Rapid and transient induction of cyclo-oxygenase 2 by epidermal growth factor in human amnion-derived WISH cells

Douglas J. PERKINS* and Douglas A. KNISS*†‡

*Department of Cell Biology, Neurobiology and Anatomy, The Ohio State University, College of Medicine, Columbus, OH 43210, U.S.A., and †Department of Obstetrics and Gynecology (Laboratory of Perinatal Research), The Ohio State University, College of Medicine, 1654 Upham Drive, Means Hall, Columbus, OH 43210, U.S.A.

The central enzyme in the prostaglandin (PG) biosynthetic cascade is PGH₂ synthase or cyclo-oxygenase (COX). At present, two distinct isoforms of PGH₂ synthase/COX have been identified: COX-1 and COX-2. In many systems, COX-1 is a constitutively expressed isoform that is responsible for normal physiological production of PGs, whereas COX-2 is an inducible isoform that responds to cytokines, endotoxin and growth factors by producing high levels of PGs. The regulation of COX-2 mRNA and protein, and the subsequent production of PGE₂, were therefore examined in amnion-derived WISH cells stimulated with epidermal growth factor (EGF). Treatment of WISH cells with EGF (0.01–100 ng/ml) elicited dose-dependent synthesis of COX-2 mRNA and protein *de novo*. In addition, stimulation of WISH cells with EGF (10 ng/ml) induced steady-state levels of COX-2 mRNA and protein that appeared within 30 min and then declined rapidly to near baseline levels within

2–4 h. In contrast, COX-1 protein was unchanged in response to treatment with EGF. PGE₂ production was also rapid and transient. Preincubation of cells with the novel COX-2 enzymic inhibitor NS-398 (10^{–5}–10^{–10} M) completely prevented PGE₂ formation in a dose-dependent manner. Preincubation of cells in dexamethasone (Dex; 0.1 µM), however, resulted in only a 31 % decrease in PGE₂ formation in response to EGF (10 ng/ml) while completely attenuating PGE₂ biosynthesis in tumour necrosis factor α (TNF-α)-stimulated cells. In addition, Dex (0.1 µM) was only partly effective at preventing EGF-induced COX-2 mRNA and protein expression *de novo*, whereas Dex completely inhibited TNF-α-promoted COX-2 mRNA and protein expression. Thus the results presented here demonstrate that EGF induces the rapid but transient expression of COX-2 mRNA and protein and the subsequent production of PGE₂ in WISH cells.

INTRODUCTION

Epidermal growth factor (EGF) is a small monomeric peptide that promotes mitogenesis in tissues of mesodermal and ectodermal origin [1]. In addition, EGF and related peptides have been implicated in the promotion of cell proliferation in wound healing and tissue repair [2–4]. In Balb/c-3T3 cells, EGF-induced DNA synthesis has been shown to involve the metabolism of arachidonic acid and linoleic acid to several bioactive derivatives [i.e. cyclo-oxygenase (COX) and 5-lipoxygenase products] [5–7]. In fact, the blockade of either COX or 5-lipoxygenase activity resulted in a dose-dependent decrease in DNA synthesis [6].

As the rate-limiting enzyme for the biosynthesis of prostaglandins (PGs) and thromboxane A₂, COX exists in two genetically distinct isoforms, COX-1 and COX-2 [8]. COX-1 is a constitutively expressed enzyme responsible for the production of PGs required for gastrointestinal, renal and vascular homeostasis, whereas COX-2 is an inducible enzyme expressed in response to some growth factors and inflammatory mediators [8–17]. EGF has been shown by a number of investigators [18–20] to elicit PGE₂ biosynthesis in amnion-derived WISH cells and primary amnion cultures. Moreover EGF has been reported to up-regulate COX-2 expression and/or the subsequent biosynthesis of PGs in a number of cell systems including Swiss fibroblast 3T3 cells [12], a rat tracheal cell line (EGV6) [21], bovine aortic endothelial cells [22,23], and guinea-pig gastric mucous cells [24].

