Early Growth Response Factor-1 Mediates Prostaglandin 
E\textsubscript{2}-dependent Transcriptional Suppression of Cytokine-induced 
Tumor Necrosis Factor-\(\alpha\) Gene Expression in Human Macrophages 
and Rheumatoid Arthritis-affected Synovial Fibroblasts* 

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Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) is a pleiotropic proinflammatory cytokine that modulates a broad range of inflammatory and immunological processes. We have investigated the potential immunomodulatory properties of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) by examining the molecular mechanism by which the eicosanoid suppresses T-cell-derived interleukin-17 (IL-17)-induced TNF-\(\alpha\) mRNA expression and protein synthesis in human macrophages and rheumatoid arthritis-affected synovial fibroblasts. Initial studies confirmed that PGE\textsubscript{2} induces egr-1 mRNA expression and protein synthesis by restricted SAPK2/p38 MAPK-dependent activating transcription factor-2 (ATF-2) dimer transactivation of the egr-1 promoter as judged by studies using wild-type (WT) and deletion mutant egr-1 promoter constructs, Northern and Western blotting, and standard and supershift electrophoretic mobility shift analyses. Using human leukemic monocyte THP-1 cells stably transfected with WT and dominant-negative mutant expression constructs of Egr-1, cotransfected or not with a WT pTNF-\textsubscript{615}SVOCAT construct, we observed that PGE\textsubscript{2} inhibition of IL-17-stimulated TNF-\(\alpha\) mRNA expression and promoter activity was dependent on Egr-1 expression, as mutants of Egr-1, alone or in combination, markedly abrogated any inhibitory effect of PGE\textsubscript{2}. Standard and supershift electrophoretic mobility shift analysis, signaling “decoy” overexpression studies, and pTNF-\textsubscript{615}SVOCAT promoter assays using WT and mutant promoter constructs revealed that IL-17-upregulated promoter activity was largely dependent on ATF-2/c-Jun transactivation. PGE\textsubscript{2} suppression of IL-17-induced ATF-2/c-Jun transactivation and DNA binding was dependent on Egr-1-mediated inhibition of induced c-Jun expression. We suggest that egr-1 is an immediate-early PGE\textsubscript{2} target gene that may be a key regulatory factor in mediating eicosanoid control of genes involved in the immune and inflammatory responses.

Inflammation and the inflammatory response represent a highly orchestrated cascade of events designed to maintain a state of homeostasis in the host following pathogenic invasion and/or tissue injury. The response involves the concerted and exquisitely timed interactions of cytokines, chemokines, growth factors, and lipid-derived mediators with inflammatory cells like macrophages, neutrophils, activated endothelial cells and fibroblasts, T- and \(\beta\)-lymphocytes, and mast cells (1–4). Among the most important classes of lipid-derived mediators in this regard are the prostaglandins of the E series that function as pleiotropic autacoids and exert modulatory effects on the immune and inflammatory responses in addition to acting as essential physiological regulators in virtually every tissue of the human organism (5, 6).

Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) is synthesized from the essential fatty acid arachidonic acid by the enzymes of the arachidonate cascade or, occasionally, by free radical-catalyzed peroxidation (7). Upon cellular activation, type IV cytosolic phospholipase A\textsubscript{2} translocates to the endoplasmic reticulum or outer nuclear membrane and releases arachidonic acid from the membrane lipids of most cell types (7, 8). The latter protein trafficking event is controlled by calcium fluxes and MAPK phosphorylation of cytosolic phospholipase A\textsubscript{2} (8). A co-localized, monotypic, integral membrane protein, prostaglandin H\textsubscript{2} synthase (also referred to as cyclooxygenase (COX), with two isoforms, COX-1 and COX-2), catalyzes the first committed, rate-limiting step by converting arachidonic acid to prostaglandin H\textsubscript{2}. The latter intermediate is rapidly metabolized by cell- and tissue-specific synthases to one of several prostanoids (e.g. PGE\textsubscript{2} by prostaglandin E\textsubscript{2} synthase) (9, 10).

The PGE\textsubscript{2} signal is transduced at the cell surface by specific cognate receptors (designated EP receptors, subtypes EP1–

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1 The abbreviations used are: PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; MAPK, mitogen-activated protein kinase; COX, cyclooxygenase; CREB, cAMP response element-binding protein; ATF, activating transcription factor; AP, activator protein; ERK, extracellular signal-regulated kinase; TNF-\(\alpha\), tumor necrosis factor-\(\alpha\); MLK, mixed lineage kinase; MKK, mitogen-activated protein kinase kinase; RA, rheumatoid arthritis; PMA, phorbol 12-myristate 13 acetate; hIL-17, recombinant human interleukin-17; PCS, fetal calf serum; RASF, rheumatoid arthritis-affected synovial fibroblast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; WT, wild-type; PGK, phosphoglycerate kinase; CRE, cAMP response element; SRE, serum response element; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CAT, chloramphenicol acetyltransferase; SAPK, stress-activated protein kinase; LPS, lipopolysaccharide; IL, interleukin; MSK, mitogen- and stress-activated protein kinase; JNK, c-Jun N-terminal kinase.
EP4 that are characterized by an extracellular N-terminal domain, seven membrane-spanning regions containing a variable cytoplasmic loop, and a cytoplasmic C terminus. Ligand binding takes place in the membrane-spanning domains, and G-protein coupling occurs through the intracellular loops and C terminus (11–13). In general, EP2 and EP4 signal through Gαs coupling, resulting in increases in cAMP levels and activated protein kinase A within seconds to minutes. The EP1 and some EP3 receptor subtypes associate with Gαi and mediate increases in intracellular calcium, whereas Gαq proteins reduce levels of cellular cAMP (EP3) (8, 11). For example, the C terminus of EP receptors also harbors serine/threonine residues (anywhere from 1 to 9) that, when phosphorylated by activated protein kinase A, induce receptor desensitization and signal attenuation (reviewed in Ref. 13).

