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Activation of the transcription factor NF-κB in human tracheobronchial epithelial cells by inflammatory stimuli

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Activation of the transcription factor NF-κB in human tracheobronchial epithelial cells by inflammatory stimuli. B. Jany, R. Betz, R. Schreck. ©ERS Journals Ltd 1995.

ABSTRACT: Recent studies have shown that surface epithelial cells play a major role in the defence and inflammatory reactions of the airways. How extracellular stimuli lead to increased gene expression in these epithelial cells is not well known. In this study, we asked whether the multiunit transcription factor, nuclear factor (NF)-κB, which regulates the expression of genes involved in defence and immune processes, is activated in airway epithelial cells following stimulation with inflammatory mediators and hydrogen peroxide. In addition, we studied whether this would be followed by upregulation of the NF-κB target gene product granulocyte-macrophage colony-stimulating factor (GM-CSF).

Activation of NF-κB in the SV40 transformed human tracheobronchial epithelial cell line 1HAEo- was measured by electrophoretic mobility shift assays. GM-CSF concentrations in cell culture supernatants were determined by enzyme-linked immunosorbent assays.

NF-κB was rapidly activated by exposure of cells to interleukin-1β (IL-1β), phorbol myristate acetate (PMA), and tumour necrosis factor-α (TNF). Exposure to H_2O_2 platelet activating factor (PAF) and lipopolysaccharide (LPS) did not lead to increased NF-κB activation. Co-stimulation of IL-1β with H_2O_2 led to augmentation and prolongation of the effect on NF-κB activation compared to stimulation with IL-1β alone. GM-CSF concentrations increased following stimulation with IL-1β and H_2O_2, and the effect of IL-1β/H_2O_2 co-stimulation on GM-CSF concentrations was additive.

These results suggest that NF-κB may represent an important transcription factor, controlling the expression of cytokine genes in airway epithelial cells.

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Chronic airway diseases, such as asthma and chronic bronchitis, are characterized by inflammation of the tracheobronchial mucosa. Histologically, the airways show a cellular infiltrate with activated eosinophils, lymphocytes and neutrophils [1, 2]. Recent findings indicate that in addition to immune cells, airway epithelial cells are able to act as immune effector cells by secreting proinflammatory mediators. *In vivo*, an increased number of airway cells in asthmatics express messenger ribonucleic acid (mRNA) coding for several interleukins and granulocyte-macrophage colony-stimulating factor (GM-CSF) [3–5]. Cytokines, such as interleukins 5, 6 and 8 (IL-5, IL-6 and IL-8), tumour necrosis factor (TNF) and GM-CSF are released from tracheobronchial epithelial cells in culture [6, 7]. An important role for GM-CSF in inflammatory airway diseases has been suggested by studies indicating that this cytokine promotes eosinophil [5, 8], neutrophil [9], and macrophage [10] accumulation in the airways, probably by enhancing their survival [11, 12].

The intracellular mechanisms leading to the increased biosynthesis of proinflammatory cytokines by airway-epithelial cells have not been well studied. Some cytokine genes, such as GM-CSF, contain recognition sequences for the multi-unit transcription factor, nuclear factor (NF)-κB [13, 14]. Cleavage of an inhibitory (I)-κB subunit from a cytoplasmic, inactive form leads to nuclear translocation, binding of the p50 and p65 subunit to the corresponding cis-acting deoxyribonucleic acid (DNA) sequence, and transcriptional activation of the respective gene [13, 15]. NF-κB may act as an important second messenger system in defence processes by activating transcription of genes of the acute phase response, immune receptors, or by inducing the interferon-γ (IFN-γ) gene [16]. NF-κB-inducing factors include TNF-α, IL-1β [17], radiation [18], lipopolysaccharide (LPS), bacteria and exotoxins [19], double-stranded ribonucleic acid (RNA), viral proteins, ultraviolet (UV) light and oxidative stress (for review see [20, 21]). The activation of the cytoplasmic form of NF-κB is probably mediated by reactive oxygen intermediates [22]. The role of NF-κB as a major intracellular transducer of a variety of external signals has been investigated mainly in immune cells and fibroblasts [16–21]. Less is known about the function of this transcription factor in normal epithelial cells.