As excessive PG release is a hallmark feature of most inflammatory diseases, understanding the molecular details of COX regulation could offer insights into opportunities for the de-

velopment of novel drugs. We have shown previously that the human amnion-derived cell line WISH offers a highly reproducible model system in which to test the control of arachidonic acid (AA) metabolism by growth factors and cytokines [17,18]. This model faithfully replicates many features of amnion cells maintained in primary culture [25,26]. Thus in the current study we examined the regulation of COX-2 gene expression and subsequent PGE₂ biosynthesis by EGF in WISH cells. Our results demonstrate that EGF induces a rapid but transient up-regulation of COX-2 protein and that, in contrast with previous reports for other cell systems, dexamethasone (Dex) was only partly effective at inhibiting COX-2 mRNA accumulation [27,28].

EXPERIMENTAL

Materials

All tissue culture media and serum were purchased from Gibco/BRL (Gaithersburg, MD, U.S.A.). Recombinant human EGF and tumour necrosis factor α (TNF-α) were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Mouse anti-human COX-2 C-terminus antibodies were obtained from Transduction Laboratories (Lexington, KY, U.S.A.), and rabbit anti-(sheep COX-1), unlabelled PGE₂ and *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulphonamide (NS-398) were from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Horseradish peroxidase-conjugated rabbit anti-(mouse IgG) and sheep anti-(rabbit IgG) were purchased from Cappel (Malvern, PA, U.S.A.). RediPrime random priming cDNA labelling kits, ECL[®] enhanced chemiluminescence reagents, Hybond-C and Hybond-N nitro-

Abbreviations used: AA, arachidonic acid; COX, cyclo-oxygenase; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; NS-398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulphonamide; PG, prostaglandin; TNF, tumour necrosis factor.

‡ To whom correspondence should be addressed.

cellulose and nylon membranes, [5,6,8,11,12,14,15(n)-³H]PGE₂ (specific radioactivity 185 Ci/mmol) and [α -³²P]dCTP (specific radioactivity more than 3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). All other reagents were from United States Biochemicals (Cleveland, OH, U.S.A.) or Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

The human amnion-derived WISH cell line was obtained from the American Type Culture Collection (CCL25) and maintained as described by Hayflick [25]. Cells were grown at 37 °C under air/CO₂ (19:1) in Ham's F12/Dulbecco's modified Eagle's medium (F12/DMEM, 1:1) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 mg/ml gentamicin and 10% (v/v) newborn calf serum. Cells were seeded on 35 mm plates at 10⁶ cells per dish for immunoblotting, 60 mm dishes at 2 × 10⁶ cells per dish for Northern blot analysis, and 24-well plates at 2 × 10⁵ cells per well for PGE₂ measurements. Before treatment with EGF, cells were rinsed with serum-free F12/DMEM media followed by incubation in serum-free F12/DMEM overnight.

Northern blot analysis

Cells were rinsed with cold PBS followed by the addition of 1 ml of lysing solution [4 M guanidium isothiocyanate/0.1 mM 2-mercaptoethanol/0.5% sarcosyl (pH 5.5)] [29]. After overnight precipitation at -20 °C, RNA was extracted with lysing solution, reprecipitated and then resuspended in ribonuclease-free deionized water. After measurement of the absorbance at 260 nm, 20 µg of total RNA was fractionated on a 1% (w/v) agarose/formaldehyde gel, transferred overnight in 10 × SSC (1 × SSC being 150 mM NaCl/15 mM sodium citrate) to a nylon membrane (Hybond-N), and cross-linked by UV irradiation. Membranes were prehybridized in 50% (v/v) formamide/200 mM NaCl/250 mM sodium phosphate (pH 6.8)/1 mM EDTA/1% (w/v) SDS/100 µg/ml sheared salmon sperm DNA. Membranes were hybridized at 42 °C with a 1.8 kb cDNA fragment encoding human COX-2 (a gift from Dr. Tim Hla, American Red Cross, Rockville, MD, U.S.A.) labelled by random priming with [α -³²P]dCTP. Hybridized membranes were then washed at 60 °C in 0.2 × SSC/0.5% SDS for 60 min. To control for loading efficiency, blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase with a 358 bp cDNA fragment labelled as described above.

