Journal of Cellular Biochemistry 65:408–419 (1997)

Prostaglandin E_2 Stimulates Insulin-Like Growth Factor Binding Protein-4 Expression and Synthesis in Cultured Human Articular Chondrocytes: Possible Mediation by Ca^{++}-Calmodulin Regulated Processes

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Abstract Insulin-like growth factor-1, IGF-1, is believed to be an important anabolic modulator of cartilage metabolism whose action is mediated by high affinity cell surface receptors and bioactivity and bioavailability regulated, in part, by IGF-1 binding proteins (IGFBPs). Prostaglandin E_2 (PGE_2) stimulates collagen and proteoglycan synthesis in cartilage via an autocrine feedback loop involving IGF-1. We determined whether the eicosanoid could regulate IGFBP-4, a major form expressed by chondrocytes and, as such, act as a modifier of IGF-1 action at another level. Using human articular chondrocytes in high-density primary culture, Western and Western ligand blotting to measure secreted IGFBP-4 protein, and Northern analysis to monitor IGFBP-4 mRNA levels, we demonstrated that PGE_2 provoked a 2.7 ± 0.3- and 3.8 ± 0.5- (n = 3) fold increase in IGFBP-4 mRNA and protein, respectively. This effect was reversed by the Ca^{++} channel blocker, verapamil, and the Ca^{++}/calmodulin inhibitor, W-7. The Ca^{++} ionophore, ionomycin, mimicked the effects of PGE_2. The phorbol ester, PMA, which activated phospholipid-dependent protein kinase C (PKC) in chondrocytes, had no effect on IGFBP-4 production. Cyclic AMP mimetics and PKA activators, IBMX, and Sp-cAMP, inhibited the expression of the binding protein as did the PGE_2 secretagogue, interleukin-1β (IL-β). The inhibitory effect of the latter cytokine was mediated by a erbstatin/genistein (tyrosine) sensitive kinase. Dexamethasone, an inhibitor of cyclooxygenase (COX-2) expression and PGE_2 synthesis, down-regulated control, constitutive levels of IGFBP-4 mRNA and protein, eliminating the previously demonstrated possibility of cross-talk between glucocorticoid receptor (GR) and PGE_2-receptor signalling pathways. The results suggest that extracellular signals control IGFBP-4 production by a number of different transducing networks with changes in Ca^{++} and calmodulin activity exerting a strong positive influence, possibly maintaining the constitutivity of IGFBP-4 synthesis under basal conditions. PGE_2 activation of the IGF-1/IGFBP axis may play a pivotal role in the metabolism of cartilage and possibly connective tissues in general. Eicosanoid biosynthesis may be a rate-limiting step in cartilage repair processes. J. Cell. Biochem. 65:408–419. © 1997 Wiley-Liss, Inc.

Key words: chondrocytes; calcium; calmodulin; binding proteins; gene expression

Growth factors like 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 stabilizes chondrocyte phenotype in pathological conditions where homeostasis is perturbed [Tesch et al., 1992; Franchimont and Bassler, 1991; Sandell and Dudek, 1988]. 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 functional-

Contact grant sponsors: Canadian Arthritis Society, Medical Research Council of Canada.

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Received 21 October 1996; accepted 22 January 1997

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ity of the IGFBPs [Lamson et al., 1993; Cohen et al., 1991].

Insulin-like growth factor binding protein-3 and -4 are secreted in the greatest abundance of all IGFBPs by human chondrocytes in culture and are produced in dramatically increased amounts in rheumatoid (RA) and osteoarthritic (OA) chondrocytes [Doré et al., 1994; Olney et al., 1996; Di Battista et al., 1996a]. The synovial fluids of OA and RA patients have markedly increased levels of IGFBP-3 and -4 vis-à-vis fluid aspirated from the joints of normal donors [Matsumoto et al., 1996; Kanety et al., 1996]. Indeed, the concentration of IGFBP-4 in inflammatory synovial fluid is significantly higher than that measured in serum [Kanety et al., 1996], suggesting that local production by joint tissues is of some consequence physiologically.

Secreted IGFBP-3 associates preferentially with the cell surface [Doré et al., 1994; Oh et al., 1993a,b] while IGFBP-4 appears to bind strongly to the extracellular matrix [Jones and Clemmons, 1995]. IGFBP-3 can potentiate the action of IGF-1 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, IGFBP-3 may act independently of IGF-1 in inhibiting cell growth of mouse embyro fibroblasts transfected with IGFBP-3 and having a targeted disruption of the IGF-1 receptor (null) gene [Valentinis et al., 1995]. In contrast, IGFBP-4 inhibits IGF-1 actions under most, if not all, experimental conditions [Jones and Clemmons, 1995; Rechler, 1995], although there are IGF-1-dependent IGFBP-4 proteases secreted by a number of cell types that regulate the bioavailability of IGFBP-4. Interestingly, IGFBP-3 can function as an IGF-1-reversible inhibitor of IGFBP-4 proteolysis [Fowlkes et al., 1995], suggesting that proteolysis is dependent on the relative proportions of the different binding proteins and the level of IGFs [Donnelly and Holly, 1996]. Thus, elucidating the mechanisms controlling IGFBP-3 and -4 expression and synthesis is important to understanding the modulation of IGF-1-mediated metabolism in both normal cartilage and diseased states.

