Superinduction of COX-2 mRNA by cycloheximide and interleukin-1β involves increased transcription and correlates with increased NF-κB and JNK activation

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Abstract Many primary response genes, including cyclooxygenase-2 (COX-2), exhibit mRNA superinduction following agonist stimulation in the presence of translational blockers such as cycloheximide. This is widely assumed to result from mRNA stabilisation. However, superinduction of IL-1β-induced COX-2 mRNA levels by cycloheximide in pulmonary type II A549 cells occurred by increased transcription and not by mRNA stabilisation. Furthermore, equivalent effects were observed on NF-κB binding to COX-2 promoter κB sites and activation of the Jun N-terminal kinases (JNK), p54 and p46. These signalling pathways play important roles in COX-2 induction and may therefore account for the observed increases in COX-2 transcription. These data are consistent with negative feed-back involving down-regulation of NF-κB by de novo IκBα synthesis and suggest that JNK activation may also be down-regulated by a cycloheximide sensitive process.

Key words: Cyclooxygenase; Prostaglandin G/H synthase; Superinduction; Cycloheximide; Jun N-terminal kinase; Nuclear factor-κB

1. Introduction

Prostaglandins (PGs) are potent paracrine and autocrine lipid mediators that are implicated in many pathophysiological processes, including inflammation. PGs are produced by many cell types, including inflammatory cells and both endothelial and epithelial cells [1]. The first dedicated step in PG synthesis is conversion of arachidonic acid, released from membrane phospholipids by phospholipase enzymes, to PGH_2 by the two cyclooxygenase (COX) enzymes [1]. COX-1 is a constitutively expressed housekeeping gene, whilst COX-2 is required for mitogen-dependent PG synthesis and generally shows a low basal expression that is rapidly induced by pro-inflammatory stimuli [1–3]. COX is the main target for non-steroidal anti-inflammatory drugs (NSAIDs) [1], whose anti-inflammatory benefits derive from COX-2 inhibition, whilst many of the undesirable side effects are due to COX-1 inhibition [1]. Use of isoform-specific inhibitors confirms this and highlights the importance of COX-2 in inflammation [4,5].

Primary response genes, which include growth factors, cytokines and transcription factors, are genes whose mRNA induction is refractory to translational blockade [6]. Furthermore, many of these mRNAs are actually induced by translational blockers such as cycloheximide and exhibit mRNA superinduction by agonist in the presence of a translational inhibitor. These genes are often associated with rapid mRNA turnover and many contain multiple AU-rich elements [7], which are thought to confer instability, in their 3′ untranslated regions (3′-UTR) [8]. Consequently, superinduction is commonly attributed to decreased mRNA turnover. However, increased nuclear signalling may also explain this effect [9].

COX-2 was cloned as a superinducible gene and in common with human bronchial epithelial cells, pulmonary type II A549 cells show increased PGE_2 release via transcriptional induction of COX-2 in response to pro-inflammatory cytokines [10–13]. We have examined COX-2 superinduction in these cells and show that increased nuclear signalling via the JNK and NF-κB pathways may play an important role.

2. Materials and methods

2.1. Cell culture

A549 cells were grown to confluency as described [12]. Cells were incubated over-night in serum-free media before changing to fresh media containing cytokines, drugs or vehicle. IL-1β (2×10^5 U/μg) (Genzyme, MA, USA), cycloheximide (Sigma, Poole, UK) and actinomycin D (Sigma) were used at 1 ng/ml, 10 μg/ml and 10 μg/ml respectively.

2.2. RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted and reverse transcription reactions and PCR for COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) performed 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 and in each case was determined as described [12]. Products were run on 1.5% agarose gels and Southern hybridisation performed with cloned cDNA probes to confirm product identity and, as all primer pairs cross introns, checked against possible amplification of genomic DNA [12]. Further aliquots were dot-blotted, hybridised 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 a ratio of COX-2 to GAPDH and relative values plotted as means ± S.E.M.

2.3. Northern blotting and hybridisation

RNA (10 μg) was size fractionated by electrophoresis on 1.0%

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Abbreviations: COX, cyclooxygenase; CRE, cAMP responsive element; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; JNK, Jun N-terminal kinase; NF-κB, nuclear factor-κB; PG, prostaglandin; RT-PCR, reverse transcriptase-polymerase chain reaction; TNFα, tumour necrosis factor-α

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agarose/formaldehyde gels in 1× MOPS. Capillary blotting and hybridisation was performed according to standard procedures [14]. Hybridisation probes for COX-2 and GAPDH were as above.

