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# Evidence for Involvement of NF-κB in the Transcriptional Control of COX-2 Gene Expression by IL-1β

Robert Newton,[1] Lieske M. E. Kuitert, Martin Bergmann, Ian M. Adcock, and Peter J. Barnes
*Department of Thoracic Medicine, National Heart and Lung Institute, Imperial College School of Medicine, Dovehouse Street, London, SW3 6LY, United Kingdom*

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**The cyclooxygenase (COX) isoforms COX-1 and COX-2 convert arachidonic acid to prostaglandin (PG) precursors and are a limiting step in PG production. Interleukin-1β (IL-1β) treatment of type II A549 cells increases $PGE_2$ synthesis via transcription- and translation-dependent induction of COX-2. IL-1β produces a 10-fold induction of COX-2 mRNA and an 8-fold increase in COX-2 transcription that was temporally preceded by activation of the transcription factor nuclear factor-κB (NF-κB). The protein-tyrosine phosphatase inhibitor phenylarsine oxide (PAO) prevented both NF-κB activation and induction of COX-2 mRNA. We show that two putative NF-κB motifs, κBu (−447/−438) and κBd (−224/−214), from the COX-2 promoter bind p50/p65 NF-κB heterodimers in an IL-1β-dependent manner and that the upstream element has the greater affinity. Finally, we demonstrate that the two NF-κB subunits, p50 and p65, synergistically activate a −917/+49 COX-2 promoter construct. We conclude that IL-1β stimulates PG production via transcriptional activation of COX-2 and provide evidence that this may involve NF-κB.** 

Prostaglandins (PGs) are lipid mediators, which are involved in many processes, including inflammation, and are produced by many cells types (1). PG synthesis involves conversion of arachidonic acid by the cyclooxygenase (COX) enzymes to the general prostanoid precursor, $PGH_2$ (1). COX-1 is constitutively expressed, whilst COX-2 has low basal expression that is rapidly induced by pro-inflammatory stimuli (1). The antiinflammatory effects of non-steroidal anti-inflammatory drugs (NSAIDs), which act via inhibition of COX, indicates the relevance of this pathway to inflammation and the use of COX-2 inhibitors specifically highlights the importance of COX-2 (1-3).

Once regarded as purely structural cells, epithelial cells play an active role in inflammation by production of various cytokines and eicosanoids (4). Human bronchial epithelial cells along with the pulmonary type II cell line, A549, show inducible $PGE_2$ release and COX-2 production in response to proinflammatory cytokines, such as IL-1β (5). In A549 cells IL-1β also activates the acute-phase transcription factor NF-κB (6), which is now recognized as an almost ubiquitous activator of inflammatory and many primary response genes (7). As COX-2 was isolated as a primary response gene (8), and has two putative NF-κB sites in the 5′-promoter region, it may also be NF-κB responsive (9-11). Here we describe the transcriptional upregulation of COX-2 and provide support for involvement of NF-κB.

## MATERIALS AND METHODS

*Cell culture.* A549 cells were grown to confluency as described (12). Cells were incubated over-night in serum-free media (SFM) before changing to new SFM plus vehicle, 1ng/ml IL-1β ($2 \times 10^5$U/μg) (Genzyme, MA, USA), 10μg/ml cycloheximide (Sigma, Poole, UK), 10μg/ml actinomycin D (Sigma) or phenylarsine oxide (PAO) (Sigma) as indicated.

*Western blot analysis.* Cells were harvested and resuspended in 100μl Hank's balanced salt solution. An equal volume of 100mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.05% bromophenol blue, 200mM DTT, 2mM PMSF was added and the sample boiled for 5 minutes. Total protein (70μg) was separated by 10% SDS-PAGE and transferred to hybond-ECL membranes (Amersham, Buckinghamshire, UK). Membranes were blocked in phosphate buffered saline (PBS), 0.1% Tween-20 (Sigma), 5% dried milk, and incubated for 1 hour with a 1 in 200 dilution of rabbit anti-human COX-2 antibody (Oxford Biomedical, Michigan, USA). Membranes were washed in PBS, 0.1% Tween and incubated for 20 minutes with horseradish peroxidase-linked anti-rabbit immunoglobulin (Amersham). After washing, detection was performed using enhanced chemiluminescence (ECL) (Amersham).

