IL-1β-dependent activation of NF-κB mediates PGE_2 release via the expression of cyclooxygenase-2 and microsomal prostaglandin E synthase

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Abstract Prostaglandin (PG) E_2 release is induced in pulmonary A549 cells by the NF-κB-activating stimuli interleukin-1β (IL-1β) and phorbol 12-myristate 13-acetate (PMA). Adenoviral over-expression of IκBαΔN, a dominant NF-κB inhibitor, prevents NF-κB-dependent transcription and was used to qualify the role of NF-κB in the release of PGE_2. IκBαΔN repressed IL-1β-induced, but not PMA-induced, cycloxygenase-2 (COX-2) and microsomal prostaglandin E synthase (mPGES) expression. These data conclusively demonstrate a substantial role for NF-κB in the co-ordinate induction of COX-2, mPGES and in the corresponding release of PGE_2 by IL-1β. However, other pathways are primarily responsible for PGE_2 release induced by PMA.

Key words: Cyclooxygenase-2; Prostaglandin GH synthase; Nuclear factor κB; Prostaglandin E synthetase

1. Introduction

Prostaglandins (PGs) are implicated in normal cellular processes as well as pathophysiologically in inflammation, oedema, bronchoconstriction, fever and hyperalgesia [1]. PG synthesis requires conversion of arachidonic acid to PGH_2 either by the constitutive cyclooxygenase (COX) 1 or by COX-2, which is induced by inflammatory and mitogenic stimuli [1]. Subsequently, PGE_2 is produced by two prostaglandin E synthases (PGESs). The microsomal glutathione-dependent PGES (mPGES) [2] is functionally linked to COX-2, whereas the cytosolic PGES is coupled to COX-1 [3,4]. As the target of non-steroidal anti-inflammatory drugs (NSAIDs), this pathway is of great pharmacological importance and it is believed that the anti-inflammatory benefits of NSAIDs derive from COX-2 inhibition [1]. However, the inhibition of COX-2 may also exacerbate inflammation by affecting resolution [5].

The transcription factor nuclear κB (NF-κB) regulates numerous inflammatory genes and is considered as a possible target for therapeutic intervention [6]. Various studies have implicated NF-κB in the transcriptional regulation of COX-2 using reporter-based assays [7–10], inhibitors [11–15], antisense [16] or decoy oligonucleotides [9]. However, an unequivocal demonstration of a role for NF-κB in inflammatory PG synthesis is currently lacking and the ATF/CRE and C/EBP sites are cited as the main regulators of COX-2 promoter activity [1]. In pulmonary A549 cells, COX-2 is transcriptionally induced by interleukin (IL) 1β and NF-κB (p50/p65) binds to putative NF-κB sites from the human COX-2 promoter [17]. As mPGES is also up-regulated by inflammatory stimuli [2,18,19], we have used A549 cells and adenoviral-mediated over-expression of IκBαΔN, a dominant inhibitor of NF-κB [20], to unequivocally address the role of NF-κB in these processes.

2. Materials and methods

2.1. Cell culture and adenoviral infection

A549 cells were grown as described in [21]. Ad5 adenoviral vectors were: a green fluorescent protein (GFP) expressing vector, Ad5-GFP (QBioGene-Alexis, Nottingham, UK), Ad5-IκBαΔN (HepaVec, Berlin, Germany), which contains an RSV promoter driving expression of an N-terminally deleted IκBα [20], and an empty Ad5-RSV vector (Null). Pre-confluent cells were infected with adenoviral vectors at the indicated multiplicity of infection (MOI) and incubated overnight prior to washing and changing to fresh serum-containing medium. Following overnight incubation, cells were treated 48 h after adenoviral infection by changing to fresh medium or media containing 1 ng/ml IL-1β (R&D Systems, Abingdon, UK) or 1×10^{-7} M phorbol 12-myristate 13-acetate (PMA) (Sigma, Poole, UK) as indicated. Cells grown on coverslips were fixed with 4% paraformaldehyde and stained with 1 μM 4′,6′-diamidino-2-phenylinole dihydrochloric hydrate (DAPI) (Sigma) prior to analysis by confocal fluorescent microscopy using a Leica confocal microscope equipped with a 488 and 514 dual-band argon laser and TCS NT software for the capture of images (Leica Microsystems, Milton Keynes, UK).