In a variety of target cell types, PGE2 modulates transcription of immediate-early genes such as junB, junD, fosB, fra-1, and c-fos, although the mechanistic details are completely undefined (14–16). These early gene products are transcription factors that, along with CREB, ATF-1, ATF-2, and AP-2, modify the expression of PGE2 target genes (16, 17). The latter are genes associated with mitogenic, apoptotic, hypertrophic, and differentiation signals in many cell types, including stem cells (18, 19). Signaling cascades mediating mitogenic activity such as the Ras/Raf-1/ERK cascade are inhibited by PGE2 secondary to phosphorylation and inactivation of Raf-1 kinase by protein kinase A (20). In addition, the p38 MAPK cascade is a primary target through which PGE2 signaling via EP4 is manifested in human synovial fibroblasts and macrophages (21).

Preliminary data from this laboratory (23) and others (22, 24) suggest that a zinc finger transcription factor, the early response gene egr-1/krox-24, may also be a target gene of PGE2, although detailed studies regarding the mechanism of egr-1/krox-24 gene induction and the specificity of cell-type responsiveness were never performed. Also known as zif268, egr-1/krox-24 was originally identified as a growth response gene in fibroblasts and B-cells (25, 26). The expression of Egr-1/Krox-24 is induced during differentiation of myeloid cells, nerve, and bone and has been shown to be essential and determinant for macrophage differentiation (reviewed in Ref. 26; Refs. 27 and 28). It is a strong nuclear DNA-binding protein that specifically recognizes the sequence 5’-GGG(T/G)-GGGG-3’ and can transactivate promoters containing the appropriate cognate site. The promoters of tumor necrosis factor-α (TNF-α) (29), both chains of platelet-derived growth factor (30, 31), and transforming growth factor-β1 (30) harbor active binding/enhancer sites for Egr-1/Krox-24. Cell type determines whether Egr-1/Krox-24 acts to activate or inhibit promoter activity of target genes, suggesting that it interacts with tissue-specific factors and/or transcription factors that associate with proximal promoter elements such as Sp1 (25, 31). In this study, we investigated in detail the regulatory mechanisms controlling PGE2-dependent induction of the egr-1 gene in human monocytes/macrophages and rheumatoid arthritis-transformed synovial cells. Our results indicate that PGE2 induces Egr-1 expression through stimulation of the MLK2/MKK3/p38 MAPK cascade with ATF-2 transactivation of the egr-1 promoter. Furthermore, using dominant-negative mutants in stably transfected cell lines, we show that PGE2 suppresses T-cell cytokine-induced TNF-α gene expression and synthesis in human macrophages (and rheumatoid arthritis (RA)-affected synovial fibroblasts) largely through Egr-1-dependent TNF-α promoter suppression. The data suggest that PGE2/Egr-1 disrupts essential components of the TNF-α promoter transactivation complex, viz. the suppression of ATF-2/c-Jun heterodimer DNA binding through inhibition of c-Jun expression.

MATERIALS AND METHODS

Chemicals—Sodium fluoride, leupeptin, aprotinin, pepstatin, phenylmethylsulfonyl fluoride, actinomycin D, dithiothreitol, sodium orthovanadate, and bovine serum albumin were products of Sigma. PGE2, SB 202190, KT5720, phorbol 12-myristate 13-acetate (PMA), ionomycin, A23187, forskolin, and rosiglitaz were purchased from Calbiochem. SDS, acrylamide, bisacrylamide, ammonium persulfate, and protein reagent were from Bio-Rad. Tris base, EDTA, MgCl2, CaCl2, chloroform, Me2SO, anhydrous ethanol (95%), methanol (99%), formaldehyde, and formamide were obtained from Fisher. Recombinant human interleukin-17 (rhIL-17) was purchased from R&D Systems (Minneapolis, MN). Dulbecco’s modified Eagle’s medium, phosphate-free and phenol red-free Dulbecco’s modified Eagle’s medium, RPMI 1640 medium, TRIzol reagent, heat-inactivated fetal bovine serum, and an antibiotic mixture (10,000 units of penicillin (base) and 10,000 µg of streptomycin (base)) were products of Invitrogen.

Cell Culture—Human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll-Hypaque density centrifugation (Amersham Biosciences) as described previously (32). Monocytes/macrophages were allowed to adhere to plastic dishes (Flow Laboratories, McLean, VA) in RPMI 1640 medium containing 10% fetal calf serum (FCS) and penicillin/streptomycin (antibiotics). THP-1 cells (2–3 × 106 cells/ml; American Type Culture Collection, Manassas, VA), a human monocytic cell line, were cultured as suspensions in RPMI 1640 medium supplemented with 50 µg/β-mercaptoethanol (Sigma). Human RA-affiliated monovial RA-affected synovial fibroblasts (RASFs) were obtained from RA patients undergoing arthroplasty who were diagnosed based on criteria developed by the American College of Rheumatology Diagnostic Subcommittee for Arthritis (33). Details of RASF isolation and enrichment have been described previously (33) and were routinely cultured in Dulbecco’s modified Eagle’s medium, 4.5 g/liter glucose, 10% fetal bovine serum (FCS, and antibiotics. NIH-3T3 and HeLa cells (American Type Culture Collection) were cultured as described above for RASFs.