SDS/PAGE and immunoblot analysis

Cells were lysed with 1% (v/v) Triton X-100/120 mM NaCl/25 mM Hepes (pH 7.4)/1 mM PMSF/10 mg/ml leupeptin/10 µg/ml aprotinin/10 µg/ml antipain. The extracts were incubated at 4 °C for 30 min and centrifuged at 12000 g for 10 min. Protein samples (15 µg) were applied to an SDS/10% polyacrylamide gel, transferred to nitrocellulose filters, blocked with Tris-buffered saline containing 0.2% Tween-20 and 5% non-fat dry milk (Blotto) and probed with a monoclonal mouse anti-human COX-2 antibody (1:2000) or a polyclonal anti-COX-1 antibody (1:1000). Membranes were washed with Blotto and incubated with a goat anti-mouse IgG or goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:1000). Immunoreactive proteins were detected by chemiluminescence with ECL[®] reagent and exposure to X-ray film.

Radioimmunoassay

PGE₂ accumulation in the culture medium was determined by specific radioimmunoassay with anti-PGE₂ antibodies and a

previously described assay [30]. For these experiments, cells were placed in serum-free F12/DMEM containing 5 µM AA.

Statistics

Radioimmunoassay data were plotted as PGE₂ produced (ng/mg of protein) and represent the means ± S.E.M. for triplicate or quadruplicate determinations. Each experiment was replicated a minimum of three times. Where appropriate, the data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference *post hoc* test with *P* < 0.001 considered statistically significant [31].

RESULTS

EGF induces COX-2 mRNA and protein expression

Previous experiments in our laboratory established that EGF stimulates PGE₂ biosynthesis in WISH cells in a dose-dependent fashion [18]. Because the recently discovered COX-2 isoform seems to synthesize PGs in the setting of cell stimulation, we examined whether this isoenzyme was responsible for EGF-induced PGE₂ accumulation in the culture medium. Cultures were incubated in serum-free medium in the presence or absence of various concentrations of human recombinant EGF (0–100 ng/ml) for 1 h and Northern blots were probed with a cDNA encoding human COX-2. As shown in Figure 1(A), EGF induced a dose-dependent increase in the accumulation of COX-2 transcripts at 10 and 100 ng/ml but not at lower concentrations. Therefore 10 ng/ml of EGF was used for the experiments described below. Because our COX-1 cDNA probe frequently hybridized to COX-2 mRNA, our Northern blots of COX-1 mRNA were difficult to interpret. We therefore used reverse transcriptase-PCR employing isoform-specific primer sets to ascertain that EGF did not alter the steady-state expression of the COX-1 isoform under any of the conditions examined in this work (results not shown).

When immunoblots of protein extracted from EGF-treated and control cells were probed with a monoclonal anti-(COX-2 C-terminus) antibody, a doublet migrating at approx. 72 kDa was detected (Figure 1B). Low levels of COX-2 protein were seen in control cells, but expression was markedly enhanced when cells

Figure 1 Dose-response induction of COX by EGF

(A) Cells were treated with 0–100 ng/ml of EGF for 1 h followed by Northern blot analysis with a ³²P-labelled COX-2 specific cDNA probe (upper panel). Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) Cultures were treated with 0–100 ng/ml of EGF for 1.5 h and protein extracts were separated by SDS/PAGE. Immunoblotting with anti-COX-2 and anti-COX-1 monoclonal antibodies was used to determine the specific expressions of COX-2 (upper panel) and COX-1 (lower panel) respectively.

Figure 2 Kinetics of EGF-promoted PGE₂ biosynthesis and COX expression

(A) Cells were stimulated with EGF (10 ng/ml) or serum-free media (controls) for 0–8 h. PGE₂ contained in the media was evaluated by radioimmunoassay. Results are presented as means $\pm$ S.E.M. ($n = 9$) in ng of PGE₂ produced/mg of protein. (B) RNA species from cells incubated for 0–8 and 24 h were probed with a COX-2-specific radiolabelled cDNA probe (upper panel). Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (C) COX-2 protein expression was determined by treatment of cells with EGF for 0–8 and 24 h followed by SDS/PAGE. Immunoblots were probed with an anti-(COX-2 C-terminus) antibody (upper panel). Identically treated cell lysates were immunoblotted with anti-COX-1 specific antibodies (lower panel). Abbreviation: hrs, h.

were incubated with 10 or 100 ng/ml EGF. When protein blots were probed with a polyclonal antibody recognizing COX-1, there was no such regulation of expression, and COX-1 was seen at similar levels despite EGF stimulation (results not shown).