In normal human chondrocyte cultures, prostaglandin E_2 (PGE_2) induces the expression and synthesis of IGFBP-3 and IGF-1 [Di Battista et al., 1996a,b]. Prostaglandin E_2 induced short-term changes in cellular Ca^{++}/calmodulin activity are responsible for this induction but there does not appear to be a cAMP-protein kinase A signalling cascade. Downstream Ca^{++}/calmodulin-regulated kinases and/or phosphodiesterases may mediate PGE_2 chondrocyte signalling since W-7 uncoupled the inductive effects but no PGE_2-sensitive phosphatases were involved. In chondrocytes, PGE_2 stimulation of matrix macromolecular synthesis (e.g., collagen) is mediated by IGF-1 through a tight autocrine feedback loop; excess production of IGFBP-3 can block this response [Doré et al., 1994; Di Battista et al., 1996b]. Physiologically speaking, PGE_2 can be found in quantity at sites of inflammation and, indeed, chondrocytes produce massive amounts when activated [Chan et al., 1995]. There is a growing appreciation for the anticatabolic and proanabolic effects of PGE_2 in terms of connective tissue metabolism and the IGF/IGFBP axis is just one of many cellular regulatory systems affected by the eicosanoid [Di Battista et al., 1996a,b; Pash and Canalis, 1996; McCarthy et al., 1996].

Though we have recently begun to elucidate the molecular mechanisms by which PGE_2 regulates IGF-1 and IGFBP-3, even less is known about eicosanoid control of IGFBP-4 synthesis in connective tissue cells. We determined whether PGE_2, and substances known to modulate the production of PGE_2 (i.e., IL-1, glucocorticoids), could influence IGFBP-4 synthesis and expression and, as such, act as hormonal modifiers of IGF-1 action at a completely different level. These experiments appeared justified on the basis of the relative abundance of IGFBP-4 and PGE_2 in arthritic synovial fluid and connective tissues, and the fact that the IGFBP-3/IGFBP-4/IGF-1 axis may be important in maintaining cartilage homeostasis and the biological activity of IGFBP-4 may depend on the presence of IGFBP-3.

MATERIALS AND METHODS

Chemicals

Anti-IGFBP-4 (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′-dideoxyadenosine (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-methoxy-benzyl)-2-imidazolidinone), calphostin-C, verapamil, W-7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl), Sp-cAMP, ionomycin, and phorbol 12-myristate-13-acetate (PMA) 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-(hydroxymethyl)-aminomethane), NaCl, $MgCl_2$, $CaCl_2$, 8-hydroxyquinoline, formamide, formaldehyde, and ethanol were from Fisher Scientific (Montreal, Quebec).

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 [Di Battista 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-4

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; Di Battista 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-4 (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, Oakville, Ontario). 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-4. In addition the immunoblots provided some indication of proteolysis.

Northern Blot Analysis of IGFBP-4 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-treated 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 a random primed Digoxigenin (DIG)-deoxy UTP labeled cDNA probe [EcoRI, HindIII fragment corresponding to 505 bp of the published coding sequence encompassing amino acid 48 to 216 [Shimasaki et al., 1990] and cloned into pBS SK+] for IGFBP-4 kindly provided by Dr. S. Shimasaki of the Scripps Research Institute,

La Jolla, CA, for 24 h at 50°C. All blots were subjected to laser scanning densitometry (GS-300 Hoefer Scientific Instruments) for semi-quantitative measurements with the relative amount of IGFBP-4 mRNA normalized to the level of 28S rRNA.

Analysis of Protein Kinase Activity

These nonradioisotopic techniques are 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 or without (phosphatase assay) 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 auto-inhibitor 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 pmol 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-4 Protein and mRNA: Effect of cAMP Mimetics

Exposure of human articular chondrocytes to PGE$_2$ (1–10,000 nmol/L) resulted in a dose-dependent increase in the amount of IGFBP-4 (at 1,000 nmol/L, 3.8 ± 0.5-fold; band at 24 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 70 nmol/L. Routinely we also detected an intense doublet at 41/38, and minor bands at 34 and 31 kDa (Fig. 1B, hyper-exposure of control medium), which we previously identified, using specific antibodies, as being IGFBP-3, IGFBP-2, and IGFBP-5, respectively [Di Battista et al., 1996a; 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%, IGFBP-2 for 1–2%, and IGFBP-5 for trace amounts (see Fig. 1B). The increase in the 29.1 kDa (glycosylated form) and 24 kDa (nonglycosylated form) bands was verified by Western analysis and gave an identical pattern of upregulation (Fig. 1C). The results also confirmed that there was little, though still detectable, proteolytically induced fragmentation (14 kDa immunoreactive fragment) of IGFBP-4 in control culture medium. The bands disappeared in the presence of increasing concentrations of PGE$_2$. The 29.1 kDa form represents no more than 4–7% of the total immunoreactive/IGF-1 binding IGFBP-4.

