

Positive and Negative Regulation of NF- κ B by COX-2

ROLES OF DIFFERENT PROSTAGLANDINS*

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The prostaglandin H synthases (PGHS) catalyze the conversion of arachidonic acid to prostaglandin H₂, the committed step in prostanoid synthesis. Two forms of PGHS exist, PGHS-1 (COX-1) and PGHS-2 (COX-2). The gene encoding the latter form is known to be inducible by a number of stimuli including several inflammatory mediators. Recent evidence indicates that the inducible cyclooxygenase may have both pro- and anti-inflammatory properties through the generation of different prostaglandins. Previous reports indicate that the transcription factor NF- κ B can function upstream of COX-2 to control transcription of this gene and that the cyclopentenone prostaglandins can inhibit NF- κ B activation via the inhibition of the I κ B kinase. Thus, it is suggested that cyclopentenones feed back to inhibit continued nuclear accumulation of NF- κ B. In this report we demonstrate COX-2 expression inhibits nuclear translocation of NF- κ B, and we confirm that the cyclopentenone prostaglandins inhibit NF- κ B. In addition, we show that prostaglandin E₂ and its analogs promote the inherent transcriptional activity of the p65/RelA subunit of NF- κ B in a manner independent of induced nuclear accumulation. Consistent with this evidence, prostaglandin E₂ strongly synergizes with the inflammatory cytokine tumor necrosis factor- α to promote NF- κ B-dependent transcription and gene expression. The data provide a molecular rationale to explain both the pro- and anti-inflammatory nature of COX-2.

namely PGJ₂ (1, 2). These prostanoids have a diverse array of effects on cellular processes, such as affecting vascular permeability, inducing hyperalgesia, and driving T helper and B cell differentiation (1, 2). However prostaglandins do not always act in a similar or coordinate manner. For example PGI₂ inhibits platelet aggregation, whereas thromboxane A₂ increases it (3). Therefore, the net effect of PGs may depend on their balance or timing within a tissue or during a physiological response.

Whereas PGHS-1 is constitutively expressed, PGHS-2 is inducibly expressed in most mammalian cells. This expression occurs rapidly following stimulation by cytokines, growth factors, and bacterial endotoxin (4, 5). PGHS-2 plays a major role in inflammatory processes, and its expression has been correlated with several diseases associated with inflammation and with colon cancer (6–11). In addition to its pro-inflammatory aspects, PGHS-2 has been shown to have anti-inflammatory properties. Thus, Gilroy, *et al.* (12) showed in a carrageenin-induced pleurisy model in rats that PGHS-2 protein peaked at 2 h which was associated with maximal PGE₂ synthesis and increasing inflammation. However, at a later time point, PGHS-2 exhibited a second increase that was associated with reduced PGE₂ synthesis but with increased levels of PGD₂ and PGJ₂ and with decreased inflammation. PGHS inhibitors reversed this trend, inhibiting inflammation at the early time point but enhancing inflammation later. These data indicate that the PGE₂ type prostaglandins are associated with pro-inflammatory mechanisms, whereas the PGD₂ and PGJ₂ forms are associated with suppression of inflammation.

The transcription factor NF- κ B has been shown to be important in inflammation, suppression of apoptosis, and in cell proliferation (13, 14). In addition, NF- κ B has been shown to control the induced transcription of the PGHS-2 gene (15, 16). The prototypical member of the NF- κ B family is a heterodimer consisting of p65/RelA and p50/NF- κ B1. Both subunits contribute to DNA binding, but only the p65 subunit has transactivation domains capable of initiating transcription. The NF- κ B heterodimer is typically localized to the cytoplasm by the inhibitory protein, I κ B. Upon stimulation of the cell, such as with TNF- α , I κ B is phosphorylated, ubiquitinated, and degraded. This allows the free NF- κ B to accumulate in the nucleus where it can activate transcription. In the nucleus NF- κ B can regulate the expression of many genes involved in inflammation such as ICAM-1, IL-2, IL-8, and complement factors (13). In fact, strong evidence indicates that chronic dysregulation of NF- κ B may underlie most inflammatory diseases and contribute to oncogenesis (17, 18).

Although most research has focused on the induction in NF- κ B activity caused by increased nuclear levels of NF- κ B, there is evidence that the transcription function of NF- κ B can be enhanced without increasing nuclear levels of the NF- κ B subunits. For example, an early report indicated that protein

Prostaglandin H synthases 1 and 2 (PGHS-1¹ and PGHS-2; or cyclooxygenase-1 and -2 (COX-1 and COX-2)) catalyze the conversion of arachidonic acid and O₂ to generate prostaglandins (PGs). PGHS-1 and -2 catalyze both a cyclooxygenase reaction in which arachidonate is converted to PGG₂ and a peroxidase reaction in which PGG₂ is reduced to prostaglandin H (PGH₂). Subsequently, PGH₂ is converted to the key prostanoids PGE₂, PGF₂, PGI₂, PGD₂, and thromboxane A₂. PGD₂ can also be converted into cyclopentenone prostaglandins,

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¹ The abbreviations used are: PGHS, prostaglandin H synthase; PG, prostaglandin; COX, cyclooxygenase; TNF- α , tumor necrosis factor- α ; EMSA, electrophoretic mobility shift assay; EP, prostaglandin E; IKK, I κ B kinase; IL, interleukin; TAD, transactivation domain; MHC, major histocompatibility complex; PBS, phosphate-buffered saline.

kinase C could stimulate the function of the transactivation domain of p65, leading to enhanced NF- κ B function (19). Recently, phosphorylation of p65 has been shown to increase transcriptional activity of NF- κ B (20–22) at least partly through the recruitment of coactivators (23). Moreover, some inhibitors of NF- κ B activity function without affecting induced nuclear accumulation (24). These data indicate that dual controls exist for NF- κ B, with one pathway functioning to increase nuclear levels of NF- κ B while another controls inherent trans-activation potential.