WISH cells, like amnion cells maintained in primary culture, do not contain detectable levels of 15-hydroxyprostaglandin dehydrogenase and thus do not degrade AA metabolites elaborated into the medium [32]. Therefore the accumulation of primary PGs in the medium reflects their rate of synthesis and release from the cell. In experiments examining the time course of PGE₂ accumulation in the medium of EGF-treated cells, we detected a rapid rise in the rate of PGE₂ release within the first hour of stimulation and a sharp decrease in the rate of synthesis thereafter (Figure 2A). This is reflected in a decrease in the slope of the time-course curve shown in Figure 2(A) at later compared with earlier time points. Additional studies confirmed that PGE₂ does not accumulate in the medium of cells stimulated with EGF for times longer than 8 h, e.g. 24 h (results not shown).

To understand the molecular control of this rapid and transient synthesis of PGE₂ after EGF treatment, we probed Northern blots with a COX-2 cDNA after extraction of RNA species from cells exposed to 10 ng/ml EGF for 0.5–24 h. As shown in Figure 2(B), COX-2 mRNA transcripts accumulated within 30 min of

Figure 3 Dose-dependent inhibition of EGF-promoted PGE₂ formation by NS-398

WISH cells were preincubated with NS-398 (10^{-5} – 10^{-10} M) or serum-free media (controls, Con) for 30 min followed by stimulation with EGF (10 ng/ml) for 4 h. Media were removed and PGE₂ formation was determined by radioimmunoassay. The results are means $\pm$ S.E.M. ($n = 9$) in ng of PGE₂ produced/mg of protein.

EGF treatment and persisted until 60 min, but rapidly declined near to background (unstimulated) levels by 2 h after EGF treatment. Immunoblots of protein extracts from EGF-treated cells (0.5–24 h) also revealed that COX-2 protein accumulation occurred rapidly (within 1 h) but was transient, declining near to control levels by 4 h (Figure 2C). In contrast, immunoblotting with an anti-COX-1 antibody revealed no such rapid change in the levels of COX-1 protein after EGF stimulation (Figure 2C, lower panel). Thus the kinetics of appearance and disappearance of COX-2 transcripts and subsequent COX-2 protein are consistent with the rapid and transient increase in the rate of accumulation of PGE₂ in the medium in EGF-treated cells. Moreover, these results indicated that the COX-2 isoform is probably responsible for much if not all of the net increase in PG formation in the WISH cells.

Inhibition of EGF-induced PGE₂ synthesis by NS-398

Non-steroidal anti-inflammatory drugs are a large class of pharmaceuticals employed in many clinical settings of acute and chronic inflammation [33,34]. However, most such currently available drugs, such as aspirin and indomethacin, are indiscriminate in their blockade of COX function. NS-398 is one of a number of experimental compounds reported to show selectivity *in vitro* and *in vivo* for COX-2 without altering the function of COX-1 [35,36]. Therefore, to provide further evidence that EGF-induced PGE₂ synthesis is a COX-2-driven event, we pre-incubated WISH cells with various concentrations of NS-398 (10^{-5} – 10^{-10} M) for 30 min followed by a 4 h stimulation with EGF (10 ng/ml). EGF alone stimulated a 3.3-fold increase in PGE₂ production over control cells (Figure 3, left panel). RIAs of culture medium revealed that NS-398 inhibited in a dose-dependent manner the increase in PGE₂ accumulation seen in cultures treated with EGF alone (Figure 3), suggesting that EGF-induced PGE₂ production is a COX-2-driven process in WISH cells. Consistent with the reported strong potency of this drug, NS-398 exhibited an IC₅₀ of 25 nM [37].

