 2 to 3 h (unpublished observations). However, no AP-1 consensus elements have been identified in the promoter region of IGFBP-3 and more studies are required to rationalize these observations. Interestingly, another tumour promoter, OKA, which is a specific inhibitor of protein phosphatases [Cohen and Cohen, 1989], has no affect on the expression and synthesis of IGFBP-3 in human chondrocytes. Unlike PMA, OKA does not activate PKC but does induce c-jun and c-fos and AP-1, SP-1, and NF-κB DNA binding activity albeit over a much different time course (8–16 h) [Thévenin et al., 1990, 1991; Schonthal, 1992]. The observation that OKA does not influence IGFBP-3 expression in the manner of PMA, stands in marked contrast to other genes like collagenase and stromelysin which are dramatically induced by both tumour promoters [Westermarck et al., 1994]. This suggests that the inhibition of the serine-threonine protein phosphatases, PP-1 and PP-2A, profoundly affects the state of signalling pathways controlling the expression of some genes but not others. This is confounding because the signalling pathways (kinases, transcription factors) appear to be very similar in those genes affected by OKA (e.g., collagenase) and those that are not (e.g., IGFBP-3). One possibility is that OKA induces the generation of H_2O_2 and superoxide free radicals [Schmidt et al., 1995] in certain cell types but PMA does not. The cytokine induced expression of *c-fos* (and collagenase) has been shown to be dependent on the production of O_2 radicals [Lo and Cruz, 1995] although to our knowledge the relationship between the generation of free radicals and IGFBP-3 expression has not been explored.

Recently, it was demonstrated that IL-1α (and TNFα) increased the production of IGFBP-3 protein by human chondrocytes in culture [Olney et al., 1995]. They proposed that the cartilage matrix changes seen in inflammatory arthritides may be due to the reduction of IGF-1 anabolic activity mediated through the cytokine-induced increase in chondrocyte derived IGFBP-3. Conceivably, IL-1 could therefore be responsible for the increased amounts of IGFBP-3 found in synovial fluid of RA and OA patients [Reboul et al., 1994]. We are not able to confirm these results and indeed there was a marked inhibition of IGFBP-3 protein production as judged by two blotting techniques. Furthermore, steady-state IGFBP-3 mRNA levels were also concomitantly down-regulated. We observed inhibition with as little as 50 pg/mL (2.5 pmol/L) of rhIL-1β and there was a dose-dependency to the response. Interleukin-1β and Il-1α have very similar affinities for the chondrocyte IL-1 receptor [Martel-Pelletier et al., 1992] and generally the cytokines induce target genes of chondrocytes at a very low receptor occupancy rate (1–2%) [DiBattista et al., 1991a]; there is therefore a significant spare-receptor phenomenon in the IL-1 receptor system. In our experiments, PGE_2 does not mediate the IL-1 induced inhibitory although the eicosanoid is a major product of IL-1 action on chondrocytes with up to hundreds of ng/mL accumulating in the culture medium after 48 h under IL-1 activation. This should be more than enough to feed back on the cell and affect IGFBP-3 production. It would seem that an erbstatin-sensitive kinase is responsible for the IL-1β induced suppression of IGFBP-3 in addition to the observation that a PKA inhibitor could at least partially reverse this action. This may not be surprising since cAMP signalling pathways have been shown to be important in IL-1β induced transcriptional regulation in 3T3 and T-helper cells [Bonin et al., 1990; Munoz et al., 1990]. Further studies

will be needed to identify this (these) kinase activity(ies) because a good deal of evidence has accumulated suggesting that PKC probably is a mediator of IL-1 action in a number of cell types [Case et al., 1990; Bunning et al., 1986; Conca et al., 1989]. One cannot rule out the possibility that there exists target gene specificity in terms of signal pathways activated by the cytokine or other cell specific factors.

Finally, it is tempting to speculate that PGE_2 induction of IGFBP-3 is mediated by IGF-1 since the latter growth factor is a known inducer of IGF binding proteins. However, while the former process occurs well within 24 h, PGE_2 stimulation of IGF-1 production requires 48–96 h with human articular chondrocytes lessening the likelihood of an IGF-1 mediated event.