[1] To whom correspondence should be addressed: Fax: (44) 171 352 8126. E-mail: robert.newton@ic.ac.uk.

Abbreviations: COX, cyclooxygenase; CRE, cAMP responsive element; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; NF-κB, nuclear factor-κB; PAO, phenylarsine oxide; PG, prostaglandin; RT-PCR, reverse transcriptase-polymerase chain reaction; SFM, serum free media; TNFα, tumor necrosis factor-α.

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*COX activity determination.* Media was removed and cells rinsed prior to addition of SFM containing 30$\mu$M arachidonic acid. After incubation for 10 minutes at 37°C, media was removed and $PGE_2$ measured by RIA using a commercial anti-$PGE_2$ antibody (Sigma) (5). Release of $PGE_2$ was taken as an index of COX activity.

*RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).* RNA was extracted and 1$\mu$g was reverse transcribed as described (12). PCR primers, conditions and cycling parameters for COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as previously described (12). The number of amplification cycles used was that necessary to achieve exponential amplification where product formation is proportional to starting cDNA. Products (10$\mu$l) were run on 1.5% agarose gels and Southern hybridization performed with cloned cDNA probes to confirm product identity and, as all primer pairs cross introns, checked against possible amplification of genomic DNA. Further aliquots (5$\mu$l) were dot-blotted, hybridized as above and quantified by Cerenkov counting. Linearity of the relationship between starting cDNA concentration and product formation using this methodology has previously been shown (12). Data are expressed as the ratio COX-2 to GAPDH and relative values plotted as means ± SEM.

*Nuclear run-on transcription assay.* Nuclei were prepared and run-on transcription reactions were performed using $5 \times 10^7$ nuclei and radiolabelled RNA extracted as described (13). Hybridizations, using 10$\mu$g of denatured plasmid DNA immobilised to Hybond-N (Amersham) and $2 \times 10^6$ cpm of radiolabelled were as described (13). Plasmids used were; pGEM3z (Promega), cloned GAPDH cDNA (12), and 2029bp of COX-2 cDNA (bases 2-2030) (14) cloned into pGEM5z (Promega). Following hybridization, filters were washed in: Buffer A (300mM NaCl, 10mM Tris-HCl, pH7.4, 2mM EDTA, 0.1% SDS), room temperature, 15 minutes; Buffer A, 50°C, 30 minutes; Buffer B (10mM NaCl, 10mM Tris-HCl, pH 7.4, 2mM EDTA, 0.4% SDS), 50°C, 30 minutes; Buffer A plus 1$\mu$g/ml RNase A and 10U/ml RNase T1, 37°C, 30 minutes; Buffer A, 30 minutes, 55°C; and finally Buffer B, 30 minutes, 55°C before autoradiography.

*Electrophoretic mobility shift assay (EMSA).* Nuclear proteins (2-10$\mu$g) were incubated with $^{32}$P labelled double-stranded oligonucleotide probe as described (6). Consensus NF-$\kappa$B oligonucleotide (sense strand) was, 5′-AGT TGA GGG GAC TTT CCC AGG-3′. Putative COX-2 NF-$\kappa$B probes (sense strand) were: $\kappa$Bu, 5′-GGA GAG GGG ATT CCC TGC GC-3′ (−452/−433), and $\kappa$Bd, 5′-GAG TGG GGA CTA CCC CCT CT-3′ (−228/−209). Mutant oligonucleotides were: $\kappa$Bu(mut), 5′-GGA GAG GCC ATT CCC TGC GC-3′, and $\kappa$Bd(mut), 5′-GAG TGG CCA CTA CCC CCT CT-3′ (mutated bases are underlined). Specificity was shown by the prior addition of 100-fold excess of unlabelled competitor consensus oligonucleotide. For supershift analysis, nuclear extracts were incubated on ice for 2 hours with Rel antisera (Santa Cruz), at 0.4$\mu$g/ml, prior to addition of radiolabelled probe. Reactions were separated on 7% native acrylamide gels before vacuum drying and autoradiography.