2.4. Nuclear run-on transcription assay

Nuclei, prepared as described by Greenberg and Ziff [15], were resuspended at 5×10^7/100 μl in 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 M Sorbitol, 2.5% Ficoll, 0.008% spermidine, 1 mM DTT, 50% glycerol and stored at −70°C. Radiolabelled RNA from run-off transcription reactions using 5×10^7 nuclei was extracted and hybridisation carried out against 10 μg of immobilised denatured plasmid DNA as described by Rousell et al. [14]. Plasmids used were pGEM3z (Promega), cloned GAPDH cDNA [12], and 2029 bp of COX-2 cDNA (bases 2-2030) [2] cloned into pGEM5z (Promega). Following hybridisation, filters were washed in: Buffer A (300 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% SDS), room temperature, 15 min; Buffer A, 50°C, 30 min; Buffer B (10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.4% SDS), 50°C, 30 min; Buffer A plus 1 μg/ml RNase A and 10 U/ml RNase T1, 37°C, 30 min; Buffer A, 30 min, 55°C; and finally Buffer B, 30 min, 55°C before autoradiography.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins (2–10 ug) were used in binding reactions as described previously [16]. After incubation on ice for 10 min, 0.0175 pmol of ^{32}P kinase labelled double-stranded oligonucleotide probe was added. Putative NF-κB elements from the COX-2 promoter (sense strand) used were: κBu, 5′-GGA GAG GGG ATT CCC TGC GC-3′, and κBd, 5′-GAG TGG GGA CTA CCC CCT CT-3′. Specificity was determined by the prior addition of 100-fold excess of unlabelled competitor oligonucleotide. Reactions were separated on 7% native acrylamide gels before vacuum drying and autoradiography.

2.6. Jun N-terminal kinase (JNK) assay

Cells in 6 well plates were harvested in 100 μl of 1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, 10 mM Tris-Cl pH 7.4, 75 mM NaCl, 10 mM EDTA, 0.5 mM PMSF, 2 mM Na-orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM Na-pyrophosphate. An equal volume of 100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.05% bromophenol blue, 5 mM β-mercaptoethanol was added and the sample boiled for 5 min. JNK kinase activity was determined using an 'in gel' renaturation assay using GST-c-Jun (1-135) as substrate [17]. Briefly, total protein was size fractionated on 10% SDS-PAGE containing 1.25 mg/ml bacterially expressed GST-c-Jun (1-135). Gels were washed in 50 mM Tris-HCl, pH 8.0, 20% isopropanol for 3×20 min to remove SDS, before further washing in 50 mM Tris-HCl, pH 8.0 5 mM βME for 3×20 min. Proteins are then denatured in 50 mM Tris-HCl pH 8.0, 6 M Guanidine HCl, 5 mM βME. Renaturation occurs during incubation at 4°C for 18 h in 5 changes of 50 mM Tris-HCl pH 8.0, 5 mM βME, 0.04% Tween. Gels were then soaked in 10 mM HEPES pH 8.0, 0.1 mM EGTA, 5 mM $MgCl_2$, 25 μM ATP, 200 mM DTT and 12.5 μCi γ^{32}P-ATP. After washing in 5 changes of 5% TCA, 1% w/v sodium pyrophosphate for 18 h, gels were vacuum dried before autoradiography.

2.7. Western blotting and immunodetection

Total protein was harvested as above, fractionated by 10% SDS-PAGE and transferred to hybond-ECL membranes (Amersham, Buckinghamshire, UK). Immunodetection of threonine 183/tyrosine 185 (TPY) phosphorylated and non-phosphorylated p54 and p46 was performed according to the manufacturer's instructions (New England Biolabs, Hitchin, UK). IκBα immunodetection was performed with 1 in 1000 dilutions of primary antibody (Santa Cruz, CA, USA) and 1 in 4000 dilutions of anti-rabbit secondary antibody (Dako Ltd, Bucks, UK) using the ECL protocol according to the manufacturers instructions (Amersham).