Preparation of Cell Extracts and Western Blotting—Cellular proteins (50–100 µg) extracted in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, µg/ml leupeptin, µg/ml pepstatin, 1% Nonidet P-40, 1 mM sodium orthovanadate, and 1 mM NaF) or in hot SDS-PAGE loading buffer from control and treated cells were subjected to SDS-PAGE on 8–12% gels (16 × 20 cm, final concentration of acrylamide) under reducing conditions and transferred onto nitrocellulose membranes.

Total cellular RNA (1 µg) was extracted using TRIzol reagent as described previously (32). Generally, 5–20 µg of total RNA were resolved on formaldehyde-containing 1.2% agarose gel and transferred electrothermally (30 V overnight at 4 °C) to Hybond-N+ nylon membranes (Amersham Biosciences) in 0.5 × sodium acetate/EDTA (pH 8.7). After prehybridization for 24 h, hybridizations were carried out at 50–55 °C for 24–36 h, followed by high stringency wash at 68 °C in 0.1 × SSC and 0.1% SDS. The following probes, which were labeled with digoxigenin-dUTP by random priming, were used for hybridization. Human TNF-α cDNA (1.3 kb), initially cloned into the HindIII and BamHI sites of pSPORT1 (American Type Culture Collection), was released by digestion. Human egr-1/krox-24 cDNA, initially cloned into the EcoRI sites of
the pMexNeo vector (an EgR-1 expression vector; provided by Dr. D. Skup, Department of Biochemistry, University of Montreal, Montreal, Canada), contains 300 bp of 5′-noncoding region and 580 bp of 3′-noncoding region of egr-1. Both the egr-1 and junB probes were 2.6- and 1.9-kb cDNA fragments inserted into pGEM2 (American Type Culture Collection). A 780-bp PsiI/XbaI fragment from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (1.2 kb; American Type Culture Collection) initially cloned into the PsiI site of the pBR322 vector served as a control for RNA loading, as GAPDH is constitutively expressed in the cells used in these experiments. All blots were subjected to a digital imaging system (Alpha Imager 2000) for semiquantitative measurements, and changes in TNF-α or egr-1 mRNA expression were always considered as the ratio of GAPDH mRNA.

Extraction of Nuclear Proteins and Electrophoretic Mobility Shift Assay (EMSA)—Confluent and treated cells (3–5 × 10⁵ cells/well) in 4-well cluster plates were carefully scraped into 1.5 ml of ice-cold E100 Buffer (10 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 150 mM NaCl, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride). Cell extracts were prepared as described previously (33).

Double-stranded oligonucleotides containing wild-type (WT) and mutant sequences were obtained from Invitrogen; annealed in 100 mM Tris-HCl (pH 7.5), 1 mM NaCl, and 10 mM EDTA at 65°C for 10 min, cooled for 1–2 h at room temperature; and finally end-labeled with [γ-³²P]ATP using T4 polynucleotide kinase (Promega, Madison, WI). The purity of the described oligonucleotides (WT and mutant sequences) was determined by gel electrophoresis and the presence of sense-strand sequences confirmed by sequencing. Nuclear extracts were then prepared by first incubating nuclei in NE-Binding Buffer (20 mM Tris-HCl, pH 7.5, 250 mM NaCl, 50 mM dithiothreitol, 10% glycerol, and 0.5 μg of poly(dI-dC)) for 30 min and then centrifuging. Reaction mixtures contained 5 μg of purified protein and 400,000 cpm of 32P-labeled oligonucleotide. Reaction mixtures were subjected to electrophoresis on 5% native polyacrylamide gel and then transferred to a Hybond-N+ nylon membrane (Amersham). Membranes were hybridized overnight at 42°C in hybridization buffer (5× SSPE, 5× Denhardt’s solution, 0.5% SDS, 1% BSA, and 0.1 mg/ml salmon sperm DNA) containing a 32P-labeled oligonucleotide specific for the Egr-1 promoter. Membranes were then washed twice in buffer containing 2× SSPE and 0.1% SDS at 55°C, followed by autoradiography.