In order to ascertain if the stimulatory pattern was manifested at the level of IGFBP-4

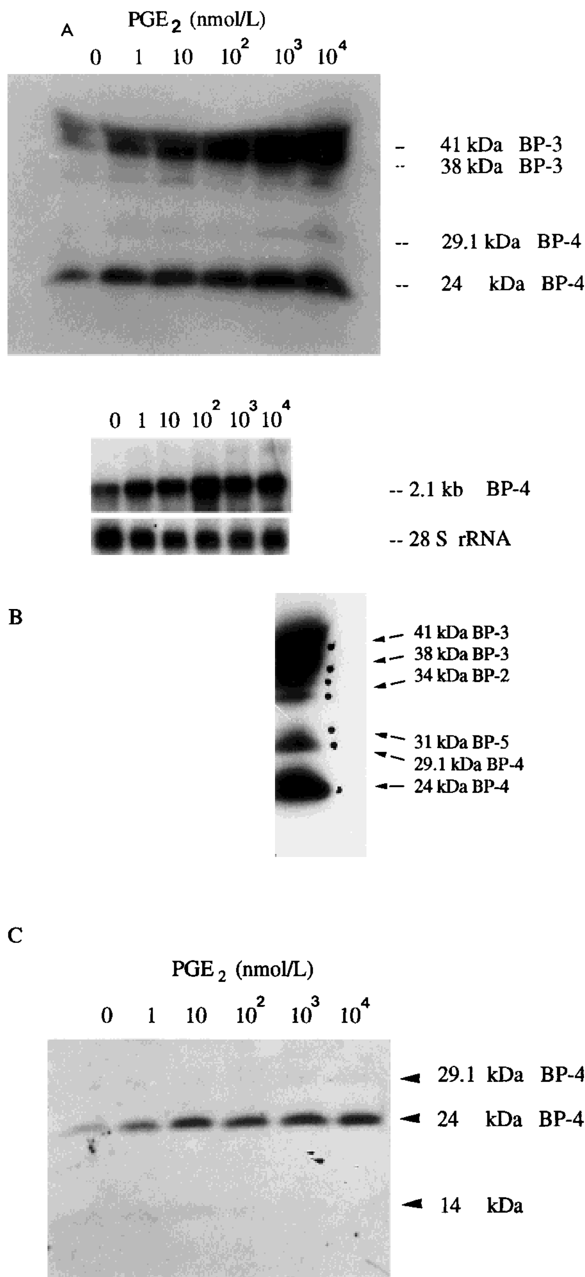
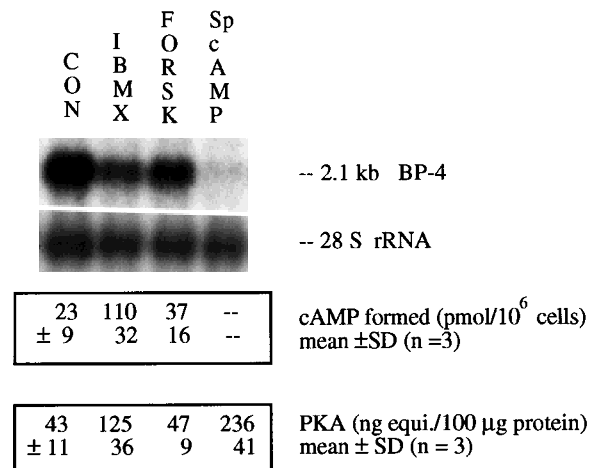


Fig. 1. **A:** Dose-dependent stimulation by PGE_2 of IGFBP-4 (BP-4) synthesis (top) and steady-state mRNA expression (bottom) using primary cultures of normal human articular chondrocytes. Cells were incubated with or without increasing concentrations of PGE_2 for 24 h. Conditioned medium was collected, concentrated, and analyzed by Western ligand blot analysis as described in Materials and Methods. Cell monolayers were extracted for total RNA and 10 μg were subjected to Northern analysis with probes for IGFBP-4 and 28S rRNA. Also illustrated is the relative abundance and regulation by PGE_2 of BP-3 compared to BP-4. **B:** A hyperexposure of a Western ligand blot of control medium illustrating the various IGF-1 binding proteins secreted by human chondrocytes. Between 38 (BP-3) and 34 kDa (BP-2) is an IGF-1 binding species that is as yet unidentified. **C:** Western immunoblot analysis of medium from A using a specific anti-human IGFBP-4 antibody. The 14 kDa band is presumed to be a BP-4 proteolytic fragment.