3. Results and discussion

3.1. Cycloheximide superinduces IL-1β-induced COX-2 mRNA

We have previously shown that the IL-1β-induced rise in COX-2 protein, activity and PGE_2 release by A549 cells is prevented by either transcriptional or translational blockers

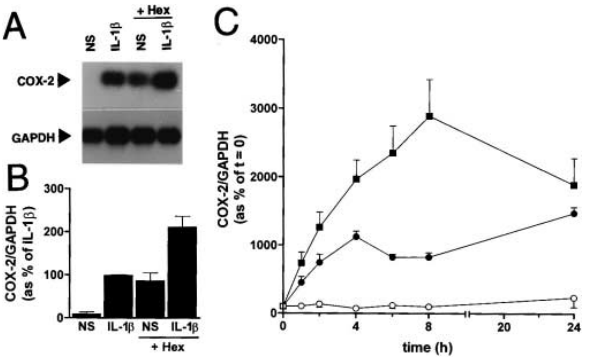

Fig. 1. Effect of IL-1β and cycloheximide on COX-2 mRNA levels. Cells were not stimulated (NS) or stimulated with IL-1β either with or without cycloheximide (Hex) as indicated. Cells were harvested at 2 h and RNA prepared for semi-quantitative RT-PCR. A: Autoradiographs showing RT-PCR products for COX-2 and GAPDH after Southern hybridisation analysis. PCR products were dot-blotted and hybridised with appropriate cDNA probes to allow quantification by Cerenkov counting. B: Data ($n=5$) were expressed as ratios of COX-2/GAPDH and plotted as a percentage of IL-1β stimulated. C: Cells were either not stimulated (○), or treated with IL-1β (●) or IL-1β+cycloheximide (■) and harvested for RNA at the times indicated. After semi-quantitative RT-PCR, data ($n=3$–4) were expressed as ratios of COX-2/GAPDH and plotted as a percentage of t = 0. All data are plotted as means ± S.E.M.

[12,13]. Furthermore, IL-1β elevates COX-2 mRNA primarily by transcriptional activation rather than via mRNA stabilisation [13,18]. As COX-2 was isolated as a superinducible primary response gene in NIH 3T3 cells [10], we investigated this effect in A549 cells. Alone, IL-1β caused a 5–10-fold increase in steady state COX-2 mRNA levels (Fig. 1A and B). Likewise, cycloheximide resulted in a substantial induction of COX-2 mRNA, whilst together with IL-1β a 2–2.5-fold superinduction over IL-1β treated levels was observed. This was most apparent at 4–8 h where the IL-1β-induced rise in COX-2 mRNA reached a plateau, corresponding to reduced transcription [13], whilst IL-1β+cycloheximide treated mRNA levels continued to rise (Fig. 1C). These data suggest that translational blockade prevents synthesis of a protein or proteins, which negatively regulate COX-2 mRNA levels.

3.2. Effect of cycloheximide on IL-1β-induced COX-2 mRNA half-life

Translational blockade is widely assumed to cause mRNA stabilisation and has previously been found to increase mRNA half-life of many genes including COX-2 in ECV304 cells [19]. However, we have previously used actinomycin D half-life studies to show that COX-2 mRNA is remarkably stable ($t_{1/2} \gg 2$ h) in A549 cells and that this stability was only marginally increased by IL-1β treatment [18]. Consequently only limited potential exists for further stabilisation of COX-2 mRNA. This was examined by treating cells for 2 h either with IL-1β alone or simultaneously with IL-1β+cycloheximide. Following addition of actinomycin D, degradation of COX-2 mRNA was monitored by Northern analysis (Fig. 2). For both treatments, COX-2 mRNA levels were found to decrease gradually for 2 h and by 6 h no further decay was observed. No substantial differences in the stability of COX-2 transcripts were observed. Indeed the gradient for the IL-1β plus cycloheximide samples was greater than for IL-1β alone.

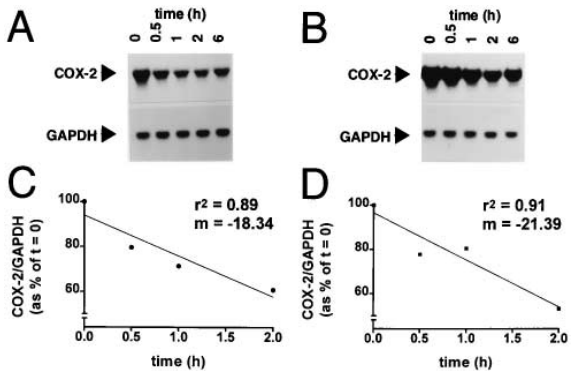

Fig. 2. Comparison of COX-2 mRNA stability from IL-1β and IL-1β plus cycloheximide treated cells. Cells were stimulated with IL-1β (A, C) or IL-1β plus cycloheximide (Hex) (B, D) and after 2 h actinomycin D was added ($t=0$). Cells were harvested at the times indicated and RNA prepared for Northern blotting. Representative autoradiographs for COX-2 and GAPDH after Northern hybridisation analysis are shown (A, B). After laser densitometry of autoradiographs data, $n=5$ for IL-1β (C) and $n=6$ for IL-1β plus cycloheximide (D), were expressed as a percentage of $t=0$ and means used for linear regression analysis.