2.2. Analysis of NF-κB DNA binding, NF-κB-dependent transcription and IκBα protein

Electromobility shift assay (EMSA) and the use of the NF-κB-dependent reporter cells, 6κBtk.luc A549s, was as described in [22,23].

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Abbreviations: COX, cyclooxygenase; DAPI, 4′,6′-diamidino-2-phenylinole dihydrochloric hydrate; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; IL, interleukin; mPGES, microsomal PGES; MOI, multiplicity of infection; NF-κB, nuclear factor κB; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; PGES, prostaglandin E synthase; PMA, phorbol 12-myristate 13-acetate

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Western blot analysis using SDS–PAGE and standard transfer protocols was carried out using an antiserum specific for the serine 32 phosphorylated form of IκBα (#9240) (New England Biolabs, Hitchin, UK) and total IκBα (Sc-371) (Santa Cruz, CA, USA).

2.3. Analysis of PGE_2 release, COX-2 and PGES expression

PGE_2 in culture media was measured by radioimmunoassay (RIA) using an anti-PGE_2 antibody (Sigma) according to the manufacturer's instructions as described in [21]. Northern blot and Western blot analysis was as described in [21]. Antibodies for Western analysis were COX-2 C20 epitope (Santa Cruz) and mPGES (Cayman).

2.4. Densitometry and statistical analysis

Densitometric analysis of X-ray films from Western blot analysis, following ECL (Amersham) visualisation, or Northern blot hybridisation, following autoradiography, was performed below the saturation level of the film using TotalLab software (Nonlinear Dynamics, Newcastle upon Tyne, UK). In all cases statistical analysis was performed using analysis of variance with a Bonferroni post-test and graphical data are presented as means ± S.E.M.

3. Results

3.1. Infection of A549 cells with Ad5 expression vectors

A549 cells were infected with various MOIs of a GFP-expressing Ad5 construct. Following DAPI staining of nuclei, the presence of GFP was analysed by confocal fluorescence microscopy (Fig. 1). Below a MOI of 0.1, only low numbers (< 7.5%) of GFP-positive cells were observed (data not shown). Between MOIs of 0.1 and 10 a concentration-dependent increase in GFP-positive cells was observed (Fig. 1). At a MOI of 10, ~90% of cells were positive for GFP and no significant effect on cell viability was observed as determined by MTT cell viability assay (Fig. 1 and data not shown).