As mentioned previously (25–28), NF- κ B can up-regulate PGHS-2 expression. Additionally, recent evidence indicates that PGHS-2 activity may also affect NF- κ B. This could lead to a positive or negative feedback control mechanism dependent on whether PGHS-2 has a positive or negative effect on NF- κ B activity. Since PGHS-2 has anti- and pro-inflammatory functions, we were interested in testing whether these distinct processes could be due to the opposing actions of PGHS-2 and its effectors on NF- κ B activation. By controlling NF- κ B activity, PGHS-2 and prostaglandins could self-regulate PGHS-2 expression and ultimately control other NF- κ B-dependent inflammatory genes. In the current study, we used the immortalized colorectal cell lines, Caco-2 and HT-29, in order to determine if the expression of PGHS-2 and the action of prostaglandins could regulate NF- κ B activity. We have found that PGHS-2 and its different effector prostaglandins can up-regulate or down-regulate NF- κ B function through distinct regulatory mechanisms.

EXPERIMENTAL PROCEDURES

Cell Lines and Treatments—HT-29 human colon epithelial cells (American Type Culture Collection (ATCC HTB 38)) were grown in McCoy's 5A growth medium supplemented with 10% fetal bovine serum and antibiotics. Caco-2 human intestinal epithelial cells (ATCC HTB 37) were grown in minimal essential medium- α mixture supplemented with 10% fetal bovine serum and antibiotics. Both Caco-2 and HT-29 cells were used during an early passage (<10 passages). Prostaglandins were obtained from Cayman Chemical (Ann Arbor, MI). 16,16-Dimethylprostaglandins were utilized for their enhanced stability as recommended by the supplier. 16,16-Dimethyl-PGE₂ binds EP receptors similarly to PGE₂ (29).

Expression Vectors—A mammalian expression vector for human cyclooxygenase-2 under control of the CMV promoter was kindly provided by Timothy Hla (Center for Vascular Biology, University of Connecticut). An expression vector encoding the NF- κ B p65 subunit has been described previously (30). Gal4-p65 and Gal4-VP16 expression vectors have been described previously (31). For the analyses pcDNA 3.1 from CLONTECH (Palo Alto, CA) was used as vector control. Recombinant adenovirus (either control or encoding the "super-repressor" form of I κ B) was prepared by the University of North Carolina Gene Therapy Center.

Luciferase Assay Vectors—The NF- κ B-responsive luciferase construct contains three tandem repeats of the κ B site from the class I MHC enhancer cloned into a luciferase expression vector (32). The Gal4-responsive luciferase construct contains five tandem repeats of the Gal4 site cloned into a luciferase expression vector (33).

Nuclear and Cytoplasmic Extracts—Caco-2 cells were plated at 1×10^6 cells per 100-mm tissue culture plate in complete medium. If required, cells were transfected the next day as described below. Cells were then treated as described with TNF- α (10 ng/ml) (Promega, Madison, WI), diclofenac (Cayman Chemical, Ann Arbor, Michigan), or prostaglandins for 24 h or as described. After treatment, cells were washed in phosphate-buffered saline (PBS), scraped from the plates, transferred to microcentrifuge tubes, and lysed on ice in 1 pellet volume of cytoplasmic extraction buffer (10 mM Hepes, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2.3 μ g/ml aprotinin, leupeptin, and pepstatin). Nuclei were pelleted, and cytoplasmic supernatants were transferred to fresh tubes. Nuclei were washed once in cytoplasmic extraction buffer lacking Nonidet P-40 and then repelleted. Nuclear pellets were resuspended by vortexing in 1 pellet volume of nuclear extraction buffer (20 mM Tris, pH 8.0, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 2.3 μ g/ml aprotinin,

leupeptin, and pepstatin). All cytoplasmic and nuclear extracts were spun and transferred to fresh tubes. Glycerol was added to cytoplasmic extracts to a final concentration of 20%. Protein concentrations were determined by the Bradford assay using the Bio-Rad protein assay dye reagent (Bio-Rad catalog number 500-0006), and extracts were stored at -70°C .

EMSA—Electrophoretic mobility shift assays (EMSAs) were performed as described previously (32). Briefly, equal amounts of nuclear extract protein were incubated for 15 min at room temperature with a ³²P-labeled NF- κ B probe containing a κ B site from a murine MHC I promoter. Reactions were carried out in binding buffer (10 mM Tris, pH 7.7, 50 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) containing 2 μ g of poly(dI-dC) (Amersham Pharmacia Biotech). Complexes were resolved on a 5% polyacrylamide gel in Tris glycine/EDTA buffer (25 mM Tris, 190 mM glycine, and 1 mM EDTA). After completion of the run gels were dried and autoradiographed.