CON	IBMX	FORSK	Sp cAMP	
23 ± 9	110 32	37 16	-- --	cAMP formed (pmol/10⁶ cells) mean ±SD (n =3)
43 ± 11	125 36	47 9	236 41	PKA (ng equi./100 μg protein) mean ± SD (n = 3)

Fig. 2. Effect of cyclic AMP mimetics on basal chondrocyte IGFBP-4 (BP-4) expression. Cells were incubated with or without (CON) the phosphodiesterase inhibitor IBMX (200 μmol/L), cAMP mimetics forskolin (60 μmol/L, FORSK), and Sp cAMP (1 μmol/L) for 24 h. Cell monolayers were extracted for total RNA and 10 μg were subjected to Northern analysis with probes for IGFBP-4 and 28S rRNA. Cellular cAMP levels and protein kinase A (PKA) activity were also measured in cell extracts as described in Materials and Methods.

message, we measured the steady-state levels of IGFBP-4 mRNA. IGFBP-4 mRNA was upregulated by PGE_2 in a dose-dependent manner but to a lesser degree (2.7- ± 0.3-fold, n = 3) than IGFBP-4, suggesting an important element of post-transcriptional control. As in other cells, one major transcript was most often apparent at 2.1 kb although, depending on experimental and/or hybridization conditions, a minor transcript at 4.0 kb was observed. The nature and regulation of this larger mRNA species was not pursued further in this study.

The synthesis and expression of IGFBP-4 are stimulated in endothelial and osteoblast-like cells by increases in cellular cAMP levels [LaTour et al., 1990; Yang et al., 1993]. PGE_2 is known to rapidly elevate cAMP levels in many cells and has been used in a variety of studies as a cAMP mimetic [Case et al., 1990; Di Battista et al., 1994, 1995]. However, this is not the case with human chondrocytes because of a highly active, constitutive, cAMP dependent phosphodiesterase (PDE), which eliminates increases in cAMP levels as a result of G-protein-receptor signalling [Di Battista et al., 1996a]. The known cAMP mimetics, IBMX (200 μmol/L), Sp-cAMP (1 μmol/L), and forskolin (60 μmol/L) were tested and only the phosphodiesterase (PDE) inhibitor, IBMX and Sp-cAMP, had a consistent and significant effect on IGFBP-4 expression (Fig. 2). As judged by

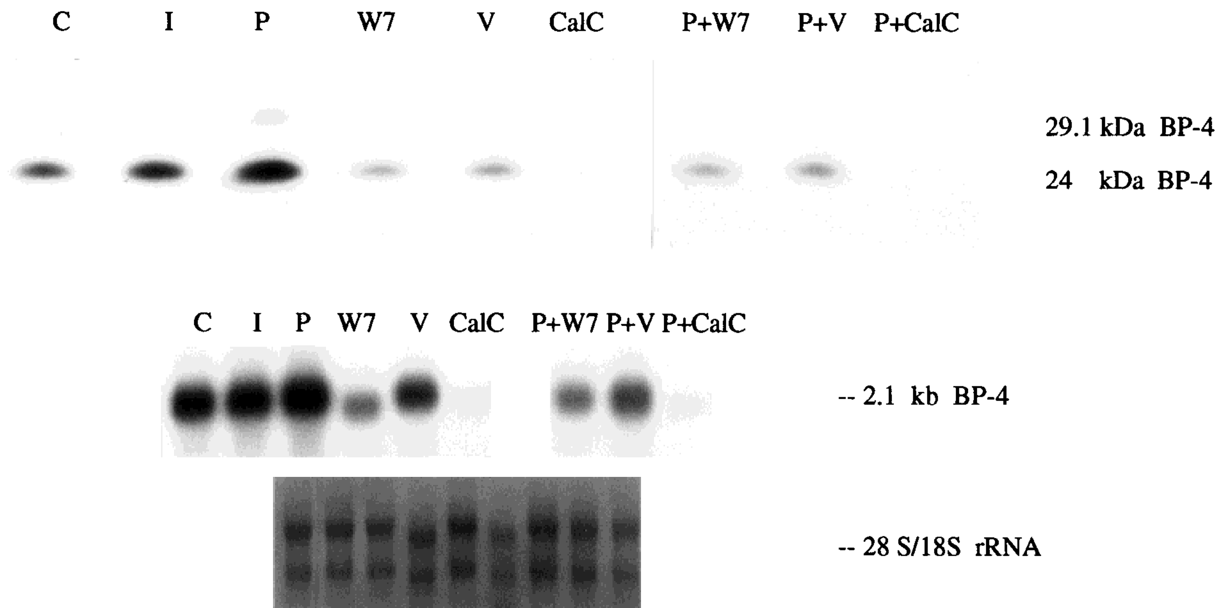


Fig. 3. Suppression of PGE_2 (P) stimulation of IGFBP-4 (BP-4) synthesis (**top**) and steady-state mRNA expression (**bottom**) in primary cultures of human articular chondrocytes by the Ca^{++}/calmodulin antagonist W7, by the Ca^{++} channel blocker (verapamil, V), and by the Ca^{++}-phospholipid dependent protein kinase C inhibitor, calphostin C (CalC). Cells were incubated without (control, C) or with PGE_2 (P, 100 nmol/L) in the presence or absence of W7 (25 µmol/L), V (50 µmol/L), and CalC (100 nmol/L) for 24 h. The latter inhibitors were added 1 h before the addition of PGE_2. Ionomycin (I, 20 nmol/L), a Ca^{++} channel ionophore, was added 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 were subjected to Northern analysis with a probe for IGFBP-4. A representative Northern blot is shown along with an accompanying ethidium bromide stain of 28S/18S rRNA.