In summary, IGFBP-3 is up-regulated by PGE_2 probably through a Ca^{++}/calmodulin sensitive process and not through cAMP signalling pathways. Consistent with these observations are the findings that the Ca^{++} phospholipid dependent PKC increases IGFBP-3 production; PKA mediates an opposing effect. Prostaglandin E_2 stimulation of IGFBP-3 may contribute to the overall anabolism of cartilage by favouring IGF-1 action although the role of IGFBP-3 in this regard in this unclear in any cell or tissue type.

ACKNOWLEDGMENTS

This research was supported by grants (J.D.B.) from the Canadian Arthritis Society and the Medical Research Council of Canada. The authors thank Dr. M. Zafarullah for his critical review of the manuscript and Dr. J.-P. Pelletier for providing us with human chondrocytes.

REFERENCES

An S, Yang J, So SW, Zeng L, Goetzl EJ (1994): Isoforms of the EP3 subtype of human prostaglandin E_2 receptor transduce both intracellular calcium and cAMP signals. Biochemistry 33:14496–14502.

Beavo JA, Reifsnyder DH (1990): Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends Pharmacol Sci 11:150–155.

Bennett A, Chen TL, Feldman D, Hintz RI, Rosenfeld RG (1984): Characterization of insulin-like growth factor I receptors on cultured rat bone cells: regulation of receptor concentration by glucocorticoids. Endocrinology 115:1577–1583.

Bichell DP, Rotwein P, McCarthy TL (1993): Prostaglandin E_2 rapidly stimulates insulin-like growth factor I gene expression in primary rat osteoblast cultures: Evidence for transcriptional control. Endocrinology 133:1020–1028.

Binoux M, Hossenlopp P, Lassare C, Segovia B (1991): Degradation of IGF binding protein-3 by proteases: physiological implications. In Spencer EM (ed.) "Modern Concepts of Insulin-Like Growth Factors." Proc. 2nd. Int. Symposium Insulin-like Growth Factors/Somatomedins, Elsevier, pp 329–336.

Bonin PD, Chiou WJ, McGee JE, Singh JP (1990): Two signal transduction pathways mediate interleukin-1 receptor expression in Balb/c3T3 fibroblasts. J Biol Chem 265:18643–18649.

Bunning RAD, Richardson HJ, Crawford A, Skjodt H, Hughes D, Evans DB, Gowen M, Dobson PR, Brown BL, Russell RG (1986): The effects of interleukin-1 on connective tissue metabolism and its relevance to arthritis. Agents Actions 18:131–152.

Burton K (1956): A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–323.

Case JP, Lafyatis R, Kumkumian GK, Remmers EF, Wilder RL (1990): IL-1 regulation of transin/stromelysin transcription in rheumatoid synovial fibroblasts appears to involve two antagonistic transduction pathways, an inhibitory, and a stimulatory, protein kinase C dependent pathway. J Immunol 145:3755–3761.

Chan CC, DiBattista JA, Zhao J, Pelletier J, Martel-Pelletier J, Kennedy BP, Brideau C, Rodger IW (1995): Induction of cyclooxygenase-2 (COX-2) in human articular chondrocytes and inhibition of PGE_2 synthesis by a selective COX-2 inhibitor. Inflammation Res 44 (Suppl 3):S273.

Chen TL, Chang LY, Bates RL, Perlman AJ (1991): Dexamethasone and 1, 25-dihydroxyvitamin D3 modulation of insulin-like growth factor binding proteins in rat osteoblast-like cell cultures. Endocrinology 128:73–80.

Cohen P, Cohen PTW (1989): Protein phosphatases come of age. J Biol Chem 264:21435–21438.

Cohen P, Graves HCB, Peehl DM, Kamarei M, Giudice LC, Rosenfeld RG (1991) Prostate specific antigen (PSA) is an IGF binding protein-3 (IGFBP-3) protease found in seminal plasma. J Clin Endocrinol Metab 73:391–407.

Conca W, Kaplan PB, Krane SM (1989): Increase in levels of procollagenase messenger RNA in cultured fibroblasts induced by recombinant interleukin-1 beta or serum follow c-jun expression and are dependent on new protein synthesis. J Clin Invest 83:1753–1756.