*COX-2 promoter plasmid.* A 966bp fragment, −917 to +49 relative to transcription start, was amplified from human genomic DNA using the primers 5′-GGA CAT TTA GCG TCC CTG CA-3′ (sense), and 5′-GAG TTC CTG GAC GTG CTC CT-3′ (antisense). *HindIII* linkers (Promega) were added prior to cloning into pUC19. Sequencing confirmed identity before subcloning into the *HindIII* site of pGL3basic (Promega) to give pGL3.C2(−917/+49).

*Transient transfections.* A549 cells were transiently transfected using Tfx50 (Promega) as described (15). For p50 and p65 overexpression, each transfection contained 0.5$\mu$g pGL3.C2(−917/+49), 0.5$\mu$g pSV-$\beta$-Galactosidase Control Vector (Promega) and variable amounts of RcCMV-p50 (16), RcCMV-p65 (16), and empty expression vector. After 24 hours, cells were harvested in 1× Reporter Lysis Buffer (Promega). Cellular debris was pelleted and total protein measured with Biorad Protein Assay reagent (Biorad, Hemel Hempstead, UK). Luciferase and $\beta$-galactosidase assays were performed using commercial kits (Promega). Luminescence was measured on a TD 20/20 Luminometer (Turner Designs, Stevenage, UK). As p65 overexpression increased expression of $\beta$-galactosidase, relative luminescence readings were normalized to total protein and expressed as fold activation relative to control ± SEM.

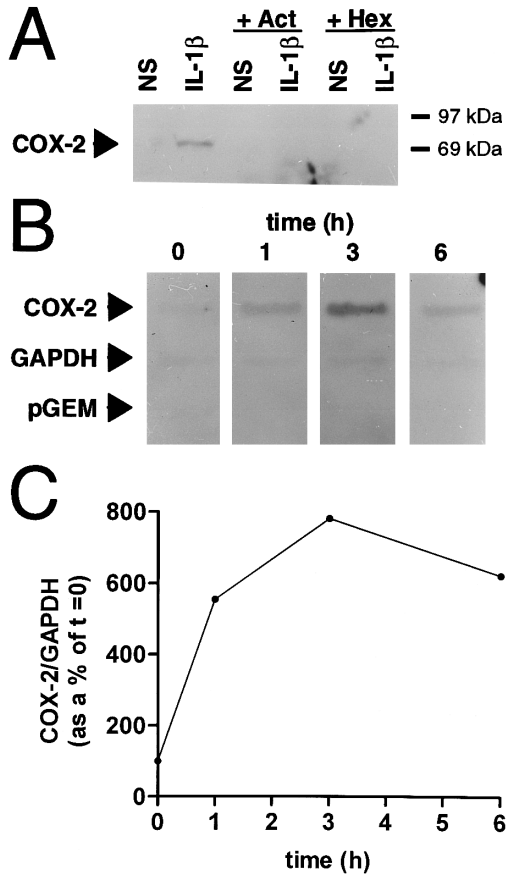


**FIG. 1.** Induction of COX-2 is transcriptionally controlled. Cells were treated as indicated and incubated for 24 hours prior harvesting for Western analysis (NS = No stimulation, Act = actinomycin D, and Hex = cycloheximide). A representative Western blot from one of three experiments is shown (A). Cells were either not stimulated (t = 0) or treated with IL-1$\beta$ and nuclei were harvested at the times indicated for nuclear run-on transcription analysis. Autoradiographs showing hybridisation of labelled run-off RNA to COX-2, GAPDH, and pGEM3z plasmids (B). Data from two experiments are shown as means of the ratio COX-2/GAPDH expressed as a percentage of IL-1$\beta$ stimulation (C).