Effect of Dex on EGF-induced COX-2 expression

Previous reports have demonstrated that glucocorticoids exert part of their anti-inflammatory actions via the suppression of COX-2 gene expression and subsequent PG biosynthesis [17,38–42]. Therefore we examined whether Dex could inhibit

Table 1 Evaluation of inhibition by Dex of EGF- and TNF- α -induced PGE₂ formation

WISH cells were seeded on 24-well plates and grown for 2 days in F12/DMEM/10% (v/v) newborn calf serum. The cells were rinsed and incubated in serum-free medium overnight and then treated with either EGF (10 ng/ml) in the presence or absence of Dex (0.1 μ M) for 4 h, or TNF- α (50 ng/ml) in the presence or absence of Dex for 12 h. After each incubation period, the media were removed and PGE₂ content was measured by radioimmunoassay. The results are expressed as means $\pm$ S.E.M. ($n = 12$); * $P < 0.001$.

PGE ₂ produced (ng/mg of protein)	
Expt. 1	
Control	7.12 $\pm$ 0.11
EGF	19.90 $\pm$ 0.60
EGF + Dex	13.80 $\pm$ 1.65
Expt. 2	
Control	4.05 $\pm$ 0.15
TNF- α	56.20 $\pm$ 4.2
TNF- α + Dex	2.19 $\pm$ 0.04*

Figure 4 Suppression of COX-2 mRNA and protein expression by Dex

(A) After preincubation of cells for 30 min with medium alone (controls) or Dex (0.1 μ M), cultures were exposed to media alone (controls), EGF (10 ng/ml) alone, or EGF (10 ng/ml) and Dex (0.1 μ M) (left panel), or media alone (controls), TNF- α (50 ng/ml) alone, or TNF- α (50 ng/ml) and Dex (0.1 μ M) (right panel). Northern blots were probed with a COX-2 cDNA and then a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. (B) Cultures were treated in identical conditions except that protein extracts were fractionated by SDS/PAGE, transferred to nitrocellulose filters and then incubated with anti-COX-2 monoclonal antibodies.

EGF-induced COX-2 expression and PGE₂ production in WISH cells. WISH cell cultures were preincubated for 30 min with Dex (0.1 mM) and then stimulated for 4 h with EGF (10 ng/ml). As shown in Table 1, Dex inhibited EGF-induced PGE₂ synthesis by only approx. 31% (EGF compared with EGF plus Dex, $P > 0.05$). In contrast, when WISH cells were preincubated with Dex (0.1 mM) and then challenged for 12 h with recombinant human TNF- α (50 ng/ml, a concentration determined by dose-response curves; D. J. Perkins, unpublished work) there was a complete suppression of TNF- α -induced PGE₂ production (TNF- α compared with TNF- α plus Dex, $P < 0.001$; Table 1). It is noteworthy that TNF- α does not stimulate a rapid increase in PGE₂ synthesis in WISH cells. The duration of TNF- α treatment was therefore extended to 12 h as this was the optimal period over which to observe maximal PG formation (D. J. Perkins, unpublished work).

WISH cells were also preincubated for 30 min with Dex (0.1 mM) and then stimulated for 60 min with either EGF (10 ng/ml) or TNF- α (50 ng/ml). Northern blots from these cells

were probed with a human COX-2 cDNA fragment. As shown in Figure 4(A), EGF and TNF- α increased the expression of COX-2 mRNA at steady state compared with cells incubated with medium alone. However, a significant difference was noted in the ability of Dex to suppress COX-2 expression in EGF compared with TNF- α -stimulated cells. Although Dex attenuated the accumulation of COX-2 transcripts after stimulation with TNF- α , no such diminution in COX-2 expression was observed in EGF-treated cells (Figure 4A, compare lane 3 in right and left panels).

Immunoblots were then prepared from identically treated cells and probed with monoclonal anti-COX-2 antibodies. Similarly to the results shown with Northern blotting, Dex treatment of TNF- α -stimulated WISH cells completely prevented the accumulation of COX-2 protein (Figure 4B, right panel, lane 3). In contrast, in EGF-treated cells, Dex had only a minimal effect on the steady-state appearance of COX-2 protein (Figure 4B, left panel, lane 3).