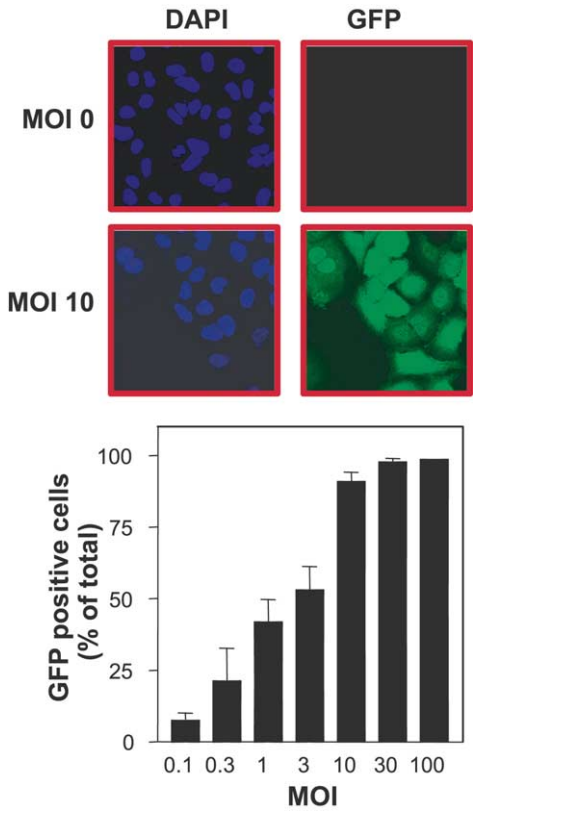

Fig. 1. Infection of A549 cells with GFP-expressing adenovirus. Cells were incubated overnight in serum containing medium with various MOIs of Ad5.CMV-GFP prior to changing to fresh medium. At 48 h post-infection cells were stained with DAPI prior to analysis by confocal microscopy. DAPI-stained nuclei (left panels) and GFP fluorescence (right panels) are shown for MOI 0 and 10. Cells showing any GFP fluorescence were counted as positive. Data from five such experiments were expressed as a percentage of the total cell number and are plotted as means ± S.E.M.

3.2. IκBαΔN inhibits NF-κB-dependent transcription and DNA binding

The IκBαΔN derivative of IκBα is a dominant inhibitor of NF-κB by virtue of the N-terminal (1–70) deletion of the region containing the serine residues, S32 and S36 [20]. Analysis of the NF-κB-dependent reporter cell line, 6κBtk A549, revealed robust responses to both IL-1β and PMA, whilst increasing MOI of Ad5-IκBαΔN decreased luciferase activity in a concentration-dependent manner (Fig. 2A). Western blot analysis confirmed increased expression of IκBαΔN with increasing viral titre and demonstrated that this was inversely related to the expression of endogenous NF-κB-dependent gene, IκBα (Fig. 2A) [24]. These data therefore provide functional validation of the effectiveness of NF-κB inhibition.

Analysis of NF-κB DNA binding revealed a strong response to IL-1β and a considerably weaker response to PMA (Fig. 2B,C). These data are consistent with our previous findings and raise the possibility that the activation of NF-κB by PMA occurs via a mechanistically distinct process [25]. As in previous studies [22,25], we find the induction of two predominant DNA binding complexes, which were both previously shown to contain p50 (NF-kB1) and p65 (RelA) subunits [25]. In each case, prior infection with an empty Ad5 vector (Null) had little effect on either basal or stimulated levels of NF-κB DNA binding, whereas infection with Ad5-IκBαΔN at MOI 10 reduced NF-κB DNA binding to below basal levels (Fig. 2B,C).

To examine the possibility that IL-1β and PMA were causing activation of distinct signalling pathways, cells treated with each stimulus were subjected to Western blot analysis for both the phosphorylated form of IκBα as well as total IκBα (Fig. 2D). In the case of IL-1β stimulation, a strong increase in IκBα phosphorylation was observed at 5 and 15 min post-stimulation. This was closely followed by the near-complete loss of IκBα within 15 min and then a gradual reappearance 1 h later. By contrast, stimulation with PMA produced no obvious increase in IκBα phosphorylation and no loss due to signal-induced degradation. Thus these data suggest that whilst the IKK pathway leading to IκBα phosphorylation and degradation is strongly activated by IL-1β, there is no such response to PMA. It is therefore likely that the transcriptional activation by PMA is occurring via other mechanisms.