The tracheobronchial epithelium is constantly exposed

to a wide spectrum of inhaled potentially noxious agents that may play a role in the pathogenesis of airway disease. We hypothesized that, in tracheobronchial surface epithelial cells, the transcription activator NF-κB could rapidly mediate the synthesis of defence and signalling proteins. Therefore, we investigated whether factors known to be active in inflammatory airway disease and oxygen radicals would lead to activation of NF-κB in human airway epithelial cells. In addition, we studied whether this results in the release of the cytokine GM-CSF in cell culture supernatants.

Methods

Cells and cell culture

Normal human tracheobronchial epithelial cells (1HAEo-) were transformed with an origin of replication-deficient SV 40 virus [23]. The criteria for characteristics of primary airway epithelial cells are: 1) the cells form tight junctions and respond to β-adrenergic agonists by elevated cyclic adenosine monophosphate (cAMP) levels; 2) phase contrast microscopy shows the typical "cobblestone-like" appearance of epithelial cells in culture; 3) cytospin preparations of these cells shows immunofluorescent staining with a monoclonal antibody to cytokeratin 19; and 4) using the polymerase chain reaction, we found expression of the MUC 2 mucin gene in this cell line, consistant with its origin from human airway epithelium (data not shown [24]). Cells were cultured at 37°C and 5% CO_2 in F12/Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% foetal calf serum and 100 μg·ml^{-1} penicillin/streptomycin (all purchased from Gibco Laboratories). Tissue culture flasks (Falcon) were coated with a mixture of fibronectin (Becton Dikinson), bovine serum albumin (Sigma) and collagen type IV (Serva) as described previously [25]. Medium was changed every 3–4 days. Exposure to inflammatory mediators for up to 48 h did not change the morphology or growth characteristics of the cells at the concentrations used (see below) for electrophoretic mobility shift assay (EMSA) or enzyme-linked immunosorbent assay (ELISA) experiments. Stimulation of cells with H_2O_2 up to 100 μM did not lead to increased lactate dehydrogenase (LDH) concentrations in cell culture supernatants, excluding toxic effects of H_2O_2 on cell membrane integrity at the concentrations used.

Electrophoretic mobility shift assays (EMSA)

1HAEo- cells were grown to a confluence of about 70% in uncoated Petri dishes [24] and incubated for 30, 90 or 180 min with phorbol myristate acetate (PMA) (50 ng·ml^{-1}; Sigma), human TNF-α (30 ng·ml^{-1}; Saxon Biochemicals), human IL-1β (30 ng·ml^{-1}; Saxon Biochemicals), LPS (10 μg·ml^{-1}; Sigma), platelet activating factor (PAF) (1 μM; Sigma) or H_2O_2 (100 μM; Merck) alone, or co-incubated with H_2O_2 and either human TNF-α, human IL-1β, LPS or PAF (same concentrations). After washing twice with phosphate buffered saline (PBS), whole cell extracts were isolated as described by SCHRECK and BÄUERLE [26]. Protein concentrations of the whole cell extracts were determined by the Bradford microassay (Biorad). The extracts were frozen in liquid nitrogen and stored at -80°C. Equal amounts of protein were used for EMSAs. Binding reactions were performed according to ZABEL *et al.* [27] for 30 min on ice with 10–15 μg protein in 16 μl of 8 mM Tris-HCl (pH 7.5), 40 mM NaCl, 0.4 mM ethylene diamine tetra-acetic acid (EDTA), 3% glycerol, 15 μg bovine serum albumin, 2 μg poly (dI-dC) (Boehringer Mannheim), 1 mM phenylmethylsulphonylfluoride (Sigma) and about 8,500 c.p.m. NF-κB oligonucleotide (Promega), labelled with T4 kinase (Gibco/BRL) and (γ-^{32}P)-adenosine triphoshate (ATP) (3,000 Ci·mmol^{-1}; Amersham). The sequence of the NF-κB oligonucleotide is as follows:

5 -AGTTGAGGGGACTTTCCCAGGC- 3
3 -TCAACTCCCCTGAAAGGGTCCG- 5

DNA-protein complexes were separated from unbound DNA on native 4.0% polyacrylamide gels in 6.8 mM Tris-HCl (pH 7.5), 3.4 mM sodium acetate and 1.0 mM EDTA (pH 8.0). Gels were vacuum dried and exposed to Kodak (X-OMAT) films at -80°C for 10–24 h.