Northern analysis, IBMX and Sp-cAMP suppressed constitutive IGFBP-4 expression by up to 71 ± 14 and 91 ± 9%, respectively (n = 3). Additionally, the inhibitor produced a significant increase in cAMP and protein kinase A (PKA) activity; Sp-cAMP is a known potent activator of PKA (see Fig. 2).

Since the results could be interpreted to mean that PGE_2 may not signal chondrocytes primarily by inducing cAMP as a second messenger, we explored the role of the Ca^{++}/calmodulin axis as a mediator of PGE_2 action [Irie et al., 1994]. The calcium ionophore, ionomycin (20 nM), induced the steady-state synthesis of IGFBP-4 by 1.9- ± 0.4-fold (n = 3) over controls (Fig. 3). The addition of W-7 (25 µM), a Ca^{++}/calmodulin antagonist, of verapamil (50 µM), a Ca^{++} channel blocker, and of calphostin C (100 nM), a PKC inhibitor, abrogated the PGE_2-induced up-regulation of IGFBP-4. Verapamil caused a relatively small reduction in terms of basal IGFBP-4 synthesis while calphostin C was a strong suppressor. The expression of IGFBP-4 mRNA was affected in a similar fashion to that of IGFBP-4 (Fig. 3).

Effect of IL-1β and Dexamethasone on IGFBP-4 Expression and Synthesis

The synthesis of PGE_2 is induced by IL-1 (secretagogue) 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 served as a mediator of the effects of IL-1 on IGFBP-4 expression; IL-1 is proposed to be a major regulator of binding protein synthesis in OA and RA cartilage [Olney et al., 1996]. Interestingly, rhIL-1β (0–1,000 U/mL; 0–570 pmoL) depressed the expression and synthesis of IGFBP-4 in a dose-dependent fashion (Fig. 4) in contrast to the effects of PGE_2. The concentration of rhIL-β necessary to inhibit the production of IGFBP-4 by 50% (IC_{50}) was 5.4 ± 1.5 pmoL (mean ± SD, n = 4) as judged by Laser scanning densitometry of multiple blots. The broad spectrum serine-threonine kinase inhibitor, staurosporine