3.3. Effect of IκBαΔN on COX-2 expression

A549 cells were infected with Ad5-IκBαΔN and null virus (as above). As IL-1β-induced COX-2 mRNA is maximal at 4–6 h post-stimulation, cells were harvested at 6 h for Northern blot analysis [21]. COX-2 mRNA expression was significantly increased by both IL-1β and PMA (both $P < 0.001$) (Fig. 3A). In each case the null virus alone had no significant effect on COX-2 expression, whereas IL-1β-induced COX-2 mRNA, but not PMA-induced COX-2 mRNA, was significantly reduced by Ad5-IκBαΔN ($P < 0.001$ for IL-1β vs IL-1β+IκBαΔN and $P < 0.05$ for IL-1β+Null virus vs

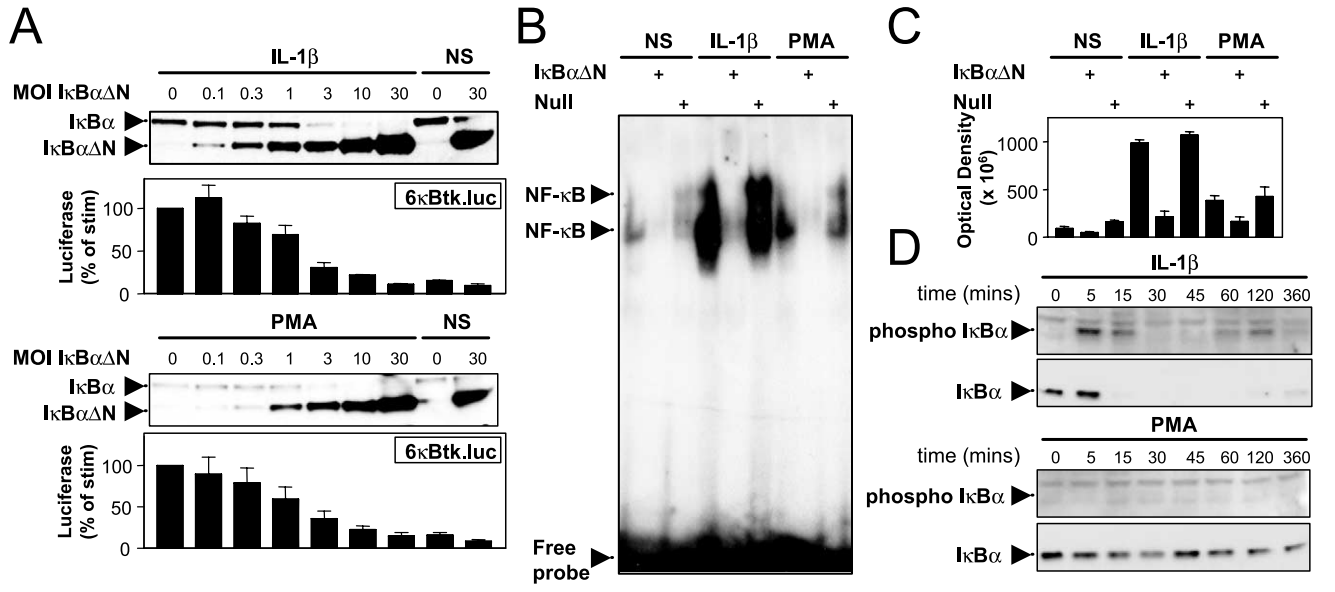

Fig. 2. Over-expression of IκBαΔN inhibits NF-κB-dependent transcription and NF-κB DNA binding. A549 6κBtk cells were either not infected or were infected with various MOI of Ad5-IκBαΔN virus. A: Cells were either not stimulated (NS) or stimulated with IL-1β (1 ng/ml) or PMA (10^{-7} M) for 6 h prior to harvesting for Western blot analysis and luciferase determination. Blots representative of three such experiments using an antiserum (IκBα C21) that detects a C-terminal epitope on IκBα are shown. Luciferase data from four such experiments were expressed as a percentage of the stimulus and plotted as means ± S.E.M. B: A549 cells were either not infected or infected at a MOI of 10 with either Ad5-IκBαΔN or null virus as indicated. After changing to serum-free media, cells were either not stimulated (NS) or treated with IL-1β (1 ng/ml) or PMA (10^{-7} M). Cells were harvested after 1 h and nuclear proteins prepared for EMSA analysis of NF-κB DNA binding. An autoradiograph representative of four such experiments is shown. The two specific DNA binding complexes were identified by cold competition and are indicated. Free probe is indicated. C: Autoradiographs ($n = 4$) from experiments in panel B were subjected to densitometric analysis and optical densities were plotted as means ± S.E.M. D: Cells stimulated with either IL-1β or PMA were harvested at the times indicated and subjected to sequential Western blot analysis for the serine 32 phosphorylated form of IκBα and total IκBα. Blots representative of two such experiments are shown.