Transfections—Caco-2 cells were transiently transfected using Fugene transfection reagent from Roche Molecular Biochemicals as described by the manufacturer. Cells were transfected 24 h after being plated. Transfections for EMSAs were carried out in 100-mm tissue culture plates, whereas transfections of luciferase assays were carried out in either 6- or 12-well dishes. For cultures prepared in 100-mm, 6-well, and 12-well dishes, 12, 1, and 0.25 μ g of DNA were transfected, respectively. Caco-2 cells were also transfected with a peGFP-C1 expression vector from CLONTECH in order to evaluate the transfection efficiency. Over 80% of transfected cells expressed GFP by fluorescence microscopy.

Luciferase Assay—Cells were transfected using Fugene as described above. Twenty four hours after transfection, cells were washed with PBS and treated with various prostaglandins, TNF- α , diclofenac, or dimethyl sulfoxide (Me₂SO). 16 h after treatment, cells were washed with PBS and lysed at room temperature for 10 min with shaking using M-PER Mammalian Protein Extraction Reagent from Pierce. Cell were transferred to microcentrifuge tubes and spun at maximum speed for 10 min. Protein concentrations were determined as described above. 100 μ g of protein was analyzed using a luminometer as described previously (34).

Northern Blot— 1×10^6 cells were plated on 100-mm dishes. The next day cells were washed with PBS and treated with media containing 10 ng/ml TNF- α , 16,16-dimethyl-PGE₂, both or neither for various time points. mRNA was obtained using the Qiagen (Valencia, CA) RNeasy mRNA preparation kit. IL-8 cDNA was used with Stratagene (La Jolla, CA) Quikhyb hybridization solution. Gene Hunter (Nashville, TN) loading dye containing ethidium bromide allowed us to verify efficient osmotic transfer from gel to blot. Adenoviral infections were performed with Ad-LacZ or Ad-I κ B-Super-repressor as described previously (35).

RESULTS

A COX-2 Inhibitor Blocks the Nuclear Accumulation of NF- κ B—Based on earlier reports of the effects of different prostaglandins on NF- κ B nuclear levels (*i.e.* inhibition by cyclopentenones and accumulation of nuclear NF- κ B by PGE₂; see Refs. 24 and 26), we examined whether PGHS-2 activity could affect NF- κ B activity. The transformed colorectal cell line HT-29 constitutively expresses an active prostaglandin H synthase 2 (36). Accordingly, we determined whether NF- κ B signaling could be altered by inhibiting PGHS-2 activity in HT-29 cells. We performed an EMSA on nuclear extracts of HT-29 cells (Fig. 1), which had been treated with either TNF- α (2nd to 5th lanes), or a PGHS inhibitor diclofenac (7th to 10th lanes), or both reagents (12th to 15th lanes). As expected TNF- α was able to stimulate nuclear translocation of NF- κ B in HT-29 cells. The PGHS inhibitor diclofenac alone did not have any significant effect on the nuclear localization of NF- κ B (Fig. 1). In contrast, when diclofenac and TNF- α were simultaneously added to the HT-29 cells, increased levels of NF- κ B nuclear translocation were observed from 4 h onward relative to TNF- α treatment alone. This result indicates that the ability of TNF- α to induce NF- κ B activation can be enhanced when the function of the two PGHS isoenzymes is inhibited and that PGHS functions to feed back and limit NF- κ B activation. It is known that TNF- α can up-regulate COX-2 expression in HT-29 cells

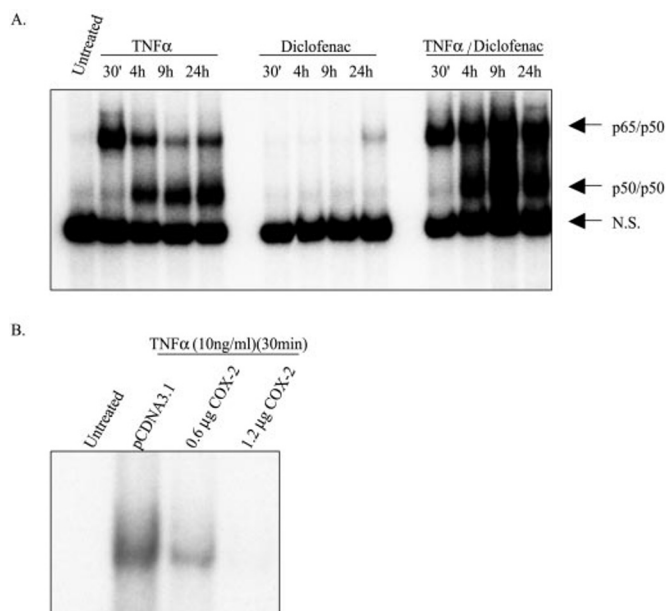


FIG. 1. COX-2 expression inhibits the ability of TNF- α to induce NF- κ B nuclear translocation. A, HT-29 cells were treated with either simultaneous addition of 10 ng/ml TNF- α and 100 μ M diclofenac (10th to 13th lanes), TNF- α (2nd to 5th lanes), or diclofenac (6th to 9th lanes) only. EMSAs of nuclear extracts were performed using a DNA probe containing an NF- κ B-binding site. Supershifts were performed to determine which NF- κ B subunits were present (data not shown). B, Caco-2 cells were transiently transfected with vector control pCDNA 3.1 (2nd lane), COX-2 expression vector (3rd and 4th lanes), or untransfected (1st lane). EMSAs of nuclear extracts were performed as described above. Data are representative of at least three independent experiments. N.S., nonspecific.

by activating NF- κ B activity (16). Therefore, our data suggest that the induction of COX-2 in response to TNF- α signaling leads to a negative feedback response to block NF- κ B nuclear accumulation.