Conti M, Catherine Jin SL, Monaco L, Repaske DR, Swinnen JV (1991): Hormonal regulation of cyclic nucleotide phosphodiesterases. Endocrine Rev 12:218–233.

Cubbage ML, Suwanichkul A, Powell DR (1990): Insulin-like growth factor binding protein-3: Organization of the human chromosomal gene and demonstration of promoter activity. J Biol Chem 265:12642–12649.

DeMellow JSM, Baxter RC (1988): Growth hormone dependent insulin-like growth factor binding protein both inhibits and potentiates IGF-1 stimulated DNA synthesis in skin fibroblasts. Biochem Biophys Res Commun 156:199–204.

DiBattista JA, Martel-Pelletier J, Wosu LO, Sandor T, Antakly T, Pelletier JP (1991a): Glucocorticoid receptor mediated inhibition of interleukin-1 stimulated neutral metalloprotease synthesis in normal human chondrocytes. J Clin Endocrinol Metab 72:316–326.

DiBattista JA, Martel-Pelletier J, Cloutier JM, Pelletier J-P (1991b): Modulation of glucocorticoid receptor expression in human articular chondrocytes by cAMP and prostaglandins. J Rheumatol 18:102–105.

DiBattista JA, Martel-Pelletier J, Antakly T, Tardif G, Cloutier J-M, Pelletier JP (1993): Reduced expression of glucocorticoid receptor levels in human osteoarthritic chondrocytes. Role in the suppression of metalloprotease synthesis. J Clin Endocrinol Metab 76:1128–1134.

DiBattista JA, Martel-Pelletier J, Kazushi I, Nagai Y, Zafarullah M, Pelletier JP (1994): Prostaglandins E_2 and E_1 inhibit the cytokine-stimulated expression of metalloproteinases in normal human synovial fibroblasts: Mediation by cyclic AMP signalling pathway. Lab Invest 71:223–270.

DiBattista JA, Abribat T, Martel-Pelletier J, Morin N, Pelletier JP (1995a): Prostaglandin E_2 up-regulation of IGF-1 and IGFBP-3 expression and synthesis in human articular chondrocytes. In Transactions of the 41th Annual Meeting of the Orthopaedic Research Society, Orlando, FL, p 374.

DiBattista JA, Pelletier JP, Zafarullah M, Fujimoto N, Obata K, Martel-Pelletier J (1995b): Coordinate regulation of matrix metalloproteases and tissue inhibitor of metalloproteinase expression in human synovial fibroblasts. J Rheumatol 22:123–128.

DiBattista JA, Doré S, Morin N, Abribat T, Pelletier JP: Prostaglandin E_2 stimulated incorporation of proline into collagenase digestible proteins is mediated by insulin-like growth factor I in human articular chondrocytes. Mol Cell Endocrinol (in press).

Doré S, Pelletier JP, DiBattista JA, Tardif G, Brazeau P, Martel-Pelletier J (1994): Human OA chondrocytes possess an increased number of binding sites to IGF-1 but are irresponsive to its stimulation: Possible role of binding proteins. Arthritis Rheum 37:253–263.

Evans CH (1993): Cytokines: Molecular keys to homeostasis, development, and pathophysiology. J Cell Biochem 53:277–279.

Franchimont P, Bassler C (1991): Effects of hormones and local growth factors on articular chondrocyte metabolism. J Rheumatol 18(Suppl 27):68–70.

Goldring MB, Suen LF (1994): Immortalized human chondrocytes: A model of osteoarthritis. Osteoarthritis and Cartilage 2(Suppl 1):33.

Harris ED Jr (1990): Rheumatoid arthritis: Pathophysiology and implications for therapy. N Engl J Med 291:1285–1292.

Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M (1986): Analysis of serum insulin-like growth factor binding proteins using Western blotting: Use of a method for titration of the binding proteins and competitive binding studies. Anal Biochem 154:138–143.