GM-CSF ELISA

1HAEo- cells were grown in coated tissue culture flasks to a confluence of about 70%. About 2 h before stimulation, culture medium was changed. Cells were incubated for 30 min, 90 min, 3, 6, 12, 24 or 48 h with either PMA, human IL-1β or H_2O_2, or co-incubated with H_2O_2 and human IL-1β as indicated. After incubation, supernatants were collected, divided into aliquots and stored at -80°C. The amount of immunoreactive human GM-CSF in the supernatants was determined by ELISA (GM-CSF ELISA; Biomar, Germany). All measurements were performed in duplicate, according to the manufacturer, using an ELISA reader (Labtek).

Results

Electrophoretic mobility shift assays

The strongest activators of NF-κB DNA binding were PMA, TNF-α and IL-1β. Incubation of a radiolabelled NF-κB oligonucleotide with cell extracts from treated cells resulted in the retardation of a single specific complex (fig. 1). This effect occurred rapidly within 30 min of stimulation (fig. 1, lanes 2–4). The signal was detectable after stimulation with PMA and TNF at about the same intensity after 90 and 180 min (fig. 1, lanes 2 and 3). NF-κB activation induced by IL-1β was a strong but transient effect that was lost after 90 min (fig. 1, lane 4).

To ensure the specificity of the DNA-protein interaction, we performed competition studies with unlabelled oligonucleotides. Addition of a 50 fold excess of unlabelled oligonucleotide resulted in complete disappearance of the

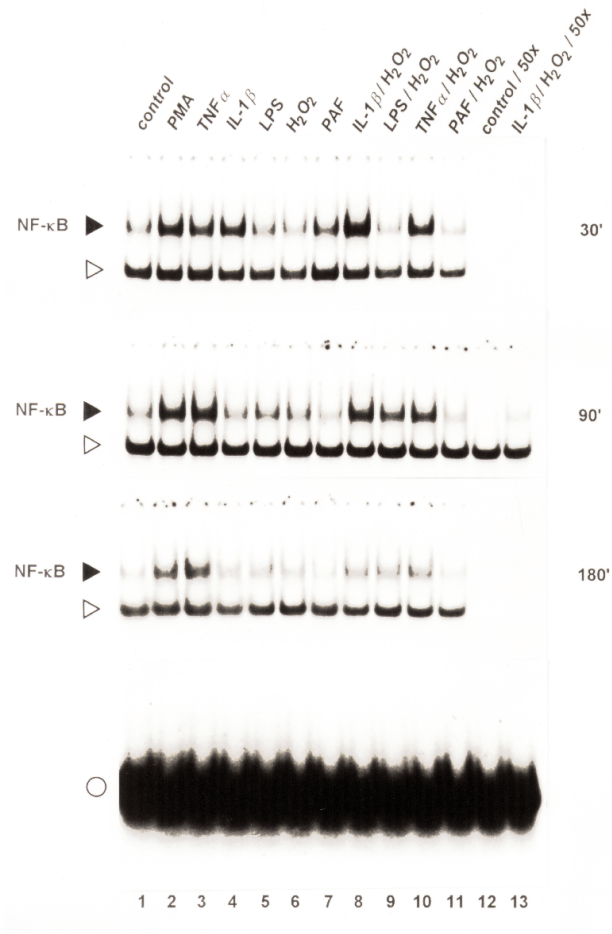

Fig. 1. – NF-κB activating effects of inflammatory stimuli in 1HAEo- cells. Cells at 70% confluency were stimulated for 30, 90 and 180 min. The figure represents a composite of the three gels after stimulation for the times indicated. Lane 1: unstimulated control cells. Lanes 2–7: incubation with single stimuli. Lanes 8–11: co-stimulation with H_2O_2. Lanes 12 and 13: DNA-protein interaction after addition of a 50 fold excess of unlabelled oligonucleotide to control cell extracts and IL-1/H_2O_2-stimulated cell extracts. This competition experiment is shown only for 90 min stimulation. Filled arrowheads: retarded NF-κB/DNA complex: open arrowheads: unspecific DNA-binding; open circle: position of unbound DNA (shown only for one gel, 90 min simulation). NF-κB: nuclear factor-κB; IL-1β: interleukin-1β; DNA deoxyribonucleic acid; PMA: phorbol myristate acetate; TNF-α: tumour necrosis factor-α; LPS: lipopolysaccharide; PAF: platelet-activating factor.