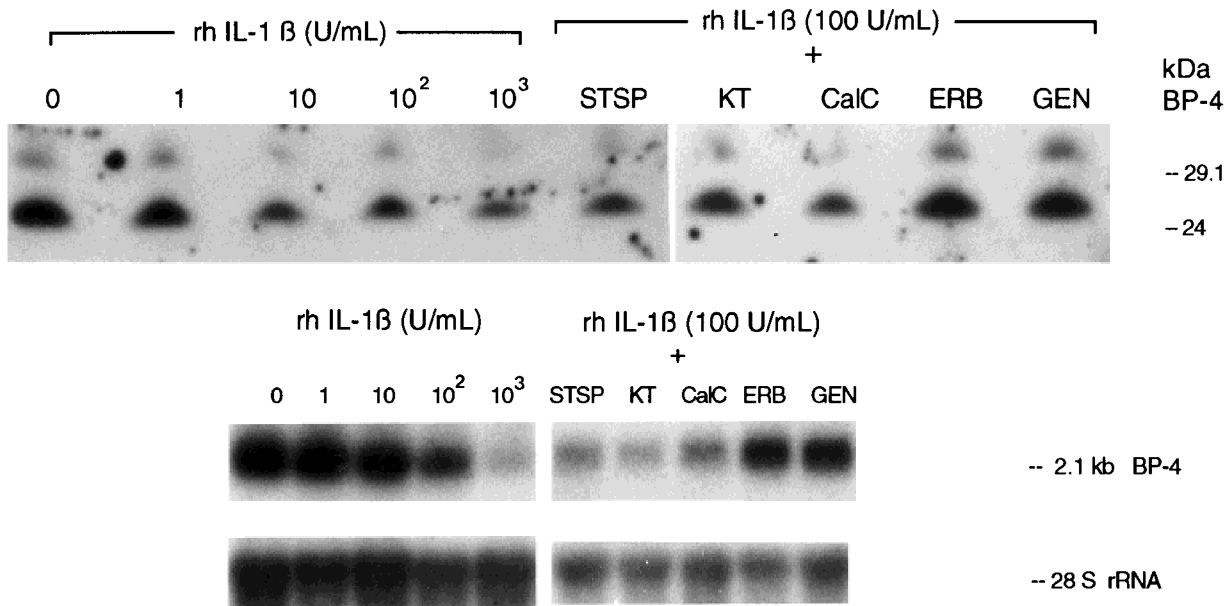


Fig. 4. Dose-dependent suppression by rhIL-1β of chondrocyte IGFBP-4 (BP-4) synthesis (**top**) and steady-state mRNA expression (**bottom**). Cells were incubated with increasing concentrations of rhIL-1β (0–1,000 U/mL) for 24 h or in the presence of 100 U/mL rhIL-1β with or without staurosporine (50 nmol/L, STSP), KT-5720 (250 nmol/L), calphostin C (100 nmol/L, Cal C), genistein (60 μmol/L, GEN), and erbstatin (50 μmol/L, ERBST) 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 were subjected to Northern analysis with probes for IGFBP-4 and 28S rRNA.

(50 nmol/L), the PKA inhibitor, KT5720 (2 μmol/L), and the specific PKC inhibitor, calphostin C (100 nmol/L), were unable to reverse the cytokine-induced effect to a significant degree while the tyrosine kinase inhibitors erbstatin (50 μmol/L) and genistein (60 μmol/L) could restablish IGFBP-4 to control levels (Fig. 4). With the exception of calphostin C, the inhibitors alone had no consistent effects on control IGFBP-4 synthesis (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 [Di Battista et al., 1991b] and, as such, it is possible that the GR may mediate some of the effects of the eicosanoid on IGFBP-4 expression. Given these facts and considering the role of the IGF-1/IGFBP axis on cartilage metabolism, we assessed the actions of the glucocorticoid dexamethasone on IGFBP-4 expression. Dexamethasone depressed the expression and synthesis of IGFBP-4 in an extremely potent and dose-dependent fashion in contrast to the effects of PGE_2 (Fig. 5). The glucocorticoid antagonist, RU486, completely reversed the suppressive effects of dexamethasone strongly supporting a role for the GR in the transactivation of the IGFBP-4 gene (Fig. 5).

Role of Protein Kinase C on IGFBP-4 Expression and Synthesis

Considering the apparent importance of cAMP and PKA mediation in the regulation of IGFBP-4, 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-4. We explored this avenue by attempting to pharmacologically activate PKC. The phorbol ester, PMA, a tumour promoter and activator of protein kinase C (PKC), had no effect on IGFBP-4 (both mRNA and protein, n = 4) although PMA potently induced PKC activity in chondrocytes by 4 h and this activity was inhibited in co-incubations with 100 nM of calphostin C (Fig. 6).

DISCUSSION

This study was prompted by, among other interests, our recent observation that PGE_2

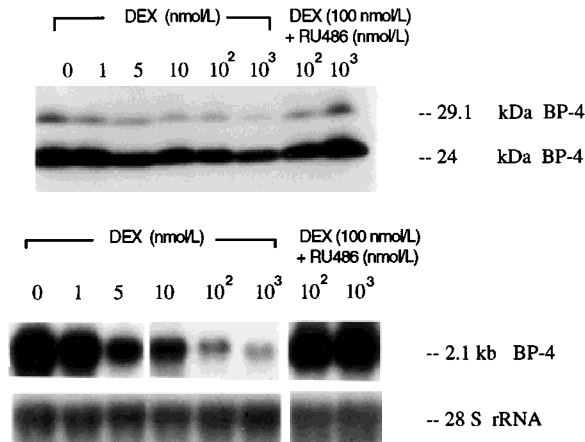


Fig. 5. Dose-response suppression by dexamethasone (DEX) of chondrocyte IGFBP-4 (BP-4) synthesis (**top**) and steady-state mRNA expression (**bottom**). Cells were incubated with or without increasing concentrations of DEX or with 100 nmol/L DEX plus the GR antagonist RU486 for 24 h. Conditioned medium was collected, concentrated, and analyzed by Western analysis as described in Materials and Methods. Cell monolayers were extracted for total RNA and 10 μg were subjected to Northern analysis with probes for IGFBP-4 and 28S rRNA.