## RESULTS

*IL-1$\beta$ induction of COX-2 is transcriptionally controlled.* Western analysis showed no detectable imunoreactive COX-2 protein in unstimulated cells, whilst 24 hours IL-1$\beta$ treatment induced COX-2 protein in a manner that was both cycloheximide and actinomycin D sensitive (Fig. 1A). Likewise COX activity at 24 hours produced an equivalent effect (No stimulation = 2.12±0.64 ng/min/well, IL-1$\beta$ = 51.7±7.30 ng/min/well, IL-1$\beta$ + actinomycin D = 0.95±0.34 ng/min/well, IL-

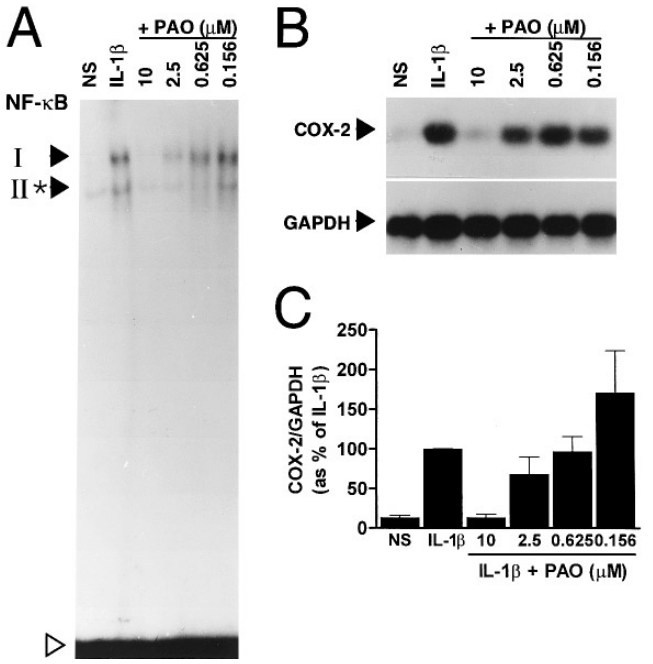


**FIG. 2.** Effect of phenylarsine oxide (PAO) on NF-$\kappa$B (A) and COX-2 mRNA (B). Cells were either not stimulated (NS) or stimulated with IL-1$\beta$ or stimulated with IL-1$\beta$ in the presence of different concentrations of PAO as indicated. After 1 hour cells were harvested and nuclear extracts prepared for EMSA. An autoradiograph representative of three EMSA using the NF-$\kappa$B consensus probe is shown (A). Specific complexes, as determined by competition analysis, are indicated by solid arrowheads. Asterisks indicate a non-specific band co-migrating with the specific complex. Unbound probe is indicated by an open arrowhead. Cells were treated as above and after 2 hours harvested for total RNA, RT-PCR, and Southern analysis. Representative autoradiographs for COX-2 and GAPDH RT-PCR products are shown (B). RT-PCR products were dot-blotted and hybridised with appropriate cDNA probes to allow quantification by Cerenkov counting. Data (n = 6) were expressed as ratios of COX-2/GAPDH and plotted as a percentage of IL-1$\beta$ stimulation as means $\pm$SEM (C).

1$\beta$ + cycloheximide = 4.78$\pm$2.95 ng/min/well). We have previously shown a 10-fold increase in steady state COX-2 mRNA levels by IL-1$\beta$ and here show a corresponding 8-fold increase in COX-2 transcription rate using nuclear run-on assay (Fig. 1B & C) (12).

*Phenylarsine oxide inhibits IL-1$\beta$-induced NF-$\kappa$B and COX-2 mRNA.* Phenylarsine oxide (PAO), a protein-tyrosine phosphatase inhibitor, prevents tumor necrosis factor-$\alpha$ (TNF-$\alpha$) induced NF-$\kappa$B activation (17). Likewise, in A549 cells, IL-1$\beta$-dependent activation of NF-$\kappa$B was inhibited in a concentration-dependent manner by PAO (Fig. 2A). As previously reported (17), this effect appeared specific to NF-$\kappa$B since binding of the transcription factors AP-1, Oct-1 and Sp1 was unaltered (data not shown). PAO also dose-dependently inhibited IL-1$\beta$-induced COX-2 mRNA levels (Fig. 2B & C).