RESULTS

PGE₂ Induces egr-1 mRNA Expression and Synthesis: Time and Dose Dependence—Before examining the role of Egr-1 in PGE₂-dependent suppression of TNF-α mRNA, we first characterized the kinetics (and mechanism) of PGE₂ induction of the egr-1 gene in clinically significant cell phenotypes. We performed both dose-response and time course studies using primary cell culture models consisting of human peripheral monocytes/macrophages and RASFs. As shown in Fig. 1A, PGE₂ induction of the egr-1 gene was dose-dependent, and the EC₅₀ for PGE₂-dependent egr-1 mRNA expression in either monocyte/macrophage or RASF cultures was ~6.1 ± 2.7 nmol/liter (mean ± S.D., n = 6). For all subsequent experimentation, 2 × EC₅₀ (90% response saturation) was chosen unless otherwise indicated. The PGE₂-dependent induction of Egr-1 protein followed a dose-response profile similar to that of egr-1 mRNA expression as judged by Western blot analysis, whereas Sp-1, a GC-box-binding transcription factor, was unaffected (Fig. 1B). Time course studies revealed rapid rises (5 min) in egr-1 mRNA (3.8 kb) expression following PGE₂ treatment, which increased to >10-fold over control levels after 15 min and attained steady state after 30 min, followed by a precipitous and rapid decay to near control levels. Modest levels of egr-1 mRNA were also observed after 4 h of PGE₂ stimulation (Fig. 1C). Specific binding to a consensus Egr-1 enhancer 32P-labeled oligonucleotide was detected by EMSA (Fig. 1D). Specific binding to a consensus Egr-1 enhancer 32P-labeled oligonucleotide was detected by EMSA (Fig. 1D).
PGE₂ stimulation of egr-1 mRNA Expression through Activation of the MMK3/SAPK2/p38 MAPK Pathway and Transactivation of 5'-Flanking Promoter Sequences—To examine for elements of transcriptional control of PGE₂-stimulated egr-1 expression, we conducted transient transfection analyses in THP-1 cells using a series of deletion mutants of the egr-1 promoter. As shown in Fig. 2A, the egr-1 promoter harbors a number of enhancer sites, including nested SRE, AP-1, AP-2, ATF-2/CRE, Egr-1, and other GC-rich sequences. Upon stimulation with PGE₂, elevated induction of CAT protein was observed with the pCAT−252 (SmaI) fragment (3.21 ± 0.63-fold; mean ± S.D. for three experiments in duplicate) and the −925 mutant fragment (3.85 ± 0.78-fold). For comparison purposes, we repeated the experiments with 100 nmol/liter PMA, a well known stimulator of Egr-1 (25, 26), and found maximal induction with the pCAT−459 construct (1.99 ± 0.14-fold). As shown diagrammatically in Fig. 2A, the pCAT−252 (SmaI) fragment has an ATF-2/CRE site at bp −138 to −131 in addition to at least one SRE site and a proximal GC box (bp −64 to −46). Because the data from Fig. 2A (see pCAT−77) suggest that the Sp-1 site was not responsive to PGE₂ (see also Fig. 1, B and D), we investigated whether ATF-2/CRE and/or SRE sites are crucial for promoter activation. As shown in Fig. 2B, promoter activation by PGE₂ was substantially decreased (>85%) when the ATF-2/CRE site was mutated. Overexpression of pMMK3 (MMK3) and, to a lesser extent, pPKA-CAT increased WT egr-1 promoter activity (pCAT−252) whereas, as in the case with PGE₂ mutating the ATF-2/CRE site abrogated the inductive response (Fig. 2B). The addition of SB 202190 (100 nmol/liter) reversed the inductive effects of PGE₂, 332 ± 51 (PGE₂) versus 65 ± 27 (SB 202190) pg of CAT protein.

In view of the presence of the SRE sites in the pCAT−252 constructs and the possibility that the serum response factor may contribute in some way to PGE₂-dependent promoter induction, we conducted further studies with transfected reporter plasmids harboring five tandem SREs. This approach gives a very sensitive measure of cell signaling and transactivation by specific transcription factors. As shown in Fig. 2C, PGE₂ did not transactivate the SRE reporter as judged by measuring luciferase activity, nor did it modify serum-induced phospho-SAPK2 (Thr69/71 and a slightly more delayed phosphorylation of ATF-2/CREB-1 at Ser133 (5 min). Furthermore, PGE₂ induced a very rapid phosphorylation of ATF-2 at Thr69/71 and a slightly more delayed phosphorylation of ATF-1/CREB-1 at Ser133 (5 min). Therefore, PGE₂ increased nuclear protein binding to the ATF-2/CRE-like oligonucleotide, and supershift analysis revealed that the principal binding species appeared to be ATF-2, with an apparently lesser changes were observed with increased Egr-1 binding (i.e. no displacement) following PGE₂ treatment.

PGE₂ induces egr-1 mRNA expression through activation of the MMK3/SAPK2/p38 MAPK pathway and transactivation of 5'-flanking promoter sequences—To examine for elements of transcriptional control of PGE₂-stimulated egr-1 expression, we conducted transient transfection analyses in THP-1 cells using a series of deletion mutants of the egr-1 promoter. As shown in Fig. 2A, the egr-1 promoter harbors a number of enhancer sites, including nested SRE, AP-1, AP-2, ATF-2/CRE, Egr-1, and other GC-rich sequences. Upon stimulation with PGE₂, elevated induction of CAT protein was observed with the pCAT−252 (SmaI) fragment (3.21 ± 0.63-fold; mean ± S.D. for three experiments in duplicate) and the −925 mutant fragment (3.85 ± 0.78-fold). For comparison purposes, we repeated the experiments with 100 nmol/liter PMA, a well known stimulator of Egr-1 (25, 26), and found maximal induction with the pCAT−459 construct (1.99 ± 0.14-fold). As shown diagrammatically in Fig. 2A, the pCAT−252 (SmaI) fragment has an ATF-2/CRE site at bp −138 to −131 in addition to at least one SRE site and a proximal GC box (bp −64 to −46). Because the data from Fig. 2A (see pCAT−77) suggest that the Sp-1 site was not responsive to PGE₂ (see also Fig. 1, B and D), we investigated whether ATF-2/CRE and/or SRE sites are crucial for promoter activation. As shown in Fig. 2B, promoter activation by PGE₂ was substantially decreased (>85%) when the ATF-2/CRE site was mutated. Overexpression of pMMK3 (MMK3) and, to a lesser extent, pPKA-CAT increased WT egr-1 promoter activity (pCAT−252) whereas, as in the case with PGE₂ mutating the ATF-2/CRE site abrogated the inductive response (Fig. 2B). The addition of SB 202190 (100 nmol/liter) reversed the inductive effects of PGE₂, 332 ± 51 (PGE₂) versus 65 ± 27 (SB 202190) pg of CAT protein.