IL-1β+IκBαΔN) (Fig. 3A). Consistent with these data, Western blot analysis of COX-2 protein expression showed a near-identical profile with the IκBαΔN virus causing significant repression of IL-1β-induced COX-2 when compared to either the IL-1β-treated samples ($P < 0.001$) and the IL-1β+null virus samples ($P < 0.01$) (Fig. 3B). Whilst in each case some degree of repression of PMA-stimulated COX-2 by IκBαΔN virus was apparent, this did not reach significance.

3.4. *Effect of IκBαΔN over-expression on mPGES expression and PGE_2 release*

Western blot analysis for mPGES at 24 h post-stimulation revealed levels of mPGES that were induced around two-fold by both IL-1β ($P < 0.05$) and PMA (Fig. 3C). The null Ad5 expression vector showed no effect on mPGES, whilst the Ad5-IκBαΔN virus reduced basal expression and prevented the induction by IL-1β ($P < 0.05$ for IL-1β vs IL-1β+IκBαΔN). Again, the PMA-dependent response was essentially unaffected.

In A549 cells, PGE_2 is the principal PG produced [26]. Supernatants from the above experiments were therefore analysed for the presence of PGE_2. Unstimulated cells produced only low to undetectable levels of PGE_2 (0.09 ± 0.05 ng/ml), whilst IL-1β and PMA resulted in significantly elevated elaboration of PGE_2 (3.99 ± 0.82 and 2.19 ± 0.56 ng/ml with $P < 0.001$ and < 0.05, respectively) (Fig. 3C, lower panel). The null virus had no obvious effect on PGE_2 release, whilst viral expression of IκBαΔN resulted in a near-complete loss of PGE_2 from IL-1β-treated ($P < 0.001$ for IL-1β vs IL-1β+IκBαΔN and $P < 0.05$ for IL-1β+null virus vs IL-1β+IκBαΔN), but not from PMA-treated cells (Fig. 3C, lower panel).

4. Discussion

A number of promoter-based studies have variously implicated the C/EBP, CRE/ATF and NF-κB binding sites in the transcriptional activation of the COX-2 promoter [7–9,27,28]. In this report, we provide compelling evidence for a major role of the transcription factor NF-κB in the induction of COX-2 mRNA, protein and the subsequent release of PGE_2 in response to IL-1β in human pulmonary A549 cells. These data are consistent with previous data showing an eight-fold induction of COX-2 transcription rate by IL-1β in A549 cells and with promoter-based studies implicating NF-κB in the activation of the COX-2 promoter in other cell lines [7,9,17]. By contrast, the induction of COX-2 expression by the phorbol ester, PMA, was essentially independent of NF-κB activation. As PMA is known to induce pathways leading to AP-1 and ATF transcriptional activation, it is likely that this response is primarily occurring via the CRE/ATF site as has been suggested for *v-src*-dependent induction of COX-2 [28]. In this respect, we also note that the PMA-dependent pathway that leads to activation of NF-κB-dependent transcription in these cells does not primarily appear to involve IKK-dependent phosphorylation of IκBα and is therefore mechanistically distinct from activation by IL-1β. Further studies are necessary to explore this result.