*IL-1$\beta$-induced NF-$\kappa$B binds to putative NF-$\kappa$B sites in the COX-2 promoter.* The ability of the two putative COX-2 NF-$\kappa$B-like sites, $\kappa$B upstream ($\kappa$Bu) and $\kappa$B downstream ($\kappa$Bd), to bind NF-$\kappa$B was tested by EMSA. Both sites showed IL-1$\beta$-inducible NF-$\kappa$B-like binding activity (Fig. 3A & B). Two complexes (complexes I & II) were observed in IL-1$\beta$ stimulated extracts and a single low intensity complex (complex II) observed in untreated extracts. Competition with excess unlabelled $\kappa$Bu or $\kappa$Bd, as appropriate, showed that these complexes were specific. EMSA using $\kappa$Bu(mut) and $\kappa$Bd(mut), which are mutated in the putative NF-$\kappa$B binding sites, produced no gel retardation in these extracts further indicating sequence specificity (data not shown). Supershift analysis was used to examine the composition of these complexes. Using $\kappa$Bu probe both

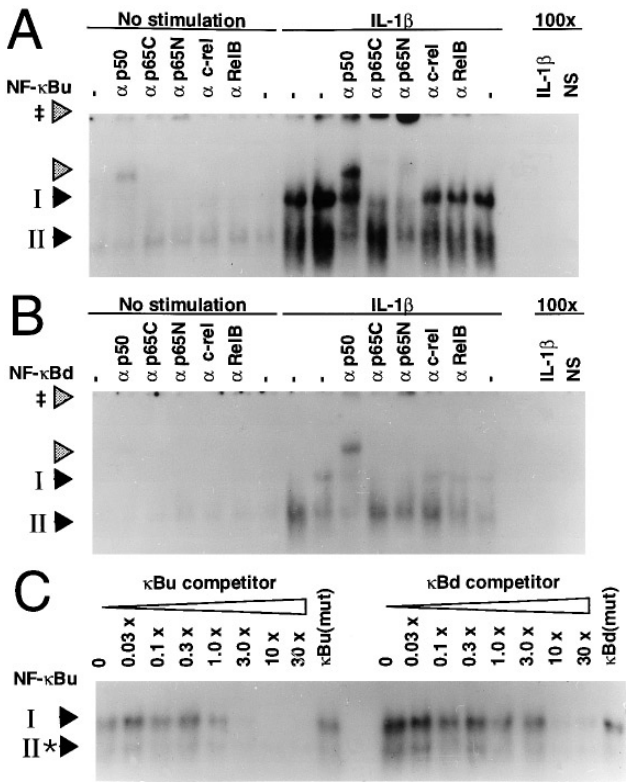


**FIG. 3.** EMSA and supershift analysis of putative COX-2 NF-$\kappa$B sites. Cells were either not stimulated (NS) or stimulated with IL-1$\beta$ as indicated. After 1 hour cells were harvested and nuclear extracts prepared for EMSA. Representative autoradiographs of EMSAs using $\kappa$Bu (A) and $\kappa$Bd (B) are shown. Specificity of binding was determined in both stimulated and non stimulated extracts by competition analysis using 100-fold (100x) excess of unlabelled probe. Supershift analysis, using nuclear extracts from both stimulated and non-stimulated extracts, was performed as indicated using antisera (Santa Cruz) raised against p50 nuclear location signal (p50), p65 carboxy terminus (p65C), p65 amino terminus (p65N), c-Rel carboxy terminus (c-Rel), or RelB carboxy terminus (RelB). Specific complexes are indicated by solid arrowheads. Supershifted complexes are indicated by shaded arrowheads and ‡ indicates the position of the wells. Nuclear extracts from IL-1$\beta$ treated cells were used for $\kappa$Bu (C) EMSA. Competition was performed with increasing fold excesses of unlabelled $\kappa$Bu or $\kappa$Bd as indicated. Competition with 100-fold excesses of the mutated probes $\kappa$Bu(mut) and $\kappa$Bd(mut) was performed as indicated.

p50 (NF-κB1) and p65 (Rel A) were identified in stimulated complexes I and II (Fig. 3A). With the κBd probe p50 was found in both IL-1β-induced complexes and p65 was only supershifted from complex I. Only p50 antisera resulted in a supershift with κBu and κBd complexes (complex II) from unstimulated cells.