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We next investigated whether PGE₂ can up-regulate and phosphorylate nuclear binding proteins that bind specifically to the ATF-2/CRE site (bp −138 to −131) using Western and EMSA analyses. Previous work from our laboratory has shown that PGE₂ rapidly activates the SB 202190-sensitive SAPK2/p38 MAPK cascade through EP4 receptor cell-surface binding in inflammatory cell phenotypes (21). As shown in Fig. 3A, PGE₂ induced a very rapid phosphorylation of ATF-2 at Thr69/71 and a slightly more delayed phosphorylation of ATF-1/CREB-1 at Ser133 (5 min). Furthermore, PGE₂ increased nuclear protein binding to the ATF-2/CRE-like oligonucleotide, and supershift analysis revealed that the principal binding species appeared to be ATF-2, with an apparently lesser

Fig. 1. Dose response (A and B) and time course (C and D) of PGE₂ stimulation of egr-1/hrox-24 mRNA and protein. Cultured confluent second passage human RASFs (1.2 × 10⁶ cells in 6-well cluster plates) were preincubated for 4 h in Dulbecco’s modified Eagle’s medium supplemented with 1% FCS plus antibiotics at 37 °C to ensure synchrony and quiescence. Cells were then treated with increasing concentrations of PGE₂ (0–20 μmol/liter) for 30 min, and monolayers were extracted for RNA (A) or protein (B). In C and D, cells were incubated with PGE₂ for varying time periods (0–16 h). Total RNA (5 μg) was analyzed for egr-1/hrox-24 mRNA by Northern hybridization using a specific digoxigenin-labeled cDNA probe as described under “Materials and Methods” (A and C), and 50 μg of protein were analyzed for Egr-1/hrox-24 protein by Western blotting using specific polyclonal antisera for Egr-1 and Sp-1 (B). In D, 15 μg of nuclear protein were subjected to EMSA using consensus and mutant (mut) ³²P-labeled Egr-1/hrox-24 oligonucleotides, and gel supershift assays were performed with specific anti-Egr-1 and anti-Sp-1 antisera as described under “Materials and Methods.” Con, control.
PGE₂ Transcriptionally Suppresses rhIL-17-induced TNF-α mRNA Expression: Role of Egr-1 and AP-1 Family Members—

Supported by the strong data on the mechanism of PGE₂ control of Egr-1 expression, we turned our attention to the putative role of Egr-1 in PGE₂-dependent suppression of cytokine-induced TNF-α expression and release. In this regard, a number of studies have shown that cAMP-elevating agents inhibit induced TNF-α expression, although the precise mechanism is complex and largely ill defined (reviewed in Ref. 35). Although PGE₂ can indeed elevate cAMP levels in a number of cell phenotypes, it also activates the p38 MAPK cascade and induces signaling cross-talk within the MAPK cascades (this work and Ref. 21). Recent results from our laboratory have provided physiological relevance to the latter reports to the extent that when freshly explanted osteoarthritis- or RA-affected synovial membranes were treated with preferential COX-2 inhibitors, a marked induction of TNF-α and interleukin (IL)-1β release was observed (36). Furthermore, lipo-polsaccharide (LPS)-induced release of TNF-α and IL-1β was augmented >4-fold in the presence of the COX-2 inhibitor. The addition of PGE₂ returned levels to the base line, and thus, strong evidence was provided that TNF-α is an eicosanoid-dependent gene.

In this series of experiments, we used our previously described paradigm in which the CD45 T memory cell-derived pro-inflammatory cytokine IL-17 was used to stimulate TNF-α expression, synthesis, and release by human monocytes/macrophages (32). As shown in Fig. 4A, increased TNF-α transcripts...
were observed after 15–30 min, reaching steady state at 2–4 h, followed by rapid decay. Immunoreactive TNF-α protein could be detected after 30 min, with levels being apparently constant after 4–8 as judged by Western analysis of conditioned medium (Fig. 4A). Co-incubations of rhIL-17-treated human monocytes/macrophages with increasing concentrations of PGE2 led to dose-dependent suppression of induced TNF-α mRNA levels. In both human macrophages and RA-affected synoviocytes, the IC50 for TNF-α suppression was 45 ± 12 nmol/liter (mean ± S.D., n = 4) (Fig. 4, B and C).