Thus stabilisation of COX-2 transcripts does not account for the superinductive effect of cycloheximide.

3.3. *Effect of cycloheximide on COX-2 transcription*

Nuclear run-off transcription assays were performed to determine whether the cycloheximide-dependent increases in steady state COX-2 mRNA were associated with increased transcription. These revealed that co-treatment of IL-1β and cycloheximide produced a 50 to 100% elevation in the rate of COX-2 transcription over that seen with IL-1β alone (Fig. 3). Alone, cycloheximide produced a marked increase in transcription to levels similar to IL-1β treatment alone. We therefore conclude from these and the half-life data that superinduction of COX-2 mRNA occurs primarily via increased transcription.

3.4. *Increased nuclear signalling by cycloheximide*

Characterisation of the COX-2 5′ promoter region has identified putative binding sites for a number of acute phase transcription factors including two NF-κB sites, C/EBP or NF-IL6 sites and a CRE [20]. In A549 cells, IL-1β rapidly induces NF-κB p50/p65 heterodimers, which bind the two COX-2 κB sites in an IL-1β-dependent manner [13,16]. In addition a −917/+49 COX-2 promoter construct is transactivated by NF-κB overexpression suggesting involvement of NF-κB in the transcriptional induction of COX-2 [13]. Other studies have also implicated the NF-κB, NF-IL6 and the CRE sites in the transcriptional induction of COX-2 [8,21–23]. Unlike classical CREs, the COX-2 CRE site behaves like an AP-1 site in binding c-Jun and is activated by MAP kinase pathways leading to activation of Jun N-terminal kinases (JNKs) [23]. We have therefore examined the effects of cycloheximide on the NF-κB and JNK signalling pathways.

The effect of cycloheximide was tested on NF-κB binding to the two COX-2 NF-κB sites. Both sites showed superinduction of NF-κB binding in nuclear extracts from cells treated with IL-1β in the presence of cycloheximide or actinomycin D and enhanced binding as a result of cycloheximide or actinomycin D alone (Fig. 4A and B). The difference in signal

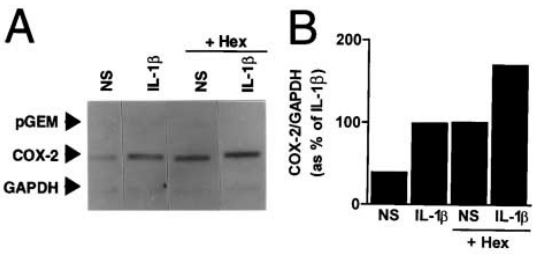

Fig. 3. Effect of IL-1β and cycloheximide on COX-2 transcription rate. Cells were not stimulated (NS) or stimulated with IL-1β either with or without cycloheximide (Hex) as indicated. Cells were harvested at 2 h and nuclear extracts prepared for run-off transcription analysis. A: Representative autoradiographs showing hybridisation of labelled run-off RNA to COX-2, GAPDH and pGEM3z plasmids. B: Data from two such experiments are shown as means of the ratio COX-2/GAPDH expressed as a percentage of IL-1β stimulation.

strength between the κBu and κBd EMSA is not due to differential loading or labelling of probe, but simply reflects the relative affinities of these two sites for NF-κB [13]. In resting cells NF-κB p50/p65 heterodimers are held inactive in the cytoplasm by inhibitor molecules known as IκB [24]. On stimulation by IL-1β or TNFα, IκB becomes phosphorylated and is targeted for rapid proteolysis to release active NF-κB, which is then free to translocate to the nucleus and activate transcription (Fig. 4C). This process is regulated by a negative feed-back loop in which NF-κB up-regulates IκBα transcription and expression to limit the response and ultimately return active NF-κB to resting levels [24]. Translational blockade by cycloheximide or transcriptional arrest by actinomycin D both prevent resynthesis of IκBα and therefore explains the superinductive effect of these inhibitors on IL-1β-induced NF-κB