In order to understand further the role of PGHS-2 in the regulation of NF- κ B activation, we next performed transient transfection experiments using a PGHS-2 expression vector. The colorectal cell line Caco-2 expresses PGHS-2 at relatively low levels and can be transfected at high efficiency (37). NF- κ B activity has not been studied previously in cells overexpressing COX-2. Therefore, we studied the effect of overexpressing PGHS-2 on the induction of NF- κ B activity in this cell line. As expected, TNF- α was able to induce nuclear translocation of NF- κ B in cells transfected with the vector alone (Fig. 1). Cells transfected with increasing levels of a PGHS-2 expression vector exhibited decreased nuclear translocation of NF- κ B (Fig. 1, lanes 3 and 4).

Distinct Classes of Prostaglandins Regulate NF- κ B Differently—The result described above indicated that the constitutive expression of PGHS-2 could negatively regulate NF- κ B activation. This response could occur through several mechanisms. Reports indicating that PGHS-2 contains perinuclear localization and integral membrane domains provide speculation regarding prostaglandin-independent mechanisms of PGHS-2 function (38, 39). However, we focused our attention on the products of the PGHS-2 enzyme, namely prostaglandins. Prostaglandins are essential to inflammation and have also been implicated in altered cell growth in some cancer models (40, 41). Although there are three classes of prostanoids, namely prostaglandins, thromboxanes, and prostacyclins, we focused on the role of prostaglandins in our analysis.

Various prostaglandins were assessed for their ability to activate or repress an NF- κ B-dependent promoter. A luciferase reporter construct containing three tandem repeats of the κ B

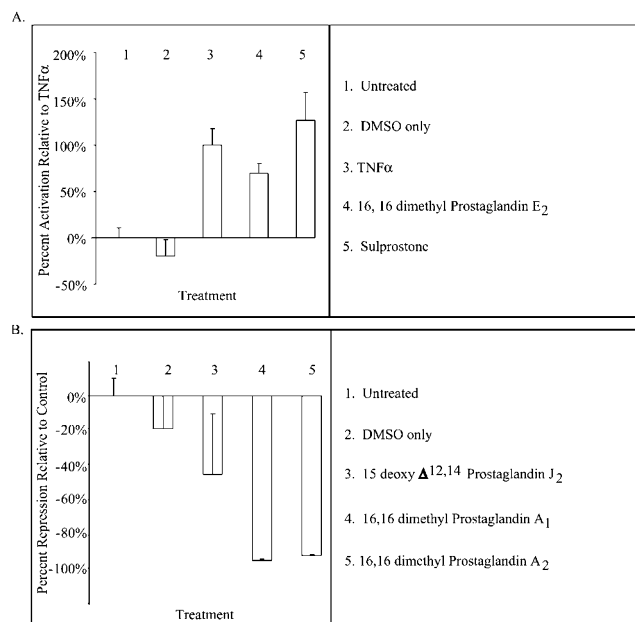


FIG. 2. Prostaglandins can activate and suppress an NF- κ B reporter. Caco-2 cells were transiently transfected with a reporter construct containing three NF- κ B-binding sites, namely the 3X- κ B reporter. Transfected cells were treated with Me₂SO (DMSO), TNF- α , or prostaglandins for 16 h, after which a luciferase assay was performed. Activation is displayed relative to TNF- α -induced activation with the untreated control being designated 0 and TNF- α -induced activation being designated 1. Negative values indicate suppression below background (untreated control). For these experiments prostaglandins were added at 100 μ M concentration. TNF- α was added at 10 ng/ml. Data are representative of triplicate wells from at least five independent experiments.

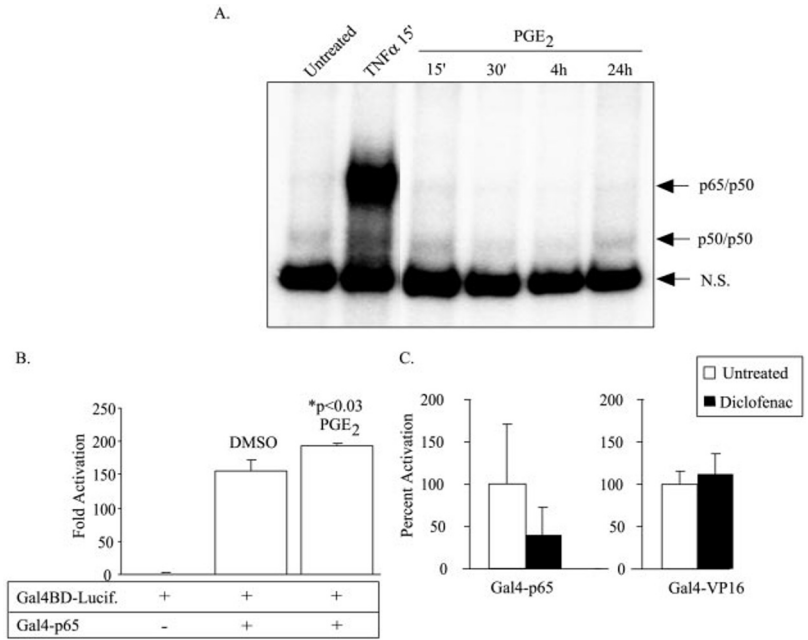
site from the class I MHC enhancer cloned into a luciferase expression vector was transiently transfected into Caco-2 cells. After 24 h, cells were treated with complete medium containing various prostaglandins. TNF- α was used as a positive control for NF- κ B activation. 16 h after treatment a luciferase assay was performed. We observed a 3–5-fold increase in promoter activity after TNF- α treatment (Fig. 2). Me₂SO alone, which was the solvent used to dissolve prostaglandins, gave no significant change in reporter activity (Fig. 2). In order to draw comparisons between experiments, prostaglandin activity is represented relative to TNF- α -induced activation.