Recent work from our laboratory has confirmed that PGE2 controls gene expression at transcriptional and post-transcriptional levels (21). As a first approach to verify whether transcriptional mechanisms are involved in the potent PGE2-dependent inhibition of rhIL-17-induced TNF-α expression, studies were conducted with the TNFα promoter pTNF-615SVOCAT in transiently transfected THP-1 cells treated with rhIL-17 with or without increasing concentrations of PGE2. Total RNAs at 2 (B) and 20 (C) μg were analyzed for TNF-α and GAPDH mRNAs by Northern hybridization.
FIG. 5. Transcriptional repression of rhIL-17-induced TNF-α gene expression by PGE₂: role of Egr-1. In A, THP-1 cells (5 × 10⁷ cells/0.5 ml), cultured in RPMI 1640 medium, 10% FCS, and antibiotics, were transfected by electroporation with 10 μg of total WT, mutant (mut) Egr-1, and mutant ATF-2/c-Jun pTNF−615SVOCAT plasmids using a 7-s pulse at 300 V and 960 microfarads. Transfection efficiencies were controlled in all experiments by cotransfection with 0.5 μg of pCMV-β-gal construct. Cells were allowed to recover for 24 h in complete medium, after which they were maintained under low serum conditions (1% for 3 h) and then treated with or without rhIL-17 (50 ng/ml) in the absence or presence of increasing concentrations of PGE₂ for 6 h. Cells were washed and lysed, and promoter activities were assessed by measuring CAT protein using a specific and sensitive enzyme-linked immunosorbent assay. β-Galactosidase (β-gal) activity was measured as described under “Materials and Methods.”

Con, control. In B, THP-1 cells (10⁷ cells/0.5 ml) or THP-1 cells stably transfected with a eukaryotic expression vector (PGK vector, PGK-Egr-1, PGK-zfEgr-1, or PGK-ΔEgr-1) were incubated with or without rhIL-17 (50 ng/ml) in the presence or absence of PGE₂ (100 nmol/liter) for 4 h, after which the cells were extracted for total RNA, and 5 μg were analyzed for TNF-α and egr-1 mRNAs by Northern hybridization using specific digoxigenin-labeled cDNA probes as described under “Materials and Methods.” In C, NIH-3T3 cells (10⁶ cells/well) stably transfected with a eukaryotic expression vector (PGK vector, PGK-Egr-1, PGK-zfEgr-1, or PGK-ΔEgr-1) were transiently cotransfected with 10 μg of WT or mutant Egr-1 site pTNF−615SVOCAT plasmid using Lipofectamine™ 2000 for 6 h according to the manufacturer’s protocol with
pletely abrogate PGE₂-dependent inhibition of induced promoter activity (Fig. 5A).

To better characterize the putative role of Egr-1 in mediating direct PGE₂ inhibition of TNF-α expression, we chose to use THP-1 cells stably transfected with eukaryotic expression constructs of WT Egr-1 and dominant-negative mutants (see “Materials and Methods”). As shown in Fig. 5F, the suppressive effects of PGE₂ on TNF-α mRNA expression were reversed significantly by the dominant-negative mutants PGK-ΔEgr-1 and PGK-zfEgr-1, and the blockade was essentially complete when both were transfected in tandem. Moreover, high level expression of WT PGK-Egr-1 in THP-1 cells inhibited rhIL-17-induced TNF-α mRNA expression as efficiently as did PGE₂ (Fig. 5B).

Because the latter experiments did not rule out the possibility that overexpressed Egr-1 constructs affect TNF-α mRNA stability (as distinct from the transcriptional control), we chose to repeat these experiments with stable NIH-3T3 transfectants expressing Egr-1 (PGK-Egr-1) and the dominant-negative mutants PGK-ΔEgr-1 and/or PGK-zfEgr-1, in which, however, the TNF-α promoter construct was transfected transiently. Our aim was to use a cell culture model that does not express TNF-α mRNA normally (eliminating low signal-to-noise ratios) and in which substantial transient transfection efficacy can be attained. PGK-Egr-1 reduced rhIL-17-stimulated WT pTNF-SVOCAT activity to near vehicle levels, mimicking to a large degree the effects of PGE₂ in this regard (Fig. 5C). The dominant-negative mutants PGK-ΔEgr-1 and PGK-zfEgr-1, alone or in combination, substantially reversed the inhibitory effects of exogenously added PGE₂. Both CAT protein and CAT activity measurements gave very similar results. However, as in the data shown in Fig. 5A, mutating the Egr-1 site did not completely reverse PGE₂/Egr-1-dependent inhibition of rhIL-17-induced promoter activity. As shown in Fig. 5D, the level of expression of all the Egr-1 and mutant constructs was quite substantial (and adequate) compared with endogenous expression of Egr-1 in untreated cells and was represented essentially stoichiometrically in cells treated with PGE₂.