Inagaki M, Gonda Y, Nishizawa K, Kitamura S, Sato C, Ando S, Tanabe K, Kikuchi K, Tsuiki S, Nishi Y (1990): Phosphorylation sites linked to glial filament disassembly in vitro locate in a non-α-helical head domain. J Biol Chem 265:4722–4729.

Irie A, Segi E, Sugimoto Y, Ichikawa A, Negishi M (1994): Mouse prostaglandin E receptor EP3 subtype mediates calcium signals via Gi in cDNA transfected chinese hamster ovary cells. Biochem Biophys Res Commun 204:303–309.

Jones JI, Clemmons DR (1995) Insulin-like growth factors and their binding proteins: Biological actions. Endocrine Rev. 16:3–34.

Kiley SC, Jaken S (1994): Protein kinase C: Interactions and consequences. Trends Cell Biol 4:223–227.

Knudsen PJ, Dinarello CA, Strom TB (1986): Prostaglandins post-transcriptionally inhibit monocyte expression of interleukin-1 activity by increasing intracellular cyclic adenosine monophosphate. J Immunol 137:3189–3194.

Kream BE, Petersen DN, Raisz LG (1990): Cortisol enhances the anabolic effects of insulin-like growth factor I on collagen synthesis and procollagen messenger ribinucleic acid levels in cultured 21-day fetal rat calvariae. Endocrinology 126:1576–1583.

Lamson G, Cohen P, Giudice LC, Fielder PJ, OH Y, Hintz RL, Rosenfeld RG (1993): Proteolysis of IGF BP-3 may be a common regulatory mechanism of IGF-1 action in vivo. Growth Regul 3:91–95.

Lo YY, Cruz TF (1995): Involvement of reactive oxygen species in cytokine and growth factor induction of *c-fos* expression in chondrocytes. J Biol Chem 270:11727–11730.

Malemud CJ (1993): Role of growth factors in cartilage metabolism. In Moskowitz RW (ed): "Rheumatic Disease Clinics in North America." Philadelphia: W.B. Saunders Company, pp 569–580.

Mankin HJ (1974): The reaction of articular cartilage to injury and osteoarthritis. N Engl J Med 291:1285–1292.

Martel-Pelletier J, McCollum R, DiBattista JA, Faure MP, Chin J, Cloutier JM, Pelletier JP (1992): The interleukin-1 receptor in normal and osteoarthritic human articular chondrocytes: Identification as the type-1 receptor and analysis of binding kinetics and biologic function. Arthritis Rheum 35:530–540.

Mauviel A (1993): Cytokine regulation of metalloproteinase gene expression. J Cell Biochem 53:288–295.

McCarthy TL, Centrella M, Raisz LG, Canalis E (1991): Prostaglandin E_2 stimulates insulin-like growth factor I synthesis in osteoblast-enriched cultures from fetal rat bone. Endocrinology 128:2895–2900.

Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA, Smithies O (1995): Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 83:473–482.

Munoz E, Beutner U, Zubiaga A, Huber BT (1990): Il-1 activates two separate signal transduction pathways in T helper type II cells. J Immunol 144:964–969.

Nicholson CD, Challiss RAJ, Shahid M (1991): Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isenzymes. Trends Pharmacol Sci 12:19–27.

Oh Y, Muller HL, Lamson G, Rosenfeld RG (1993a): Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. Cell surface binding, growth inhibition. J Biol Chem 268: 14964–14971.

Oh Y, Muller HL, Pham H, Rosenfeld RG (1993b): Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. J Biol Chem 268:26045–26048.

Okazaki R, Riggs BL, Conover CA (1994): Glucocortioid regulation of insulin-like growth factor binding protein expression in normal human osteoblast-like cells. Endocrinology 134:126–132.

Olney RC, Wilson DM, Mohtai M, Fielder PJ, Smith RL (1995): Interleukin-1 and tumor necrosis factora increase insulin-like growth factor-binding protein-3 (IGFBP-3) production and IGFBP-3 protease activity in human articular chondrocytes. J Endocrinol 146:279–286.

Pelletier JP, Martel-Pelletier J (1989): Protective effects of corticosteroids on cartilage lesions and osteophyte formation in the Pond-Nuki dog model of osteoarthritis. Arthritis Rheum 2:181–193.