Different prostaglandins were able to both enhance or inhibit NF- κ B activity. Prostaglandin E₂ and its analogs, which bind the EP receptor family, were able to activate NF- κ B (Fig. 2). The most effective NF- κ B activators were 16,16-dimethyl-PGE₂ and sulprostone, both of which are PGE₂ analogs. 16,16-Dimethyl-PGE₂ and sulprostone increased promoter activity 2- and 3-fold, respectively, which represents a 70 and 127% increase relative to TNF- α stimulation. Even at the lowest concentrations (1 μ M) of 16,16-dimethyl-PGE₂ tested, we continued to observe increased promoter activity (data not shown). Since increases in luciferase indicate enhanced transcriptional activity of NF- κ B, this result indicates that PGE₂ and its analogs can up-regulate the ability of NF- κ B to stimulate gene expression.

Although the EP receptor agonists were able to up-regulate NF- κ B promoter activity, we also found several prostaglandins that down-regulated promoter activity. Since Caco-2 cells have a high basal level of NF- κ B-dependent luciferase activity, prostaglandins could also be assessed for their ability to suppress NF- κ B activity. 15-Deoxy- Δ ^{12,14}-PGJ₂, 16,16-dimethyl-PGA₁, and 16,16-dimethyl-PGA₂ all inhibited promoter activity (Fig. 2). The PGA analogs inhibited basal promoter activity by

FIG. 3. 16,16-Dimethyl-PGE₂ affects NF- κ B activity by increasing the function of the p65 transactivation domain but without affecting nuclear levels.

A. Caco-2 cells were treated with either TNF- α (10 ng/ml) or 16,16-dimethyl-PGE₂ (100 μ M) for various times. Nuclear extracts were prepared as described under "Experimental Procedures." EMSAs of nuclear extracts were performed using a DNA probe containing an NF- κ B-binding site. **B.** Caco-2 cells were transiently transfected with the Gal4-Luciferase construct and either the Gal4-p65 or control vector. 24 h after transfection, cells were treated with Me₂SO (DMSO) or 16,16-dimethyl-PGE₂ for 16 h. Luciferase assays were performed. Data were analyzed with the Student's *t* test. **C.** Caco-2 cells were transiently transfected with the Gal4-Luciferase constructs and either the Gal4-p65 or Gal4-VP16 construct. 24 h after transfection, cells were treated with 100 μ M diclofenac or untreated for 16 h. Luciferase assays were performed. Data are representative of duplicate wells from at least three independent experiments.



greater than 10-fold, whereas a 2-fold decrease was observed after 15-deoxy- $\Delta^{12,14}$ -PGJ₂ treatment. This represents a decrease of 47 and 95% over basal activity by 15-deoxy- $\Delta^{12,14}$ -PGJ₂ and PGA, respectively. Therefore, our results indicate that there are both stimulatory and inhibitory prostaglandins with respect to NF- κ B activity.

PGE₂ Does Not Induce Nuclear Accumulation of NF- κ B—In order to examine the mechanism whereby PGE₂ up-regulates NF- κ B activity, we performed an EMSA of nuclear extracts from Caco-2 cells treated with PGE₂. Another study had reported that PGE₂ can lead to the translocation of NF- κ B into the nucleus of T cells (25); however, stimulation with PGE₂ did not induce nuclear translocation of NF- κ B in Caco-2 cells, whereas TNF- α did (Fig. 3A). We also performed a Western blot of PGE₂-treated Caco-2 cells in order to examine the levels of cellular inhibitor of NF- κ B, I κ B α . Although the expected degradation of I κ B α after TNF- α treatment was clearly observed, PGE₂ treatment did not lead to such degradation (data not shown). These data are consistent with a lack of induction of nuclear accumulation of NF- κ B after PGE₂ treatment.

PGE₂ Induces the Transactivation Potential of the p65/RelA Subunit—Although increases in NF- κ B transcriptional activation are typically associated with increasing nuclear levels, recent reports suggest that alternative pathways exist by which NF- κ B transcriptional function can be enhanced. For example, the phosphorylation of the p65 transactivation domain at serine 529 has been shown to be important for TNF- α -induced NF- κ B activation (20). Additionally, coactivators, which are important for some of the functions of NF- κ B, have been shown to be dependent on the phosphorylation of NF- κ B (21). Therefore, we used a Gal4 DNA-dependent reporter assay to examine the transcriptional activation of NF- κ B independent of nuclear translocation. The Gal4-p65 construct contains the Gal4 DNA binding domain fused to the first transcriptional activation domain (TAD-1) of p65 (residues 521–551). Consequently, when the transcriptional activation mediated by p65 TAD-1 is increased, transcription of the luciferase gene driven by a promoter containing Gal4 DNA binding domains will be increased.