Thus, the previous results presented us with a conundrum: on the one hand, the data provided strong evidence that Egr-1 mediated the inhibitory effects of PGE₂ on induced TNF-α mRNA expression, whereas on the other, PGE₂-stimulated Egr-1 binding to its cognate site in the TNF-α promoter was not wholly adequate for complete (induced) promoter suppression. We thus explored the possibility that PGE₂/Egr-1 may disrupt the rhIL-17-stimulated TNF-α transcriptional enhancer complex. In preliminary experiments using NIH-3T3 cells overexpressing “decoy” constructs (see Ref. 33 using a human synovial fibroblast cell strain) that harbor the transactivation domains of ATF-2, c-Jun, CREB-1, Elk-1, and c-Fos fused to the Gal4 DNA-binding domain, we observed that rhIL-17 induction of the TNF-α promoter CAT activity was blocked by pSV40-Gal4-ATF-2 (1–96) and pSV40-Gal4-c-Jun (1–223) (compare 0.71 ± 0.22 (control) and 6.3 ± 0.79 (rhIL-17) normalized CAT protein at ng/µg of cellular protein versus 0.65 ± 0.19 (ATF-2; p < 0.0002); 3.1 ± 0.75 (c-Jun; p < 0.043), 5.4 ± 1.09 (CREB-1), 6.6 ± 1.2 (c-Fos), and 6.1 ± 2.1 (Elk-1). Gel shift experiments were undertaken to substantiate the latter findings; and as shown in Fig. 6A, rhIL-17 increased the binding to an ATF-2/c-Jun-like 32P-labeled oligonucleotide of the human TNF-α promoter. Substantial supershifted low mobility banding was observed with anti-phospho-ATF-2 (Thr²⁰²⁷) and anti-phospho-c-Jun (Ser³²³³) antibodies, but not with anti-phospho-CREB-1 (Ser³²³) antibody (Fig. 6A, upper panel). The addition of PGE₂ and overexpression of PGK-Egr-1 blocked rhIL-17-stimulated binding to the ATF-2/c-Jun-like 32P-labeled oligonucleotide to a substantial degree (Fig. 6A, lower left panel). The inhibitory effect of PGE₂ was markedly abrogated in cells stably transfected with the dominant-negative mutants PGK-ΔEgr-1 and PGK-zfEgr-1, alone or in combination (Fig. 6A, lower right panel).

We next examined the possible role of Egr-1 in the mediation of PGE₂ effects on c-Jun (AP-1) using NIH-3T3 transformants expressing Egr-1 (PGK-Egr-1) and the dominant-negative mutants PGK-ΔEgr-1 and PGK-zfEgr-1, alone or in combination. As shown in Fig. 6B, rhIL-17-induced c-jun mRNA expression was suppressed by PGE₂, an effect mimicked by Egr-1, but compromised by the Egr-1 dominant-negative mutants. In tandem comparative studies, junB, a wholly inducible gene and potent transcriptional repressor of c-Jun transactivation activity (37), was not responsive to rhIL-17 treatments, but was up-regulated by Egr-1 overexpression. Furthermore, PGE₂ markedly up-regulated the steady-state expression of junB mRNA, and the effect was reversed by the Egr-1 dominant-negative mutants. JunB was not detectable in gel shift experiments (data not shown).

**DISCUSSION**

There is now wide agreement that TNF-α is a key cytokine mediator in the immune and inflammatory responses, initiating a cascade of processes that ultimately serve to eliminate the invading pathogen and to re-establish a state of homeostasis in the organism (4, 5). However, in chronic and systemic inflammatory states, such as those seen in RA, excess TNF-α production is believed to be associated with disease pathogenesis, and reme-}

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Note: The text continues with more detailed scientific discussion and analysis, including experimental results and conclusions, but is not fully transcribed here due to length constraints. The full document would be needed for a comprehensive understanding of the research findings.
Egr-1/Krox-24-mediated PGE₂-dependent Suppression of TNF-α

future research. Here, we have reported that the T-cell-derived pro-inflammatory cytokine IL-17 up-regulated TNF-α expression and synthesis in human macrophages and RASFs largely through ATF-2/c-Jun-dependent transactivation at a 5'-TGAGCTCA-3' palindrome of the TNF-α promoter. In previous reports, IL-17 stimulated ATF2/CRÉB1-dependent transactivation of the COX-2 promoter at a 5'-TTTCGTCA-3' site, and the cytokine cell signaling profile was also restricted to the p38 MAPK cascade in a number of different fibroblast and mesenchymal phenotypes (33). As an example of the complexity and subtlety of cell signaling, the PGE₂-targeted site of the egr-1 promoter (5'-TCACGTCA-3') binds preferentially, although not exclusively, ATF-2 dimers, the latter phosphorylated by p38 MAPK, arguing that signaling intermediates may be “selected” at the level of the promoter sequence.

Several studies suggest that IL-17 signals through NF-κB, and this may be of some significance because the TNF-α promoter has three putative NF-κB-like binding sites (39, 40). However, in all cell types we are concerned with, IL-17 activation of NF-κB is modest, delayed, or inhibited, whereas others have shown that these putative enhancer sites do not mediate the induction of the human TNF-α promoter by substances known to activate NF-κB (i.e. LPS) (see Refs. 32, 40, and 41). In support of this, it was recently shown that CREB-binding protein/p300 mediates LPS induction of TNF-α through assembly of an enhancer complex that includes Ets-1/Elk-1 binding to an NF-AT site, ATF-2/c-Jun dimer binding to the ATF-2/CRE site, and GC box-binding transcription factors in murine monocytic J774/P338D cells (41). It would be of interest, however, to determine whether the latter response is species-specific, as LPS signals through Toll-like receptor-4 in human macrophages and activates the NF-κB cascade (42). Interestingly, TNF-α controls its own synthesis (autoregulation), and a putative palindrome that resembles an ATF/CRE site (bp −125 to −82) apparently mediates TNF-α-dependent promoter induction (43); the transactivation factors were identified as exclusively c-Jun-related proteins. The observed discrepancies may be the result of cell context, and it seems likely that a gene can respond to multiple signaling cascades through the recruitment of different proteins to similar enhancer elements. It should also be mentioned that there are data demonstrating ATF-2/c-Jun transactivation at AP-1 sites of certain promoters as well (44).