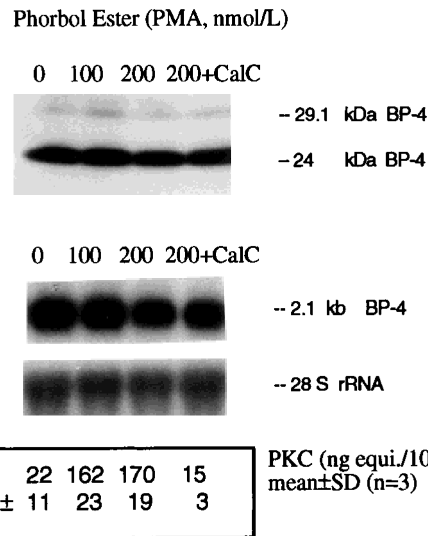


Fig. 6. Effects of the phorbol ester, PMA, on chondrocyte IGFBP-4 (BP-4) synthesis (**top**) and steady-state mRNA expression (**bottom**). Cells were incubated with or without increasing concentrations of PMA (0–200 nmol/L) or with 200 nmol/L of PMA plus 100 nmol/L calphostin C (CalC) for 24 h. Conditioned 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 were subjected to Northern analysis with probes for IGFBP-4 and 28S rRNA. Total cellular protein kinase C (PKC) activity was also measured in cell extracts as described in Materials and Methods.

stimulates the expression and synthesis of IGF-1 and the incorporation of proline into collagenase-digestible proteins in human articular chondrocytes [Di Battista et al., 1996a]. Furthermore, the eicosanoid-stimulated incorporation was mediated through an autocrine feedback loop involving IGF-1. Since IGF binding proteins (IGFBPs) are specific modifiers of IGF-1 action, we focussed our attention on whether PGE_2 could also influence the expression and synthesis of IGFBP-3 [Di Battista et al., 1996b] and, in this study, IGFBP-4. The maintenance of human chondrocyte phenotype by PGE_2, in terms of collagen type II synthesis, 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 indicates that elements responsive to IL-1 and PGE_2 resided in the upstream promoter region of COL2A1. However, no canonical PGE_2 (i.e., putative cAMP) response elements have been identified in the promoter region of the gene [Ryan et al., 1990; Wang et al., 1991]. Inasmuch as IGF-1 favours collagen type II expression in human cartilage [Sandell and Dudek, 1988] while IGFBP-3 and -4 modulate, in one way or another, this effect by sequestering IGF-1, chondrocyte phenotype may be influenced by the ability of the eicosanoid to control the IGF-1/IGFBP axis. Given the avascular nature of cartilage, there is limited ability for repair and, thus, local factors that maintain homeostasis are extremely important for the integrity and quality of cartilage [Malemud, 1993]. Among these factors, it seems likely that we must include prostaglandins and components of the IGF/IGFBP axis.

Prostaglandin E_2 may modulate IGFBP-4 production in human chondrocytes, at least in part, by transcriptional means since changes in steady-state message levels accompanied changes in the amount of IGFBP-4 released into the medium. However, the magnitude of the increase in IGFBP-4 mRNA was smaller than increases in IGFBP-4, suggesting that the mechanism of action of PGE_2 might be complex, involving more than one level of control. Indeed, IGFBP-4 is subject to regulation by proteolysis particularly in bone cells, and the presence of IGF-1 would seem to be a critical factor [Fowlkes and Freemark, 1992; Conover et al., 1993, 1995]. We detected very small amounts (though detectable) of extracellular IGFBP-4-protease activity (as judged by comparing data

from Western and Western ligand blots) that might influence the levels found in control medium. What little activity there was disappeared in the presence of PGE_2. Most of the IGFBP-4 specific proteolytic activity from bone cells has been identified as being serine and/or metal (i.e., metalloproteases) dependent enzymes [Fowlkes et al., 1995; Olney et al., 1996]. PGE_2 is a powerful inhibitor of the expression and synthesis of metalloproteases produced by connective tissue cells [Di Battista et al., 1994] and might serve to mask chondrocyte derived proteolysis. Furthermore, though PGE_2 upregulates IGF-1 (chondrocytes), which could potentially promote IGFBP-4 degradation, the eicosanoid also stimulates IGFBP-3, which has been recently shown to be an inhibitor of IGF-dependent IGFBP-4 proteolysis [Fowlkes et al., 1995]. Technical revisions may be necessary to pursue the study of this chondrocyte-derived proteolysis.

As mentioned earlier, PGE_2 upregulates the expression of IGF-1 and IGFBP-3 in chondrocytes by a cAMP-independent pathway probably involving Ca^{++}/calmodulin activated signalling systems. Ionomycin, a divalent cation ionophore, upregulated IGFBP-4 expression and synthesis possibly as a result of changes in intracellular Ca^{++}/calmodulin activity. This response was qualitatively similar to that brought about by incubations with PGE_2. IGFBP-4 induction by PGE_2 was reversed by W7, a calmodulin antagonist that inhibits Ca^{++}-calmodulin-dependent phosphodiesterase and myosin light chain kinase [Hidaka et al., 1981]. The induction was also inhibited by verapamil, a blocker of voltage-regulated Ca^{++} channels. Whereas IGFBP-3 is upregulated severalfold by PMA, an activator of Ca^{++}-phospholipid dependent protein kinase C (PKC), IGFBP-4 expression and synthesis are unaffected. It should be noted that 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 [Di Battista et al., 1996a]. 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). Somewhat confoundingly, the promoter region of the IGFBP-4 gene has putative phorbol ester response elements (AP-1) [Gao et al., 1993] while that of the IGFBP-3 gene does not [Cubbage et al., 1990]. It would seem that the IGFBP-3 and -4 genes share common upstream regulatory signals but there may be some downstream divergence. Alternatively/additionally, one cannot rule out post-transcriptional/translational regulatory systems like hitherto undefined specific proteases accounting for our observations. Calphostin C binds to the regulatory domain of PKC at the same site as PMA (and DAG) and acts as a specific competitive inhibitor of the PMA activation of PKC [Dekker et al., 1995]. This does not help to explain why, however, PMA has no effect on IGFBP-4 while calphostin C dramatically suppresses the expression and synthesis of the binding protein; interestingly, an effect reversed by co-incubation with PMA (see Fig. 6). Preliminary results in our laboratory suggests that calphostin C behaves in a similar manner to OKA with regards to apoptosis in chondrocytes and provokes the expression of a similar set of genes affecting cell viability. Indirectly, the data point to a pivotal role for PKC in apoptosis as has been suggested by others [Lucas and Sanchez-Margalet, 1995].