Cells transiently cotransfected with the Gal4-Luciferase and Gal4-p65 (TAD) constructs were treated with either Me₂SO or 16,16-dimethyl-PGE₂. We observed a consistent 30% increase in fold activation after PGE₂ treatment. Although the increase

reflected a high luciferase response, the lack of a high fold activation is likely due to high basal levels of Gal4-p65 (TAD) in Caco-2 cells (Fig. 3B). Therefore, since it is known that Caco-2 cells express detectable levels of PGHS-2, we tested whether inhibiting PGHS-2 with diclofenac could decrease the basal Gal4-p65 (TAD) activity (42). Indeed, treatment with diclofenac decreased the high basal levels of Gal4-p65 (TAD) by over 50% but had no effect on a Gal4-VP16 construct (Fig. 3C). Consistent with these results, PGE₂ stimulated expression of the Gal4-p65 reporter severalfold in WiDr cells, another colorectal cell line, which have lower basal levels of Gal4-p65 luciferase activity (data not shown).

PGE₂ Strongly Synergizes with TNF- α to Induce NF- κ B-dependent Reporter Activity as Well as Endogenous, NF- κ B-regulated, IL-8 Gene Expression—Since no increase in nuclear translocation of NF- κ B after PGE₂ treatment was observed despite an increase in transcriptional activation of p65, we tested whether prostaglandins could synergize with TNF- α to stimulate NF- κ B activity. It was hypothesized that PGE₂ could utilize enhanced levels of NF- κ B to stimulate gene expression. Sulprostone treatment synergized with TNF- α to activate NF- κ B-dependent luciferase activity in transfected Caco-2 cells (Fig. 4). Sulprostone and TNF- α together lead to a 2-fold activation compared with Me₂SO and TNF- α . Because TNF- α /Me₂SO leads to a 5-fold activation, this indicates that TNF- α /sulprostone caused a 10-fold activation over untreated cells. As expected from previous data PGJ₂ and PGA₁ both inhibited TNF- α induced NF- κ B activation. These results indicate that PGE₂ can synergize with TNF- α in order to activate NF- κ B.

Since our data indicated that PGE₂ could up-regulate NF- κ B activity, we next examined whether PGE₂ could up-regulate NF- κ B-dependent, endogenous gene expression. We chose to examine IL-8, which is an important NF- κ B-regulated gene expressed during acute inflammatory processes and in some disease states. TNF- α treatment alone could up-regulate IL-8 mRNA transcripts over time (Fig. 5). 16,16-Dimethyl-PGE₂ alone caused low levels of IL-8 mRNA transcription. However TNF- α and 16,16-dimethyl-PGE₂ treatment, added simultaneously to cultures, led to enhanced levels of IL-8 mRNA compared with either treatment alone. Between 4 and 6 h TNF- α treated cells exhibited a large decrease in IL-8 mRNA levels, whereas cotreatment of PGE₂ and TNF- α showed a minimal decrease over this period. Moreover, at 10 h IL-8 transcripts

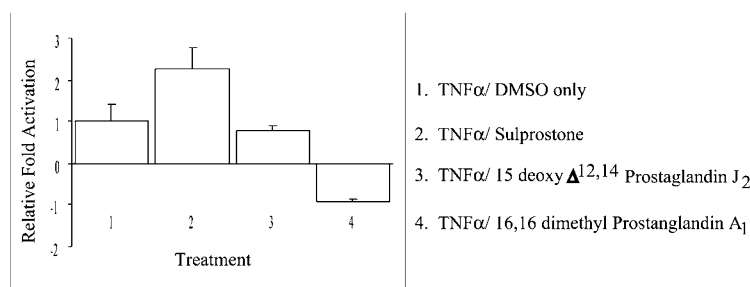


FIG. 4. **PGE₂ can synergize with TNF- α to activate NF- κ B-mediated transcription.** Various prostaglandins were tested for their ability to enhance or inhibit NF- κ B activity induced by TNF- α . Caco-2 cells were transiently transfected with the 3X- κ B reporter luciferase construct. After 24 h, cells were treated with TNF- α and either Me₂SO (DMSO) or prostaglandin. Activation is displayed relative to TNF- α -induced activation. For these experiments prostaglandins were added at 100 μ M concentration. TNF- α was added at 10 ng/ml.

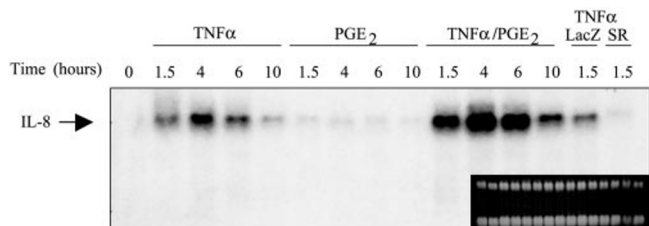


FIG. 5. **PGE₂ can synergize with TNF- α to up-regulate endogenous IL-8 expression.** Northern blot of Caco-2 mRNA using an IL-8 cDNA probe. Caco-2 cells were treated as indicated for various times. mRNA was purified and Northern blots performed for IL-8 expression. For a control Caco-2 cells were infected with either β -galactosidase (LacZ) control or super-repressor (SR)-encoding adenoviruses. Inset is the ethidium bromide-stained Northern blot.