*Relative binding affinities of COX-2 NF-κB sites.* Autoradiography of κBd EMSAs required 5-10 fold longer exposure times than for the κBu probe. As this was not due to differential probe labelling, we examined the relative affinities of these probes in crude nuclear extracts. Each labelled probe was incubated with IL-1β-stimulated nuclear extracts and competition assays performed using serial dilution's of cold homologous or heterologous oligonucleotide (as indicated). Using κBu probe, a 1:1 ratio of homologous labelled to unlabelled oligonucleotide resulted in decreased binding whereas a 10-fold excess of heterologous κBd competitor was required (Fig. 3C). This binding was not competed out even with a 30-fold excess of κBd suggesting a greater affinity of NF-κB for κBu than κBd. The converse experiment, where less heterologous κBu than homologous κBd competitor was required to compete out retarded κBd complexes, confirmed this result (data not shown). Inability of 100-fold excesses of the mutated sequences, κBu(mut) and κBd(mut), to compete out binding by the native sequences further indicates sequence specificity.

*Functional activation of the COX-2 promoter by NF-κB.* Initially we wished to use transient transfection assays as a means to identify IL-1β responsive elements in the COX-2 promoter. However, use of various transfection conditions and stimulation times failed to give IL-1β-dependent activation of the −917/+49 construct in A549, human airway epithelial BEAS2B and mouse LA-4 cells (data not shown). Furthermore a −2307/+49 construct as well as shorter constructs down to −85/+49 bp were also tested in A549 cells and failed to show either IL-1β or phorbol ester-dependent inducibility (data not shown).

We therefore examined the effect of overexpression of the NF-κB subunits p50 and p65 on the −917/+49 construct. Incubation for 24 hours following cotransfection with either RcCMV-p50 or RcCMV-p65 expression vectors, which give constitutive high level expression of p50 and p65 respectively (16), produced dose-dependent activation of this construct (Fig. 4A & B). The greatest transactivation was, as expected, observed with RcCMV-p65, whilst co-expression of p50 and p65 produced a marked synergistic effect (Fig. 4C).

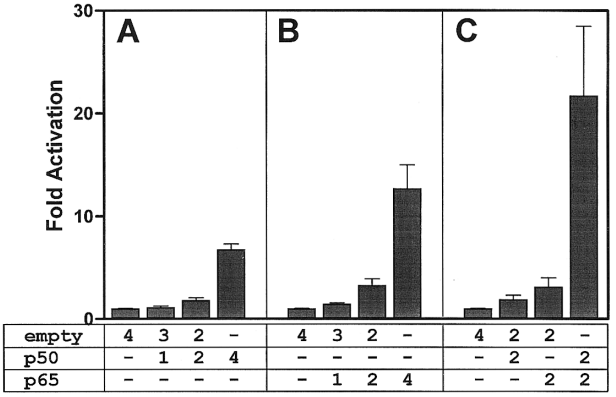


**FIG. 4.** NF-κB p50 and p65 subunits activate the COX-2 promoter. Transfections were carried out using a total of 5μg DNA/well with varying amounts (μg) of RcCMV-p50 (p50) (A) or RcCMV-p65 (p65) (B) or both together (C) and empty expression vector (empty) as indicated. Cells were harvested 24 hours later. Data are expressed as a percentage of control (empty vector) as means ±SEM of three (A, B) or two (C) experiments performed in duplicate.