retarded DNA-protein complex in unstimulated control cells (fig. 1, 90 min, lane 12). A faint band was still visible when a 50 fold excess of unlabelled oligonucleotide was used in cell extracts of IL-1/H_2O_2 stimulated cells (fig. 1, 90 min, lane 13). Independent of the time of incubation, LPS (10 μg·ml^{-1}) alone did not activate NF-κB (fig 1, lane 5). Co-stimulation of LPS and H_2O_2 resulted in a transient NF-κB activation after 90 min (fig. 1, lane 9). The lipid mediator PAF had a minor and transient effect (fig. 1, 30 min, lane 7). When the cells were co-stimulated with PAF and hydrogen peroxide, no activation of DNA-binding could be detected (fig. 1, lane 11). Incubation with H_2O_2 alone at the concentrations tested (up to 100 μM) had no effect. Interestingly, in the case of IL-1β, co-stimulation with H_2O_2 resulted in a markedly prolonged and stronger NF-κB activation compared to IL-1β incubation alone (compare fig. 1, lanes 4 and 8). The activation of NF-κB induced by IL-1β together with H_2O_2 was the strongest of all combinations tested. Taken together, these experiments demonstrate that the transcription factor NF-κB is activated by different inflammatory stimuli in tracheobronchial epithelial cells.

In all experiments, NF-κB activation was clearly dependent on the cell confluency. Confluent cultures showed lower activation of NF-κB DNA-binding compared to a confluency of about 70%. This was determined in a series of preliminary experiments (data not shown).

GM-CSF ELISA

1HAEo- cells were incubated with IL-1β or H_2O_2 alone and co-incubated with IL-1β/H_2O_2 at the same concentrations used in the EMSAs. Immunoreactive human GM-CSF was measured in cell culture supernatants up to 48 h after stimulation by ELISA. Stimulation with IL-1β led to GM-CSF concentrations above the detection limit as early as 90 min following incubation (fig. 2). There was a linear increase of GM-CSF concentrations up to 48 h following stimulation. Incubation with H_2O_2 led to increased GM-CSF concentrations after only 6 h. This delay compared to IL-1β lasted up to 12 h. H_2O_2 stimulation reached the IL-1β induced values at 24 and 48 h. Co-

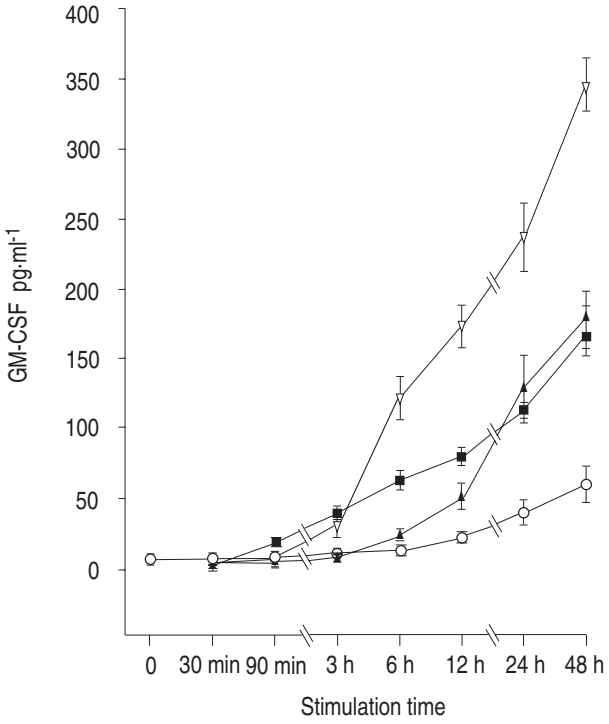

Fig. 2. – Effect of IL-1β, H_2O_2 and combination of IL-1β/H_2O_2 on the secretion of GM-CSF by 1HAEo-cells. Cells were grown to a confluence of 70% and incubated with 30 ng·ml^{-1} human IL-1β, 100 μM H_2O_2 or combined IL-1β/H_2O_2 using the same concentrations. At the different time-points indicated, cell supernatants were tested for human GM-CSF by ELISA. Data points represent the median±SD of three independent sets of experiments. All measurements were performed in duplicate. IL-1β: interleukin-1β; GM-CSF: granulocyte-macrophage colony-stimulating factor; ELISA: enzyme-linked immunosorbent assay. —▽— : IL-1β/H_2O_2; —▲— : H_2O_2; —■— : IL-1β; —○— : control.

stimulation with IL-1β/H_2O_2 showed an additive effect after 6 h, that continued up to 48 h of cell culture.

Discussion

In this report we show that exposure of human airway epithelial cells *in vitro* to inflammatory stimuli known to be active in inflammatory chronic airway disease leads to the activation and DNA-binding of the transcription factor NF-κB. This is followed by an increased secretion of the NF-κB responsive gene product GM-CSF.