Pelletier JP, Roughley PJ, DiBattista JA, McCollum R, Martel-Pelletier J (1991): Are cytokines involved in osteoarthritic pathophysiology? Semin Arthritis Rheum 20:12–25.

Raisz LG, Fall PM, Gabbitas BY, McCarthy TL, Kream BE, Canalis E (1993): Effects of prostaglandin E_2 on bone formation in cultured fetal rat calvariae: Role of insulin-like growth factor I. Endocrinology 133:1504–1510.

Reboul P, Tavera C, Abribat T, Pelletier JP, Brazeau P, Martel-Pelletier J (1994): Variations of the IGF/IGF binding protein system in the synovial fluid of OA and RA patients. Osteoarthritis Cartilage 2(Suppl 1):33.

Robinson DR (1985): Low molecular weight mediators of inflammation: Prostaglandins, leukotrienes, and cyclic nucleotides. In Kelly WN, Harris ED Jr, Ruddy S, Sledge CB (eds): "Textbook of Rheumatology." Philadelphia: W.B. Saunders Company, pp 71–82.

Ryan MC, Sieraski M, Sandell L (1990): The human type II collagen gene: Identification of an additional protein-coding domain and location of potential regulatory sequences in the promoter and first intron. Genomics 8:41–48.

Sandell LJ, Dudek EJ (1988): Insulin-like growth factor I stimulates type II collagen gene expression in cultured chondrocytes. In Transactions of the 35th Annual Meeting of the Orthopaedic Research Society, Atlanta, p 300.

Schmidt KN, Britta-Mareen Traenckner E, Meier B, Baeuerle PA (1995): Induction of oxidative stress by Okadaic Acid is required for activation of transcription factor NF-κB. J Biol Chem 270:27136–27142.

Schonthal A (1992): Okadaic acid: A valuable new tool for the study of signal transduction and cell cycle regulation. New Biol 4:16–21.

Sette C, Iona S, Conti M (1994) The short-term activation of a rolipram-sensitive, cAMP specific phosphodiesterase by thyroid-stimulating hormone in thyroid FRTL-5 cells is mediated by a cAMP-dependent phosphorylation. J Biol Chem 269:9245–9252.

Tesch GH, Handley CJ, Cornell HJ, Herington AC (1992): Effects of free and bound insulin-like growth factors on proteoglycan metabolism in articular cartilage explants. J Orthop Res 10:14–22.

Thévenin C, Kim SJ, Rieckmann P, Fujiki H, Norcross MA, Spron MB, Fauci AS, Kehrl JH (1990): Induction of nuclear factor-κB and the human immunodeficiency virus long terminal repeat by okadaic acid, a specific inhibitor of phosphatases 1 and 2A. New Biol 2:793–800.

Thévenin C, Kim SJ, Kehrl JH (1991): Inhibition of protein phosphatases by okadaic acid induced AP1 in human T cells. J Biol Chem 266:9363–9366.

Valentinis B, Bhala A, DeAngelis T, Baserga R, Cohen P (1995): The human insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-1 receptor gene. Mol Endocrinol 9:361–367.

Wang L, Balkir R, Horton WEJ (1991): Identification of a cis-acting sequence in the collagen II enhancer required for chondrocyte expression and the binding of a chondrocyte nuclear factor. J Biol Chem 266:19878–19881.

Westermarck J, Lohi J, Keski-Oja J, Kahari VM (1994): Okadaic acid-elicited transcriptional activation of collagenase expression in HT-1080 fibrosarcoma cells is mediated by JunB. Cell Growth Differ 5:1205–1213.

Wood WI, Cachianes G, Henzel WJ, Winslow GA, Spencer SA, Hellmiss R, Martin JL, Baxter RC (1988): Cloning and expression of the growth hormone-dependent insulin-like growth factor binding protein. Mol Endocrinol 2:1176–1185.

Yano T, Taura C, Shibata M, Hirono Y, Ando S, Kusubata M, Takahashi T, Inagaki M (1991): A monoclonal antibody to the phosphorylated form of glial fibrillary acidic protein: Application to a non-radioactive method for measuring protein kinase activities. Biochem Biophys Res Commun 175:1144–1151.