DISCUSSION

The experiments in this study demonstrate that EGF induces COX-2 expression and the subsequent release of PGE₂ in WISH cells with rapid but transient kinetics. The ability of the COX-2 selective drug NS-398 to abolish EGF-induced PGE₂ production suggests that all or at least much of the rapid rise in the rate of accumulation of PGE₂ in the medium can be attributed to the expression of COX-2 *de novo*. This point is strengthened by the complete lack of up-regulation by EGF of the constitutively expressed COX-1 isoenzyme. As such, our results corroborate and extend the work of Herschman and colleagues [12] showing that EGF can induce TIS10, the murine equivalent of COX-2. Furthermore our Northern blotting results with a COX-2-specific probe and our immunoblotting studies with isoform-specific antibodies provide definitive evidence for EGF-driven COX-2 induction alluded to by Casey et al. [20] and Pash and Bailey [23] before the cloning of the COX-2 cDNA. COX-2 therefore fits the operational definition of an immediate-early gene whose expression is tightly coupled to cellular activation, rapid in onset, and transient [43].

Because WISH cells have no demonstrable 15-hydroxyprostaglandin dehydrogenase activity and therefore do not catabolize primary AA metabolites, these cells have served as an excellent model system for studying the induction of the COX-2 gene by bioactive substances such as cytokines and growth factors [17]. In WISH cells stimulated with EGF, a tight temporal link exists between the initial accumulation of COX-2 mRNA transcripts, the translation of nascent protein and the subsequent synthesis and release of AA metabolites into the extracellular medium. Results presented here demonstrate that the entire process was initiated within 30 min, was maximal by 1 h and then declined rapidly to almost baseline levels by 4 h. This indicates that WISH cells have a high rate of COX-2 turnover, as suggested by others in different cell systems [44,45]. Indeed, in the present study we were able to show that the initial change in the rate of accumulation of PGE₂ in the medium follows very closely the kinetics of induction of the COX-2 mRNA and protein (see Figure 2), and probably accounts for the new synthesis of PGE₂ seen in EGF-stimulated cells.

The rapid disappearance of COX-2 after EGF stimulation was unexpected. Previous investigators have reported the rapid induction of COX-2 mRNA by EGF in Swiss 3T3 and rat tracheal epithelial cells [12,21]. However, because no data were presented about the decay of COX-2, it is difficult to determine

whether our data differ from the work of Herschman and colleagues [12] or Hamasaki et al. [21].

Previous studies from our laboratory have shown that stimulation of WISH cells with proinflammatory cytokines such as interleukin 1 β or TNF- α induces the rapid appearance of COX-2 transcripts followed by a lag phase before the appearance of COX-2 protein and the subsequent formation of PGE₂ ([17], and D. J. Perkins, unpublished work). In fact, peak levels of COX-2 protein were not seen until 24 h after interleukin-1 β treatment in WISH cells [17]. This is not surprising, however, because steady-state expression of COX-2 mRNA persisted throughout this time frame. Thus the results shown in the present paper compared with previous reports illustrate that the persistence of COX-2 expression in WISH cells is greatly influenced by the stimulus employed.

Our studies with Dex, showing only minimal suppression of EGF-induced COX-2 mRNA accumulation and PGE₂ synthesis, are in contrast with reports of several investigators [27,38,39]. However, those groups did not measure Dex effects on the EGF-induced expression of COX-2 transcripts; comparisons between our results and previous reports are therefore difficult. It is generally accepted that glucocorticoids exert part of their anti-inflammatory activity by suppressing COX-2 gene expression [28,46]. Thus we were surprised to find that Dex suppressed EGF-induced PGE₂ synthesis by only 31% and had a limited effect on COX-2 mRNA and protein expression. In contrast, Dex completely abolished TNF- α -induced PGE₂ production and COX-2 gene expression in WISH cells. Although at present the molecular mechanisms accounting for differential expression/stability of the COX-2 isoform are unknown, it is likely that signal transduction cascades for EGF and TNF- α diverge at one or more points on the way to the COX-2 gene promoter. Current investigations in our laboratory are attempting to elucidate the pathways utilized by EGF and proinflammatory cytokines in activating the COX-2 gene.

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