## DISCUSSION

The two main control levels for PG synthesis are the availability of arachidonic acid, via $PLA_2$ activity, and conversion to $PGH_2$ by COX (1). In A549 cells increased $PGE_2$ release in response to IL-1β requires *de novo* transcription and translation (12). We now show the same for COX activity and COX-2 protein expression. IL-1β also produces a rapid and prolonged induction of COX-2 mRNA, which is primarily due to increased COX-2 transcription rather than mRNA stabilization (12, 15). Together with low uninducible expression of COX-1, these data identify COX-2 transcription as a major control point.

NF-κB activation in A549 cells occurs within 15 minutes of IL-1β treatment and is therefore consistent with a role in the transcriptional activation of COX-2 (6). The protein-tyrosine phosphatase inhibitor, PAO, was used to further examine this relationship. PAO dose dependently inhibited IL-1β-induced NF-κB. Steady state COX-2 mRNA levels were similarly affected indicating the need for tyrosine phosphatases and suggestive of a causal link between NF-κB and COX-2 induction.

We therefore tested two putative NF-κB binding sites from the COX-2 promoter for their ability to bind NF-κB. Both sites showed basal and IL-1β-dependent binding to the κB-like motifs. Supershift analysis identified p50 in the single unstimulated complex, whilst both p50 and p65 were present in IL-1β-induced complexes suggesting that p50 homodimers may be involved in basal expression, whilst the transcriptionally more active p50/p65 heterodimers may be important in induction of COX-2 (7). Comparison of the COX-2 5′-promoter regions from human (9-11) and mouse (18) revealed that these NF-κB sites are conserved, supporting the hypothesis that NF-κB has an important role in control of COX-2 transcription. The mouse equivalent of the upstream element, κBu, was found to be 100% homologous to the human sequence whereas the downstream element, κBd, was less conserved. These data are consistent with the competition studies showing a higher affinity of the upstream site over the down-

stream site for NF-κB and suggest that the upstream site may play the predominant role.

In this system the −917/+49bp COX-2 promoter linked to a luciferase reporter gene was not IL-1β-inducible. This result was surprising since −1432/+59 and −327/+59 constructs conferred LPS plus PMA inducibility (19), whilst those containing the ATF/CRE site were *v-src* inducible (20, 21) and a −891/+9 promoter was PMA inducible (11). Additionally a 621bp mouse COX-2 promoter gave TNFα-dependent reporter expression primarily through the NF-κB and NF-IL6 (C/EBPβ) sites (22). However, except one study that showed a 2 fold induction of reporter activity in response to hypoxia (23), no human COX-2 promoter constructs have to date been reported which support inducible expression by physiological stimuli in transient transfection assays using human cells. Clearly present systems do not faithfully mimic the *in vivo* situation. Reasons for this are presently unclear, but may involve critical regulatory elements either proximal or distal to transcription start. Indeed up to 16 kb of the human iNOS promoter was needed to confer maximal cytokine inducibility in human cells (24). There also remains the possibility that the effect reported here is cell-type specific as the −917/+49 construct was also unresponsive in human BEAS2B and mouse LA-4 epithelial cells.

Despite the lack of IL-1β inducibility, the −917/+49 construct conferred p65 and, to a lesser extent, p50 inducibility when co-transfected with expression vectors over-expressing these NF-κB subunits. Additionally, co-expression of both p50 and p65 produced a strong synergistic activation demonstrating the functional ability of NF-κB to activate this promoter. However, given the inability of IL-1β to activate this or other COX-2 promoter constructs, the true role of NF-κB is likely to be complex and full transcriptional activation may require more proximal or distal elements. Additionally, factors such as chromatin structure, which can profoundly affect NF-κB-dependent transcription (25), may play a major role in conferring cytokine inducibility and may be inappropriately modelled by transient transfection analysis (26).

In conclusion, we have used A549 cells as a model of airway epithelial cells to show the transcriptional upregulation of COX-2 in IL-1β-induced $PGE_2$ synthesis. We find that the tyrosine phosphatase inhibitor, PAO, prevents activation of NF-κB and induction of COX-2 mRNA. In addition two putative NF-κB sites from the COX-2 promoter bind p50/p65 in a IL-1β-dependent manner and in transient transfections NF-κB is able to activate the COX-2 promoter suggesting a role for NF-κB.

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