Through the manipulation of cellular cAMP levels by different pharmacological means, it would appear that the expression of IGFBP-4 is also negatively regulated by elevations in the levels of cAMP. Noncanonical cAMP response elements have been located in the promoter region of the IGFBP-4 gene although how they function to control gene expression is not well-defined [Gao et al., 1993]. Our observations, however, are in contrast to other studies that demonstrate a marked increase by cAMP of IGFBP-4 levels in osteoblasts [Yang et al., 1993; LaTour et al., 1990]. Tissue specific trans-acting/transcription factors may be responsible for these differences. PGE_2, though often considered a cAMP mimetic, does not increase cellular levels of cAMP or increase protein kinase A (PKA) activity in human articular chondrocytes and we showed that this insensitivity may be due to a "constitutively" activated cAMP-dependent phosphodiesterase IV(PDE IV) [Di Battista et al., 1996a]. Hormonally induced, time-dependent increases in PDE IV activity with a resultant decrease in cellular cAMP levels and subsequent cellular desensitization is now a well-accepted phenomenon [Conti et al., 1991; Sette et al., 1994]. Whether the latter mechanism is responsible for the "constitutivity" of PDE IV in chondrocytes awaits further investigation.

Our data show that dexamethasone (DEX), a potent antinflammatory glucocorticoid, substantially diminished the expression and synthesis of IGFBP-4. 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 (i.e., collagen) in bone cells and certain organ culture systems and these effects has been ascribed to the ability of these steroids to inhibit the expression and synthesis of IGFBPs [Kream et al., 1990; Chen et al., 1991]. We showed that PGE$_2$ could induce a progressive upregulation in the levels of GR in connective tissue cells [Di Battista et al., 1991b] and it was thought that this would render the cells more sensitive to glucocorticoids [Di Battista et al., 1993] in terms of the expression of glucocorticoid sensitive genes like collagenase (MMP-1). Thus, we speculated that there may be cross-talk between the two signalling systems and that for some genes the GR mediated the effect of PGE$_2$. The latter scenario would not seem to be the case for the IGFBP-4 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.

Recently, it was demonstrated that IL-1α (and TNFα) increased the production of IGFBPs (e.g., 3 and 4) by human chondrocytes in culture [Olney et al., 1995]. The authors 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 IGFBPs. Conceivably, IL-1 could therefore also be responsible for the increased amounts of IGFBP-3 and -4 found in synovial fluid of RA and OA patients [Matsumoto et al., 1996; Kanety et al., 1996]. We are not able to confirm these results with IL-1β and, indeed, there was a marked inhibition of IGFBP-4 protein production as judged by two blotting techniques. Furthermore, steady-state IGFBP-4 mRNA levels were also concomitantly downregulated. 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 may conceivably activate a different set of genes but they 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%) [Di Battista et al., 1991a]; there is therefore a significant spare-receptor phenomenon in the IL-1 receptor system. It would seem that an erbstatin/genistein-sensitive (tyrosine) kinase is responsible for the IL-1β induced suppression of IGFBP-4. Further studies will be needed to identify this (these) kinase activity(ies) because a good deal of evidence has accumulated suggesting that kinases of diverse substrate specificities probably mediate IL-1 action in a number of cell types [Case et al., 1990; Bonin et al., 1990; Munoz et al., 1990]. 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.

In summary, IGFBP-4 is upregulated by PGE$_2$ probably through a Ca^{++}/calmodulin sensitive process and not through cAMP signalling pathways. Consistent with these observations are the findings that Ca^{++}/calmodulin agonists increase IGFBP-4 production while antagonists reverse the PGE$_2$-induced upregulation; PKA activation mediates an opposing effect with respect to PGE$_2$. Prostaglandin E$_2$ activation of the IGF-1/IGFBP axis suggests that eicosanoids play a pivotal role in the metabolism of cartilage and possibly connective tissues in general. Eicosanoid biosynthesis may be a rate-limiting step in cartilage repair processes.

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.

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