As a transcription factor, Egr-1 induces the promoter activity of prostaglandin E synthase directly by binding to tandem GC boxes in the proximal promoter (10), but can act in combinatorial fashion with other transcription factors, as in the case of its own expression in granulosa cells subsequent to follicle-stimulating hormone and luteinizing hormone stimulation (46). In the latter system, Egr-1 associates with Sp-1 to bind a proximal GC box and together with an enhancer complex involving CREB and serum response factor to drive egr-1 mRNA expression. This paradigm is not well suited to our results, as PGE₂ stimulation of Egr-1 protein was delayed by ~30 min, whereas PGE₂-dependent transcriptional induction of egr-1 mRNA was rapid. Our data favor the rapidly induced p38 MAPK pathway with ATF-2/CRÉB transactivation of the egr-1 promoter at cognate sites, although the increase in egr-1 mRNA seen at 4 h may be Egr-1-driven. In stromal cells, up-regulated macrophage colony-stimulating factor gene expression by TNF-α or IL-1β involves Sp-1-dependent promoter transactivation, and this occurs subsequent to Egr-1 phosphorylation and dissociation from Sp-1, resulting in higher levels of free, transcriptionally active Sp-1 (47). In contrast, Sp-1 and Egr-1 have opposing effects on the regulation of the multidrug resistance gene MDR1, where the latter transcription factor suppresses MDR1 promoter activity, and the former activates it (48). Sp-1 expression in our cell culture models is constitutive, and its binding to GC-rich sequences (Egr-1 or TNF-α promoters presumably) appears to be unchanged under the treatments we used. We can not rule out the possibility, however, that Sp-1 transactivation activity may be modified by phosphorylation/glycosyla-
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RATIONALE

Egr-1, a member of the Krox/Er81 family of transcription factors, is involved in the development of the adrenal gland, the differentiation of the thymus, and the cardiovascular system (52). Egr-1 expression is induced within seconds by a variety of extracellular stimuli, including cytokines, growth factors, hormones, and environmental toxins. In human RA-affected synovial membranes, CD45 T-lymphocyte-derived IL-17 activates a resident macrophage/synovial cell to produce TNF-α through an EP4 receptor-mediated negative feedback loop. Egr-1 activates the MLK3/MKK3/p38 MAPK and protein kinase A signaling pathways. This results, more or less simultaneously, in the suppression of induced TNF-α mRNA expression (28, 49). The culture system afforded us the opportunity to monitor the effects of Egr-1 on TNF-α mRNA expression in a more physiological setting, as opposed to transient transfection experiments, which can be laborious and inefficient. We propose that PGE$_2$, acting via feedback or feed-forward mechanisms, interferes with the pro-inflammatory response of cytokines by shutting down cytokine-activated cascades, i.e., p38 MAPK via MKK3 cascade with strong ATF-2/ATF-2:ATF-2/CREB-dependent promoter transactivation of the target promoter. However, PGE$_2$ also stimulates MEK1/2 and ERK1/2 phosphorylation, but is delayed by 1 h in our cell culture models (16), and preliminary results suggest that the eicosanoid drives ERK1/2 ubiquitination and proteasomal processing, as if to terminate an extraneous signal. We propose that PGE$_2$, acting via feedback or feed-forward mechanisms, interferes with the pro-inflammatory response of cytokines by shutting down cytokine-activated cascades, i.e., p38 MAPK via MKK3 cascade with strong ATF-2/ATF-2:ATF-2/CREB-dependent promoter transactivation of the target promoter. However, PGE$_2$ also stimulates MEK1/2 and ERK1/2 phosphorylation, but is delayed by 1 h in our cell culture models (16), and preliminary results suggest that the eicosanoid drives ERK1/2 ubiquitination and proteasomal processing, as if to terminate an extraneous signal. We propose that PGE$_2$, acting via feedback or feed-forward mechanisms, interferes with the pro-inflammatory response of cytokines by shutting down cytokine-activated cascades, i.e., p38 MAPK via MKK3 cascade with strong ATF-2/ATF-2:ATF-2/CREB-dependent promoter transactivation of the target promoter. However, PGE$_2$ also stimulates MEK1/2 and ERK1/2 phosphorylation, but is delayed by 1 h in our cell culture models (16), and preliminary results suggest that the eicosanoid drives ERK1/2 ubiquitination and proteasomal processing, as if to terminate an extraneous signal.

Whether Egr-1 has a role to play in the acute or chronic inflammatory response remains a subject for further research; however, a number of observations may be informative. The RA-affected synovial membrane/pannus expresses markedly elevated levels of egr-1 mRNA (53), whereas other oncogenes such as c-jun and c-fos, for example, are not detectable. COX-2 protein and ambient PGE$_2$ levels are extremely high, whereas pro-inflammatory cytokine production is very low until, as mentioned above, preferential COX-2 inhibitors suppress PGE$_2$ production (36). Egr-1-null mice have compromised pituitary-adrenal axis function (45), presumably leading to a suboptimal...
endogenous anti-inflammatory response. Thus, PGE$_2$/Egr-1 may form a critical axis involved in the modulation of the immune (27) and inflammatory responses and may be essential for the programming of cellular differentiation.

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