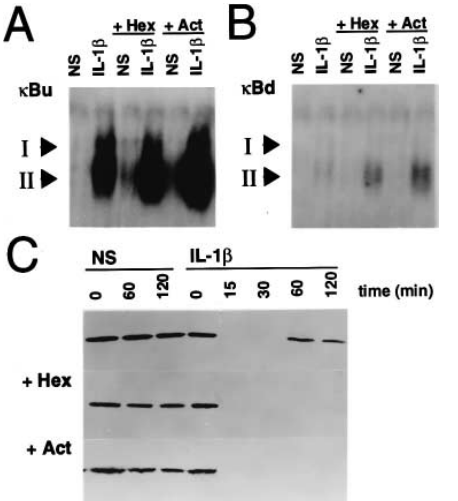

Fig. 4. Effect of IL-1β, cycloheximide and actinomycin D on NF-κB binding by κBu and κBd. Cells were not stimulated (NS) or stimulated with IL-1β either with or without cycloheximide (Hex) or actinomycin D (Act) as indicated. Cells were harvested at 1 h for nuclear extracts. Autoradiographs of EMSAs, representative of three experiments, for κBu (A) and κBd (B) are shown. Specific complexes, as determined by competition analysis, are indicated by solid arrows. Cells were either not stimulated (NS) or treated with IL-1β both with and without cycloheximide (Hex) or actinomycin D (Act) for the times indicated and total protein used for SDS-PAGE and immunoblotting for IκBα performed (C). Blots representative of three such experiments are shown.

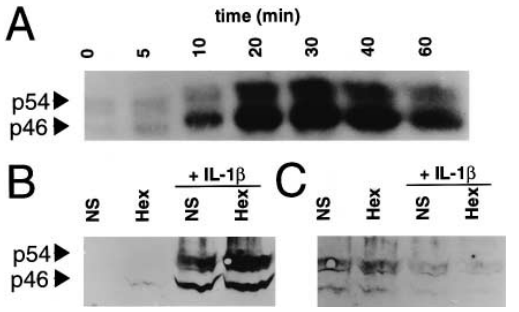

Fig. 5. Effect of IL-1β and cycloheximide on JNK activation. A: Cells were treated with IL-1β for the times indicated and proteins harvested for in gel JNK kinase assay. A representative blot of two such experiments is shown. B: Cells were treated as indicated and harvested after 1 h for total protein. After SDS-PAGE immunoblotting was performed for threonine/tyrosine phosphorylated JNK (B). The blot from (B) was stripped and reprobed for total JNK protein (C). (B) and (C) are representative of four such experiments. The positions of JNK p54 and p46 proteins are indicated.

(Fig. 4C). The appearance (on overexposed blots), at 120 min post-IL-1β+cycloheximide treatment, of low levels of IκBα indicates incomplete block of protein synthesis by cycloheximide and may explain the consistently greater NF-κB superinduction produced by actinomycin D. Furthermore, cycloheximide and actinomycin D alone result in appreciable loss of IκBα, accounting for the inductive effect of these compounds on NF-κB.

Similarly, IL-1β rapidly induced p54 and p46 JNK activity (Fig. 5A). This peaked around 30 min post-stimulation and had markedly declined by 1 h suggesting the presence of negative regulatory mechanisms. Qualitatively similar results were obtained following immunoblotting for phospho-JNK (data not shown). As JNK activity correlates with p54 and p46 phosphorylation, the effect of cycloheximide was assessed by Western blotting for phospho-JNK (Fig. 5B) and total JNK (Fig. 5C) [25]. This revealed some degree of JNK phosphorylation by cycloheximide treatment and a dramatic superinduction of IL-1β-induced JNK phosphorylation by cycloheximide. Recently, a protein phosphatase, which negatively regulates the ERK MAP kinase pathway, has been identified that is Ca^{2+}-dependent, requires ERK activation and de novo synthesis for full activity [26]. The data presented here suggest the existence of similar regulatory mechanisms for the JNK pathway and are consistent with down-regulation of JNK1/2 by the MAP kinase phosphatases, MKP1 and MKP2 [27].

In conclusion, we show induction and superinduction of COX-2 mRNA by cycloheximide in A549 cells. These effects are primarily mediated at the level of transcription and not by mRNA stabilisation. Furthermore, we present data showing that cycloheximide results in activation of two major signalling pathways, both of which are known to play key roles and may act co-operatively in the transcriptional activation of COX-2. We therefore conclude that in these cells superinduction by translational blockers is the result in increased nuclear signalling and may occur via activation of the NF-κB and/or JNK pathways.

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