Firstly, we focused on the possible NF-κB activating role of the inflammatory mediators IL-1β and TNF-α, since IL-1 receptors have been demonstrated on human airway epithelial cells [6]. IL-1 and TNF may play a key role in allergic airway disease; a selective IL-1 receptor antagonist is able to prevent airway hyperreactivity and eosinophil accumulation in a guinea-pig model [28]. In our model, both cytokines lead to a strong activation of NF-κB in human airway epithelial cells. This supports the role of these factors in the regulation of the inflammatory response of epithelial cells. In addition, NF-κB may represent an important trans-acting factor in these cells, leading to upregulation of responsive genes. In fact, we were able to show that one NF-κB target gene product, GM-GSF, is released by these cells upon stimulation, following the activation of NF-κB. This strongly suggests that NF-κB activation is one of the cytoplasmic-nuclear signalling pathways used in airway epithelial cells in response to inflammatory stimuli.

In contrast to Jurkat T-cells, mouse fibroblast and mouse pre-B-cells [21], LPS and H_2O_2 alone failed to activate NF-κB in human airway epithelial cells. The reason for this is unclear. It is conceivable that in airway epithelial cells higher levels of enzymes regulating the intracellular concentrations of reactive oxygen intermediates, such as superoxide dismutase [29], the glutathione (reduced form) GSH peroxidase/GSH system, catalase or peroxidases, prevent H_2O_2 and other resulting reactive oxygen intermediates from activating the latent cytoplasmic form of NF-κB compared to cells of the immune system. Alternatively, longer exposure times or higher concentrations of H_2O_2 may be required to activate NF-κB.

Although PAF has a wide spectrum of proinflammatory effects and may play a role in mediating the effects of ozone in the airways [30], this mediator had a very minor effect on NF-κB activation. A combination of inflammatory mediators different to those used in our experiment may lead to activation of NF-κB, since cytokine-cytokine interaction, like the synergy caused by IL-1 and TNF [31], plays an important role in several biological systems [32].

LPS co-stimulation with H_2O_2 led to transient NF-κB DNA-binding after 90 min. In pre-B-cells, PMA leads to a much faster NF-κB activation than LPS, similar to the kinetics in our experiments [17]. Interestingly, co-stimulation with H_2O_2 and IL-1β led to a longer lasting effect compared to IL-1β alone. This may be relevant to the *in vivo* situation, since in the presence of airway inflammation, the additional appearance of oxygen radicals from any source may lead to prolonged and additive inflammatory effects, mediated in part through the transcription factor NF-κB. How this synergistic effect is mediated, remains unknown. The variations of the effect of the different agents on NF-κB activation may reflect qualitative or quantitative differences in the components constituting the signalling pathway.

GM-CSF may play an important role in inflammatory airway diseases [5]. In addition, GM-CSF may be involved in the inflammatory changes in human airways following ozone exposure [33]. A role for GM-CSF in airway disease has been further emphasized by a number of *in vitro* studies [7, 9, 10, 33]. We found that human airway epithelial cells release the NF-κB target gene product GM-CSF into the culture medium upon stimulation with IL-1β. This effect was additive when the cells were co-stimulated with IL-1β and hydrogen peroxide, comparable to results in human primary airway epithelial cell cultures [8]. The effects of IL-1β/H_2O_2 co-stimulation on GM-CSF secretion are strikingly similar to the pattern of NF-κB activation. Treatment of cells with NF-κB anti-sense oligonucleotides inhibited GM-CSF gene expression [34], supporting the role of NF-κB in regulating the biosynthesis of this cytokine. Taken together, our findings strongly suggest that GM-CSF secretion by tracheobronchial epithelial cells is mediated, at least in part, by the activation of the transcription factor NF-κB.

In conclusion, we have shown that the transcription factor NF-κB, known to regulate the expression of a variety of genes involved in defence and immune reactions, is activated in human airway epithelial cells by inflammatory stimuli. The activation of NF-κB is followed by secretion of the NF-κB target gene product GM-CSF. Hydrogen peroxide has a modulating effect on the NF-κB activation induced by IL-1β. Hence, NF-κB may play a major role in tracheobronchial epithelial cells as a second messenger system following stimulation by inflammatory mediators. Investigation of compounds capable of inhibiting the activation of NF-κB may lead to the development of new treatment strategies for chronic inflammatory airway diseases, such as chronic bronchitis and asthma.

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