continued to be elevated in cotreated cells, but not after treatment with TNF- α or PGE₂ alone (Fig. 5). Finally, in order to establish that the IL-8 mRNA expression was dependent on NF- κ B activity, we utilized the super-repressor form of I κ B α . The I κ B-SR construct encodes a mutant form of the inhibitor of NF- κ B, I κ B, which can bind NF- κ B but cannot be degraded. Infection of Caco-2 cells with control adenovirus encoding LacZ did not affect the ability of TNF- α to up-regulate IL-8 expression. However, IL-8 levels were significantly reduced in cells infected with an adenovirus encoding the I κ B-SR. This indicated that the induced IL-8 expression was dependent on NF- κ B activity. RNA loading was similar in all lanes as evident by ethidium bromide staining of the RNA gel and blot. Overall, these results indicate that PGE₂ can enhance the increase in IL-8 gene expression caused by TNF- α stimulation.

DISCUSSION

The physiology and pathophysiology of inflammation has become an area of intense research as it has been realized that much disease morbidity is due in large part to the inflammatory process. There are both pro- and anti-inflammatory mediators of inflammation. These mediators must be kept in balance by complex regulatory, homeostatic mechanisms; otherwise disease or cell injury may occur. Therefore, in order to better control inflammation in a clinical setting, we must understand which mediators are pro-inflammatory and which are anti-inflammatory. Recently it has been shown that PGHS-2 can function both in a pro-inflammatory and an anti-inflammatory manner (12). In this study we examined how the effectors of prostaglandin H synthase-2, namely prostaglandins, affect the activation of NF- κ B. We have observed that different prostaglandins can function both as positive as well as negative regulators of NF- κ B. Thus, we found that PGA₁, PGA₂, and PGJ₂ were all able to suppress the activation of NF- κ B. Recent reports indicate that cyclopentenone prostaglandins can directly inhibit the I κ B kinase (IKK), thereby inhibiting NF- κ B activation (26–28), which is consistent with our

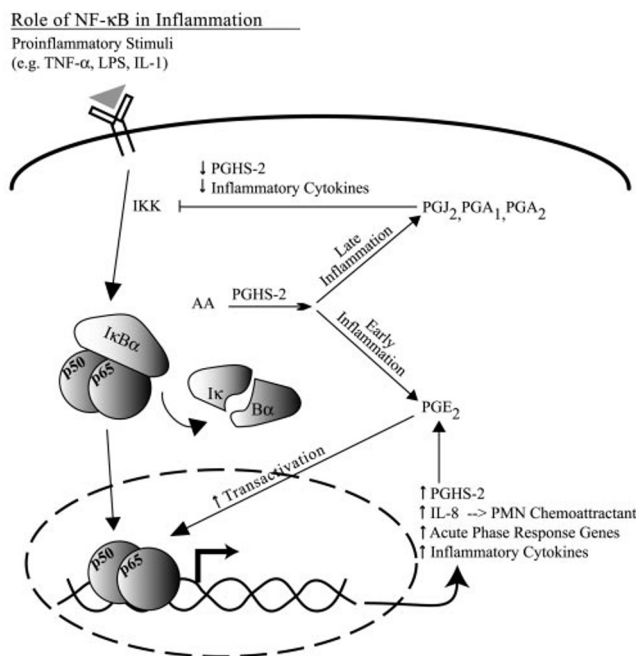


FIG. 6. **Model for NF- κ B in inflammation.** Inflammatory stimuli initiate signaling cascades that increase NF- κ B nuclear levels. This in turn leads to the induction of PGHS-2. The PGHS-2 enzyme catalyzes the conversion of arachidonic acid into various prostaglandins. Initially PGE₂ is most abundant. PGE₂ increases the transactivation function of NF- κ B, which enhances the expression of NF- κ B-dependent inflammatory genes, such as IL-8. Eventually a shift occurs in which PGE₂ becomes less abundant while cyclopentenone prostaglandins are increased. The cyclopentenone prostaglandins inhibit NF- κ B activity at least partly through IKK inhibition. This restricts NF- κ B to the cytoplasm and decreases NF- κ B-dependent inflammatory gene expression. This negative feedback loop leads to reduced PGHS-2 gene expression due to decreased NF- κ B activity. Ultimately these events lead to the resolution of inflammation.

findings. We have also observed in this study that cyclopentenone prostaglandins can decrease basal levels of NF- κ B activity and inhibit TNF- α -induced NF- κ B activation in Caco-2 colorectal cells. The PGA prostaglandins had a greater inhibitory effect on NF- κ B activity in Caco-2 cells than other cyclopentenone prostaglandins.

Our data also indicate that several agonists of EP receptors were able to up-regulate NF- κ B activity. Thus, 16,16-dimethyl-PGE₂ and its analog sulprostone were able to enhance basal levels of NF- κ B activity in Caco-2 cells by 2–3-fold. However, EP receptor agonists do not cause the nuclear accumulation of NF- κ B or the degradation of I κ B and therefore do not activate IKK. Our results indicate that PGE₂ activates the transcriptional activation domain of p65 in order to regulate NF- κ B activity. Moreover, when nuclear levels of NF- κ B are increased

with TNF- α stimulation, sulprostone enhances NF- κ B activity by more than 2-fold (245%), and importantly, this increase in NF- κ B activity leads to enhanced levels of IL-8 mRNA.