In addition to association and coordinate regulation with COX-2, the downstream mPGES gene is functionally linked

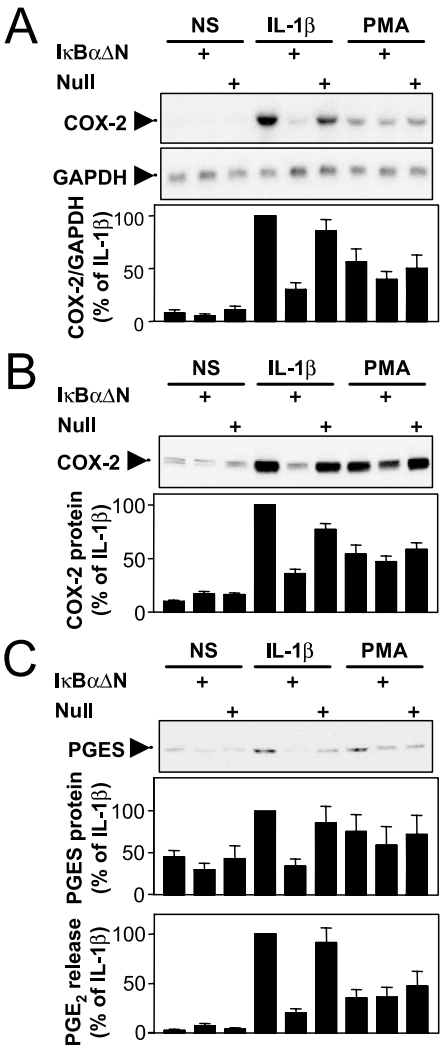

Fig. 3. Effect of IκBαΔN on COX-2 expression, mPGES protein and PGE_2 release. A549 cells were infected with Ad5-IκBαΔN or null virus and were either not stimulated (NS) or stimulated with IL-1β (1 ng/ml) or PMA (10^{-7} M) as indicated prior to harvesting at either 6 or 24 h. A: Total RNA was prepared from cells harvested at 6 h and Northern blot analysis was performed for COX-2 and GAPDH. Representative autoradiographs are shown. Following densitometric analysis, data from eight such experiments were expressed as a ratio of COX-2/GAPDH and are plotted as a percentage of IL-1β stimulated as means ± S.E.M. B,C: Cells harvested at 24 h were subjected to Western blot analysis for COX-2 and PGES. In each case representative blots are shown and data from nine experiments were expressed as a percentage of IL-1β-treated and are plotted as means ± S.E.M. C(lower panel): Supernatants from cells harvested at 24 h were analysed by RIA for PGE_2. Data from seven experiments were expressed as a percentage of IL-1β stimulated and are plotted as means ± S.E.M.

to the production of PGs via the COX-2 pathway and may be induced by inflammatory stimuli, including IL-1β [3,4,18,19,29]. Previous studies have implicated binding of the transcription factor Egr-1 to GC boxes located close to transcription start in the transcriptional activation of the mouse mPGES promoter by PMA and IL-1β+TNFα [30]. By contrast, the data reported here support a role for NF-κB in the induction of the human mPGES gene in A549 cells in response to IL-1β. In common with the effect on COX-2, phorbol ester-induced mPGES appears to be independent of NF-κB and could therefore be more reliant on GC box binding factors. Further promoter analyses will be required to address the location of NF-κB-responsive sites and the possible role of the GC boxes in the human mPGES promoter.

Taken together, the data reported here indicate a major role for the transcription factor NF-κB in the co-ordinate regulation of the COX-2/mPGES pathway in the production of PGE_2 in inflammatory situations. Given the intense interest in the pharmacological inhibition of NF-κB as a therapeutic target in inflammatory diseases such as asthma, these data certainly demonstrate, like the effect of glucocorticoids, the efficacy of inhibiting NF-κB to prevent inflammatory gene expression. However, the existence of conditions such as aspirin-sensitive asthma and the fact that COX-2 may play a role in resolution of inflammation indicates that not all the effects of NF-κB inhibition are necessarily beneficial in diseases such as asthma [5]. Further studies therefore are required to effectively weigh up the relative benefits and adverse effects of NF-κB inhibitors as novel anti-inflammatory agents.

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