Several reports indicate that the regulation of transactivation function is an important mechanism for controlling the level of activity of NF- κ B. For example, cAMP-dependent protein kinase has been shown to phosphorylate p65, which stimulates its interaction with the CREB-binding protein transcriptional coactivator (21). Moreover, phosphorylation of p65 occurs in response to TNF- α signaling and regulates NF- κ B function (20). In these cases it is presumed that the activation of NF- κ B transactivation is caused by enhanced association with transcriptional coactivators (23, 43) in response to phosphorylation of NF- κ B subunits. Additionally, the serine/threonine kinase Akt has been shown to stimulate NF- κ B activity through induction of p65 transactivation function although there is no evidence that Akt directly phosphorylates p65 (44). Interestingly, PGE₂ has recently been shown to activate Akt in the colorectal cell line, LS-147 (41). However, the Akt pathway is not likely to be relevant in mediating the activation of NF- κ B by PGE₂ in Caco-2 cells since an inhibitor of phosphatidylinositol 3-kinase, which is upstream of Akt, did not block the ability of PGE₂ to activate the NF- κ B reporter (data not shown).

Testing the ability of PGE₂ to activate the first transcriptional activation domain (TAD1) of p65 led to a significant but fairly modest 30% increase in transcriptional activity. High basal levels of luciferase activity were consistently observed when using the Gal4-p65 construct in Caco-2 cells. Because Caco-2 cells constitutively express PGHS-2, although at low levels, we hypothesized that the high level of basal luciferase activity observed with the Gal4-p65 construct was due to an increase in stimulatory prostaglandins produced by PGHS-2 in Caco-2 cells. This likely obscured the ability to measure a strong effect of PGE₂ on the Gal4-p65 reporter. To address this hypothesis, we inhibited COX-2 with a PGHS inhibitor, diclofenac, which reduced basal levels of Gal4-p65 by more than 50%.

A recent study by Gilroy *et al.* (12) sheds light on our data. These authors showed that early in the inflammatory process after the induction of PGHS-2 when levels of PGE₂ are highest, PGHS-2 functions in a pro-inflammatory manner. Later in the inflammatory response, when PGJ₂ levels are highest, PGHS-2 function is anti-inflammatory. Inhibiting PGHS-2 early leads to a decrease in inflammation, but at later times inhibition of PGHS-2 actually leads to the exacerbation of inflammation. Our findings regarding the effects of prostaglandins on NF- κ B activity are consistent with these results. Since PGE₂ is increased early in PGHS-2 activity, NF- κ B activity would be expected to be high in order to up-regulate pro-inflammatory genes such as IL-8. IL-8 is known to be important in early inflammation when neutrophils are most abundant (45–47). Therefore, PGE₂ alone may only initiate a minimal inflammatory response, due to low basal levels of NF- κ B. However, in the context of other inflammatory stimuli, such as TNF- α or IL-1 β , PGE₂ could enhance the function of nuclear NF- κ B and significantly increase inflammation (see model, Fig. 6). PGE₂ may enhance p65 transactivation by increasing the phosphorylation of p65. This could occur at the same site used by TNF- α to increase transactivation or it may occur at a different residue. Alternatively, PGE₂ may act by a mechanism other than the phosphorylation of p65. Additionally at later time points of PGHS-2 activity when PGJ₂ is increased, NF- κ B activity would be down-regulated. This would lead to a decrease in sensitivity to TNF- α , IL-1 β , and other inflammatory mediators (Fig. 6).

Exactly how the switch occurs from the production of PGE₂ to PGJ₂ is not understood. Nevertheless, PGHS-2 may be linked to the synthesis of multiple prostaglandins in a temporal

manner in order to regulate the activity of NF- κ B accordingly. This would enhance the ability of the cell to control inflammation. Dysregulation of NF- κ B activation is thought to be involved in several chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (48–52). This dysregulation may be partially due to a defect in PGHS-2-mediated prostaglandin production.

It is also interesting that PGE₂ has been shown to promote cell growth in some cancer models. It has been shown that PGE₂ can cause decreased programmed cell death in HCA-7 human colonic cancer cells (40). Moreover when *Min* mice, a mouse model for colorectal cancer, had EP receptor signaling blocked with the synthetic EP receptor antagonist ONO-8711, they formed fewer adenomatous polyps (53). Recently, PGE₂ has been shown to increase growth and motility of the human colorectal carcinoma cell line, LS-174 (41). These studies indicated that PGE₂ may be important in colon carcinogenesis. Importantly, it is proposed that NF- κ B is important for oncogenic transformation, at least partly through its ability to block apoptosis (18, 54, 55). Thus, it is possible that PGE₂ exerts its anti-apoptotic and oncogenic effects by up-regulating NF- κ B.

In conclusion we have proposed a mechanism by which EP receptor agonists can activate NF- κ B through its transactivation domain and by which cyclopentenone prostaglandins can inhibit NF- κ B. This correlates with the inducible form of prostaglandin H synthase functioning in both pro- and anti-inflammatory roles. Depending on the prostaglandins that PGHS-2 produces, the transcriptional activity of the transcription factor NF- κ B can be controlled during an inflammatory process. Ultimately the activity of NF- κ B is likely to be essential to controlling the extent of an inflammatory response.

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